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Transcriptomic and functional approaches to unveil the interaction between a biocontrol yeast and a postharvest fungal pathogen on host fruit

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"*Nuestras horas son minutos*

cuando esperamos saber,

y siglos cuando sabemos

lo que se puede aprender …"

Antonio Machado

SUMMARY

Biocontrol strategies are a promising alternative to achieve food safety and food security. The aim of this study was to decipher the molecular interactions involving the biocontrol agent (BCA) yeast *Papiliotrema terrestris* strain LS28, the postharvest pathogen *Penicillium expansum*, and *Malus domestica*. RNAseq analysis was performed during both their dual and tritrophic interactions to identify the differentially expressed genes of BCA, pathogen, and host. Analysis of transcriptome changes in the BCA revealed overexpression of genes involved in nitrogen catabolite repression and oxidative stress response, regardless of the presence of the pathogen, suggesting that these pathways are crucial for the BCA to rapidly colonize the ecological niche (fruit wounds) and outcompete the pathogen. In the absence of *P. expansum*, BCA genes involved in metabolism and transport of carbohydrates and branched-chain amino acids were highly represented, suggesting a different nutritional requirement of *P. terrestris* when it does not compete with the pathogen. To confirm transcriptomic data at phenotypic level, targeted mutants of the BCA were generated for several overexpressed genes. The in vitro phenotypic characterization and biocontrol assay revealed a crucial role of a putative amino acid transporter in the biocontrol activity of *P. terrestris* against *P. expansum*. The transcriptomic analysis of *P. expansum* revealed that genes involved in transcription, oxido-reductive processes, transmembrane transport, and amine and peptide metabolism were the most represented GO categories, regardless of the presence of the BCA. Whereas in the absence of the BCA there was only enrichment of oxido-reductive processes-related transcripts, in its presence transcripts involved in metabolic processes of polysaccharides, aminoglycan and glucosamine-containing compounds were strongly enriched, suggesting a substantial nutritional rewiring of the pathogen to directly outcompete the BCA. Analysis of the transcriptomic changes of the host *M.*

domestica revealed overexpression of genes involved in host defense signaling pathways in the presence of both the BCA and the pathogen, and a prevalence of pathogen triggered immunity (PTI) and effector trigger immunity (ETI) host genes overexpressed only during interaction with *P. expansum*. This comprehensive analysis contributed to advance the knowledge on the molecular mechanisms that underlie biocontrol activity and the tritrophic interaction of the BCA with the pathogen and the host.

SOMMARIO

L'utilizzo dei mezzi di lotta biologica al fine di contenere lo sviluppo dei patogeni fungini e ridurre le perdite di derrate alimentari consente anche di sostituire progressivamente l'impiego di fungicidi di sintesi riducendo l'impatto che questi ultimi hanno sull'ambente e sulla nostra salute.

La presente attività di ricerca è stata incentrata sull'analisi dei dati di un esperimento di *RNA-sequencing* che coinvolge tre organismi in una stretta interazione reciproca (interazione tritrofica). Oggetto di studio sono l'agente di biocontrollo *Papiliotrema terrestris* ceppo LS28, l'agente patogeno *Penicillium expansum* ceppo 7015 entrambi inoculati in ferita di mela (*Malus domestica*).

L'obiettivo di tale studio è l'approfondimento riguardo la comprensione dei meccanismi di azione che sono alla base del biocontrollo, ossia dell'attività antagonistica del ceppo LS28 nel contrastare lo sviluppo dell'agente patogeno *Penicillium expansum* in ferita di mela. A tal fine, sulla base dell'interpretazione dei dati ottenuti dallo studio di espressione genica sul ceppo LS28, è stata stilata una lista di geni che sono stati mutati mediante l'applicazione della *target* mutagenesi (mutagenesi sito-specifica). Attraverso la delezione del gene prescelto (sulla base dei dati ottenuti dallo studio di trascrittomica), ne è stata caratterizzatala relativa perdita di funzione *in vitro* e *in vivo*. Inoltre sono stati valutati, interpretati e confrontati con studi simili i principali processi biologici coinvolti nell'espressione genica del patogeno (*Penicillium expansum*) durante la colonizzazione della mela con/senza il ceppo LS28, e nell'ospite (*Malus domestica*) durante l'interazione con entrambi (interazione tritrofica) e con ciascuno di essi.

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1 Introduction

1.1 World challenges and food losses, an introduction

Nowadays food losses represent a critical issue that is strongly exacerbated by climate change. Therefore, over the past few years, scientific production related to this issue *sensu lato* has been steadily increasing in a wide range of disciplines (Figure 1, source: Web of Science). In order to address such a global challenge many plant scientists and, in particular, plant pathologists have focused their research activity on the comprehension of molecular, epidemiological and ecological bases of plant diseases with the aim of developing effective and long-lasting solution.

According with this scenario, FAO urged Countries to reduce by half the food loss and waste, thus increasing food availability and promoting sustainability (Porat et al., 2018).

The quantification of food loss and waste is not an easy task, mainly because data availability has several gaps, as highlighted by the scientific community. As regards postharvest losses, in particular, these are not restricted within a specific stage along the food supply chain. However, it must be pointed out that when *per capita* measure unit are used, losses of fruit and vegetables predominate at the postharvest stage (Xue & Liu, 2019).

1.2 Postharvest fungal pathogens

The harvesting can be considered “the ridge” between pre-harvest and post-harvest process. The assessment of fruit ripening to identify the optimal moment for the harvest could affect downstream postharvest system and diseases management. In addition, in many cases field fungal infections may keep latent or quiescent, and blast out in the postharvest phase (Adaskaveg, Förster, & Thompson, 2000; Emery, Michailides, &

Scherm, 2000; Prusky, 1996). Species belonging to genera *Colletotrichum*, *Alternaria*, *Botrytis*, *Lasiodiplodia*, *Phomopsis* and *Botryosphaeria* were reported to have a quiescent phase in the host tissue until the fruit ripen.

Symptoms caused by postharvest fungal pathogens become visible in the majority of cases during storage. Three are the main penetration mechanisms generally exploited by postharvest fungal pathogens to colonize the host tissues: (i) through wounds caused during the harvest by handling; (ii) through natural opening like lenticels or stem and at the pedicels-fruit interface (also cuticular cracking, *i.e*. breaches of cuticle that physiologically occur during fruit growth, ought to be included); (iii) by degrading host cuticle (Gibert et al., 2009; Prusky & Lichter, 2008). Wounds and natural openings are the preferential points of access of many pathogens, such as *Penicillium* spp. and *Botrytis* spp., which are defined as wound pathogens (although *Botrytis* spp. are also able to find their way through direct penetration by degrading the host cuticle). For fungi as *Monilinia laxa* and *M. fructicola*, the causal agents of brown rot of stone fruit, the infection process can take place in bloom, thud causing blossom blight, then the infection can proceed in the green fruit, which may rot and decay on the tree or later during storage, especially at ripening (Emery et al., 2000).

Many signal exchanges drive the infection during the pathogen interaction with the host fruit, and different strategies are used by fungal pathogens to survive in the host tissues. A few examples are reported below.

Colletotrichum spp. is organized phylogenetically as a complex of species having a broad hosts range, furthermore, *Colletotrichum gleosporioides sensu stricto* was isolated on *Citrus sinensis* (type strain, isolated in Italy), *Carya illinoinensis*, *Ficus* sp., *Mangifera indica*, *Pueraria lobata* and *Vitis vinifera* (Weir, Johnston, & Damm, 2012) is one of the

most important postharvest pathogens, known to have a broad range of hosts. The pathogen penetrates directly the host cuticle by forming an appressorium and an infection peg. Through dendritic-like structures and swollen hyphae it then colonizes the epidermis cell layer in unripe stage of tomato fruit artificially inoculated by Alkan and colleagues. Afterwards, *C. gloeosporioides* the biotrophic-like phase on the unripe fruit turns into a necrotrophic one, when the fruit approaches ripening (Noam, Alkan et al., 2015).

Aspergillus niger Tiegh is the most common agent of black rot of fruit, and is also able to accumulate mycotoxin (mainly ochratoxin A) that contaminate fruit and fruit-derived products. It is particularly common on citrus stored at high temperature (Abarca et al., 1994; Ladaniya, 2008). Its occurrence on pomegranate fruit, mainly as a wound pathogen, is also important and may be confused with *Alternaria* rot. In Greece it was reported that 20% of pomegranate fruit infected with *A niger* and with *A. tubingensis* are contaminated either with ochratoxin A and Fumonisin B_2, furthermore the disease incidence was high in the pre-harvest phase (Kanetis et al., 2015). On grape, *A. niger* can be frequently detected in association with *Alternaria* spp., *Penicillium* spp. and *Rhizopus* spp.; on berry, rots are exacerbated by insect damage. Another pathogen such as *A. flavus* was reported as a fruit decay agent in some region with drought climate, furthermore, its mycotoxigenic activity exacerbate serious concerns about processed food product contamination (Michailides & Thomidis, 2007).

When the fruits and vegetables are stocked in piles or bins with high moisture, infections caused by *Rhizopus stolonifer*, which is one of the fastest growing species belonging to the phylum Z*ygomycota*, require mechanical wounds or cracking to develop and cause the devastating disease known as soft rot. The soft rot appearance is due to the high activity of polygalacturonases that are considered the first hydrolases of *R. stolonifer* involved in the softening process (Bruton et al., 1998). Another Z*ygomycete, Mucor*

pyriformis can cause soft rot on apple and pear, in 2016 was also reported on mandarin (Saito, Michailides et al., 2016). The main difference between these two pathogens relies on the growth temperature; while *R. stolonifer* grows slowly at temperature lower than 5°C, *M. pyriformis* tolerates low temperature, thus causing soft rot of fruits in cold storage. Macroscopic signs of the fungus don't allow the identification of the two pathogens, both develop high amount of aerial mycelium, however fruits attacked by both pathogens present a surface covered by a fluffy cotton-like fungal structure. At microscopic level the sporangiophores of *Rhizopus* spp. present rhizoid structure while those of *Mucor* spp. they don't.

For a successful host colonization fungal pathogen must overcome its defense. One important aspect is the capability of pH modulation by the pathogen. By this dynamic adaptation, each genus of fungi is able to tune the gene expression and secretion of pathogenicity factor on its own benefit, in response to the host pH at the court of infection. Prusky and Lichter (2008) described two main "routes" in pH modulation involved in pathogenetic process, host-alkalinizing fungi and host-acidifying fungi. Alkalinization is carried out by many *Colletotrichum* species such as *C. gloeosporioides, C. acutatum, C. higginsianum, C. graminicola,* and *C. coccodes* (Alkan et al., 2008; Diéguez-Uribeondo, Förster, & Adaskaveg, 2008; Itay Miyara et al., 2010; O'Connell et al., 2012), *A. alternata* (Eshel et al., 2002), and *Fusarium oxysporum* (Miyara et al., 2012).

Conversely, there are several acidifying fungal pathogens such as *P. expansum, P. digiatum*, P. *italicum* (Prusky et al., 2004), *Phomopsis Mangiferae* (Davidzon et al., 2010), *Aspergillus niger* (Ruijter, van de Vondervoort, & Visser, 1999), *B. cinerea* (Manteau et al., 2003), and *Sclerotinia sclerotiorum* (Bateman & Beer, 1965).

The mechanism by which filamentous fungi modulate the pH is governed by *PacC* (*RIM101* in yeasts), a gene that codifies for a zinc finger transcription factor able to carry out a dual modulation - activation and repression - in alkalinizing and acidifying fungal pathogens, respectively. Furthermore, the expression of genes belonging to the same gene family may be not strictly regulated by acidic or alkaline pH value; hence *PacC*, allowing the regulation of the gene expression in function of the local pH (Alkan et al., 2013). Alkalizing fungi produce pathogenicity factor that may be affected by pH values, such as pectate liase (*pelB*) in *C. gloeosporioides*, polygalatturonase (PG1 and PG5) in *F. oxysporum* and endoglucanases in *A. alternata*. Acidification is mediated by the secretion of organic acid as gluconic acid in *P. expansum*, while alkalinization by ammonium secretion in *C. gloeosporioides*. At biochemical level this was achieved by the involvement of two genes. In particular, the gene responsible of alkalinization is mainly glutamate dehydrogenase (*GDH2*), catalyzes the oxidation of glutamate to α-ketoglutarate and the release of ammonium; while acidification is mediated by *GOX2*, which catalyzes the oxidation of glucose to gluconic acid (Hadas et al., 2007).

In agreement with these findings several studies suggest that sugar content may drive pH modulation. Indeed, *GDH2* expression was induced by low sucrose level and *GOX2* expression was induced during growth in high sucrose level (Bi et al., 2016).

In summary, seems that the balance between acidification and alkalinization is the result of differential carbon utilization ability in different fungal pathogens. The central role of carbon during the host colonization process by the fungal pathogen is not so surprisingly. Sugar was stored in the vacuole compartment that may reach also the 30% of the total nutritional content of the fruit. Anyway, during fruit growth and maturation permeability of the tonoplast and plasma membrane to glucose and fructose increase (Ofosu-Anim & Shohei, 1994), enable fungal nutrient acquisition.

1.3 Biocontrol agents applied in postharvest

In the post-harvest phase several fungal pathogens, mostly wound pathogens, can compromise the shelf life of fruits and vegetables. Since the eighties of last century, the application of antagonistic organisms such as yeasts and bacteria has been tested against postharvest pathogens (Roberts, 1994; Wisniewski et al., 2016; Wisniewski & Wilson, 1992; Wisniewski et al., 1988).

Among bacteria, the Gram-negative *Pseudomonas syringae* is one of the first antagonists that has been studied and commercialized (Biosave), since it was able to prevent infections caused by *P. expansum* and *B. cinerea* on apple (Janisiewicz & Jeffers, 1997). Furthermore, the Gram-positive *Bacillus subtilis* was commercially developed for pre and postharvest treatments to reduce decay symptoms of green and blue mold on citrus fruit (Demoz & Korsten, 2006; Obagwu & Korsten, 2003). Although a lot of studies report the biocontrol properties of bacterial BCAs (*e.g.* for their capacity to induce plant defense responses and for their host growth promotion), (Compant et al., 2005) several authors proposed yeasts as more suitable biocontrol agents against postharvest diseases because, contrarily to bacterial BCAs, they do not produce antibiotics (Dukare et al., 2019; Pimenta et al., 2009; Raaijmakers, Vlami, & de Souza, 2002).

Yeasts are able to colonize different habitats and ecological niches and naturally occur on fruits and leaves surface; the yeast community inhabiting the carposphere vary over time depending on the ripening stage of the fruit (Peter & Rosa, 2006). Several yeasts isolated from different matrices were selected and studied for their ability to counteract postharvest pathogens. Strains of the yeasts *Rhodotorula glutinis* and *R. mucillaginosa* were formulated and patented for their use against postharvest rots caused by *P. expansum*, *B. cinerea* and *Mucor* spp. (Shanmuganathan, 1996). Later on, other yeasts

were formulated and developed as biocontrol products. The ascomycetous yeast *C. oleophila* and the basidiomycetous yeast *Pseudozyma flocculosa* were commercialized for postharvest purpose (Aspire®) and for field application in vineyard against *Uncinula necator* (Sporodex ®), respectively.

The yeast-like fungus *Aureobasidium pullulans* was tested on strawberries, table grape berries and kiwifruit and showed a significant protection against major storage rot agents such as *Botrytis cinerea* and *Rhizopus stolonifer* by Lima and colleagues (1997); in particular, this study remarks that BCA application in preharvest on strawberries floral tissue increase the efficacy of the antagonist to counteract the infection, as is the case of *A. pullulans* that was commercialized as Boni protect® and developed for postharvest pathogen (Ippolito & Nigro, 2000; Lima et al., 1997; Weiss, Mögel, & Kunz, 2006). Several scientific contributions have shown the efficacy of an integrated approach for disease management of postharvest diseases, through the combination of biocontrol yeasts with different types of additives and fungicides (Lima et al., 2011; Lima et al., 2005; Spadaro & Gullino, 2004).

Application of biocontrol yeasts turns out to be also effective in significantly decreasing mycotoxin contamination, as in the case of patulin and ochratoxin accumulation in apples and in wine grape, respectively; furthermore, the ability to degrade patulin may be pursued by different pathways, and mainly two are very common among the subphylum *Pucciniomycotina* red yeasts (Castoria et al., 2005; Castoria et al., 2008; De Curtis et al., 2012; de Felice et al., 2008; Ianiri et al., 2013). Interestingly, the presence of the biocontrol yeast *Rhodotorula kratochvilovae* LS11 (previously known as *Rhodosporidium kratochvilovae*) in apple wounds stimulates the specific rate of patulin biosynthesis – measured as ng patulin/μg fungal DNA – by the mycotoxigenic pathogen *P. expansum*, yet the overall contamination of apples is decreased (Zheng et al., 2017).

Furthermore, other biocontrol yeasts that can degrade mycotoxins, LS11 is able to degrade patulin *in vitro* (De Curtis et al., 2012; de Felice et al., 2008). However, patulin degradation by LS11 has not been demonstrated *in vivo*, so the above-mentioned decrease of patulin contamination of apple fruits seems to be mainly due to the limitation of growth of the pathogen biomass operated by the BCA. Patulin degradation by *R. kratochvilovae* leads to the formation of less toxic desoxypatulinic acid (Castoria et al., 2011). This degradation pathway appears to be common within the *subphylum* Pucciniomycotina since it has also be shown for *Sporobolomyces* sp. (Ianiri et al., 2013).

A strain of the marine yeast *R. Paludigenum* (Fell & Tallman 394084) isolated in South East China is a promising BCA and was tested also the ability to degrade patulin (Zhu et al., 2015). Two papers reported that strains of the low temperature-adapted yeasts *Leucosporidium scottii* and *Cryptococcus laurentii* were highly effective in their biocontrol activity on apple and tomatoes inoculated with *B. cinerea* in cold storage conditions (Hu et al., 2017; Silvana Vero et al., 2013); these studies report labor-saving methods for the isolation of cold adapted BCAs for application on fruits (in this case on cold-stored fruit) with a reducing scale of resources, most important confirm that Basidiomycete yeast belonging to genera *Cryptococcus* were the most frequently isolated cold-adapted yeasts (De García et al., 2007) .

During the isolation of a BCA it is better to perform multiple samplings because is known that field management and abiotic factors may affect biocontrol properties of a potential BCA isolated in a single location, as specified for a strain of *Metchnikowia pulcherrima* isolated from apples by Janisiewicz and collegues; this study shows as different strains of the same species isolated over the time from the same orchard differ in their biocontrol potential (Janisiewicz et al., 2001).

In conclusion, yeast BCAs represent a concrete opportunity to accomplish the need of an eco-friendly strategy in order to reduce the losses and chemical residues in the postharvest compartment.

1.4 Main known modes of action of Yeast Biocontrol Agents

Using yeasts as biocontrol agent in order to counteract postharvest fungal diseases has several aspect of interest: (i) they are ubiquitously found in the phytobiome and there is a high degree of biodiversity that allows the discovery of natural and specific antagonisms, (ii) they are environmental friendly microorganisms and Generally Recognized as Safe (GRAS) for humans and animals and therefore safe to manipulate and (iii) the production of biomass for their application is easy to obtain with a low resource expense (Freimoser et al., 2019) . In general, more than one yeast antagonistic mechanism of action may take place at the same time, resulting in the control of the pathogen. The following mechanisms have been described: competition for nutrients and space because of their ability to rapidly reproduce on simple nutrients and to colonize surfaces; secretion of antifungal substances (*i.e.*, cell wall degrading enzymes, antibiotics, killer toxins and volatile compounds); and induction of the plant immune response (Fernandez et al., 2021).

1.4.1 Production of VOCs and killer toxins

Antibiosis (from the Greek words *ἀντι*, and *βίος* that collectively mean “against life”) is generally considered as the property of a biocontrol agent to inhibit the growth or kill another microorganism through the production of diffusible or volatile antibiotic com-

pounds with a variable target spectrum. The most common antibiotics have a natural origin and new molecules have been discovered over the years (Hutchings, Truman, & Wilkinson, 2019).

Antibiotics application on plants is generally not allowed, although some BCAs able to produce these secondary metabolites (Raaijmakers et al., 2002); The opportunity of using antibiotics-producing BCAs is still debated, due to issues related to the possible onset of antibiotic resistance in microbial species that are potentially harmful to humans. Current strategies mainly pursue the selection of BCAs that do not produce antibiotics for their use on aerial parts of the plant, and particularly on edible ones (*e.g*. fruit and vegetables) (Di Francesco et al., 2012). On the other hand, the use of antibiotics-producing BCAs is considered to be more tolerable in the rhizosphere against soilborne pathogens (Raaijmakers et al., 2009). Bacterial species belonging to *Pseudomonas* and *Bacillus* were the most studied for antibiotics production and much scientific literature is available on this topic (Dimkić et al., 2013; Haas & Keel, 2003; Pretorius et al., 2015; Weller, 2007). Other bacterial genera as *Streptomyces*, *Burkholderia*, *Serratia*, *Pantoea*, *Lysobacter* and *Enterobacter* were known as antibiotics producer with antibacterial and antifungal properties (*e.g*. iturin lipopeptides produced by *Bacillus* spp.) and have been tested against postharvest fungal pathogens (Brescia et al., 2021; Kadir et al., 2008; Meziane et al., 2006; Pacios-Michelena et al., 2021; Poppe, Vanhoutte, & Höfte, 2003; Waewthongrak, Pisuchpen, & Leelasuphakul, 2015). Among filamentous fungi, different *Trichoderma* species are known to produce antibiotic compounds active against a wide range of microorganisms (Harman et al., 2004; Verma et al., 2007). Volatile antimicrobials compounds (VOCs) are low-molecular weight molecules and like antibiotics work in a concentration-dependent manner having a cross kingdom spectrum of activity (Schulz-Bohm, Martín-Sánchez, & Garbeva, 2017).

However, for yeasts and bacterial BCAs VOCs production includes several classes of chemicals with antimicrobial activities. The VOCs that are involved in the biocontrol are alcohols, esters, aldehydes, ketones, terpenes and lactones. The majority of the scientific contributions available that relies on VOCs treat the VOCs production as good screening parameters for the selection of postharvest BCAs. Tests made on these BCAs were effective in controlled storage conditions, but these encouraging results were not obtained in the field (Choińska et al., 2020; Song & Ryu, 2013). Production of KTs by yeasts was described first in the sixties (Bevan and Makower 1963) and later on were discovered the ability of each strain to secrete more than one KT (Farkas et al., 2012). Structurally KTs are glycoprotein or proteins with variable spectrum of activity, however the list of yeasts producers of KTs is constantly updated (Giovati et al., 2021).

Well documented is the production of KTs by a killer strain of *S. cerevisiae* able to synthesize several KTs such as K1 and K2 (also called ionophoric KTs), that bind β-1-6-D-glucan, thus able to create ion channels in plasma membrane affecting the electrochemical gradient across the membrane. While a different mechanism was discovered for K28 toxin that binds to α-1-3 linked mannose residues of the cell wall, interacting with plasma membrane specific receptors; than traveling the secretion pathway in reverse, the toxin is able to reach the nucleus arresting at G1/S phase the cell cycle of the host blocking irreversibly DNA synthesis (Schmitt & Breinig, 2002).

Several BCAs were able to produce KTs as *Debaryomyces hansenii* KI2a, *D. hansenii* MI1a and *Wickerhamomyces anomalus* BS91, which were tested against *Monilinia fructigena* and *M. fructicola in vitro* and *in vivo* (Grzegorczyk et al., 2017). Yeast KTs like bacteriocins produced by bacteria give to the producer strain (self-immune to their own) an advantage in terms of natural competition. The genetic information for KTs production in yeasts may be harbored on plasmids, but often it is based on cytoplasmic

inheritance by satellite dsRNA mycovirus, or coded in the genome (Brown, 2011; Fredericks et al., 2021; Schmitt & Breinig, 2006). Furthermore, as main features, it seems that chromosomally encoded KTs have a broad spectrum of activity against many fungal pathogens, like those produced by *Pichia* spp. (Walker, 2011). *Wickerhamomyces anomalus* (formerly *Pichia anomala* and *Hansenula anomala*) synthesizes panomycocin. Panomycocin, is a 49 KDa monomeric glycoprotein with an exo-β-1,3-glucanase activity that binding glucans at cell wall level and by glucans degradation kills the susceptible host. Furthermore, the given efficacy against dermatophytes causal agent like *Candida* spp., panomycocin was proposed for topical application as antifungal (Giovati et al., 2021). Among BCAs another species that bring the killer phenotype (K+) in its repertoire is the yeast-like fungus *Aureobasidum pullulans*, characterized for its antagonistic activity against postharvest fungal pathogens (Moura et al., 2021).

1.4.2 Competition

In the microbial communities, the competition for nutrients and space is crucial since the availability of nutrients, space and other physical resources is generally limited (Ghoul & Mitri, 2016). In the case of biocontrol of postharvest wound pathogens of fruit, wound competence, *i.e*. the ability of the BCA to rapidly colonize fruit wounds, plays a key role in preventing pathogen attack (Jia Liu et al., 2013). Actually, fruits manipulation at harvest and during transportation may cause wounds, where the production of reactive oxygen species (ROS) occurs as a consequence of wounding (Janisiewicz et al., 2016). In fact, BCA must be able to cope with the oxidative stress during apple wound colonization, as prerequisite to exert biocontrol activity (Castoria et al., 2003; Macarisin et al., 2010). The growth of the BCA *Papiliotrema terrestris* LS28 in apple wounds is

affected by ROS and makes it necessary the expression of genes involved in resistance to ROS-generated oxidative stress. This was first suggested by Castoria and colleagues (2003), recently corroborated with a functional genetic approach by knocking out the gene encoding the oxidative stress-responsive transcription factor YAP1; the deleted mutants displayed a significant reduction of biocontrol activity (Castoria et al., 2021). This was remarked also in other species, in fact, the pretreatment of the biocontrol yeast *Candida oleophila* with H_2O_2, considered as mild stress (Jia Liu et al., 2012), can improve the biocontrol activity of the BCA against *P. expansum* and *B. cinerea*.

The BCAs compete with pathogen mainly for carbohydrate and nitrogen given that free forms of these macronutrients are available for microbial growth on fructoplane and on phyllosphere (Spadaro & Droby, 2016). On the other side, within micronutrients competition iron seems to be crucial according to several studies between BCAs (Segarra et al., 2010; Van Loon, 2000). At this purpose yeast BCAs *Metschnikowia pulcherrima* and *M. fructicola* are able to compete for iron through the production of the siderophore pulcherriminic acid crucial for the control of *P. expansum*, *B. cinerea* and *A. alternata* (Saravanakumar et al., 2008). The BCA *Rhodotorula glutinis* is able to sequestrate iron for its own growth in apple wound by the production of Rhodotorulic acid (Calvente, Benuzzi, & de Tosetti, 1999). Furthermore, the biocontrol of *Monilinia laxa* by *A. pullulans* is mediated by siderophore production, that is independent of the presence of the pathogen (Di Francesco & Baraldi, 2021).

A general recap on the competition and combination of BCAs plus macronutrients and micronutrients was done by Sare, Jijakli, and Massart (2021). One of the first study on competition for nutrient and space was carried out on *Papiliotrema terrestris* strain LS28 (Castoria et al., 1997). Later, by SEM observation carried out by Alessandra Di Francesco and colleagues (2017), the competition for nutrient and space by two strains

of *Aureobasidium pullulans* is involved in the biocontrol of *Monilinia laxa* on peaches. Anyway, this mode of action is based on the active metabolism of the BCA and may affect the less competitive pathogen through many ways (Köhl, Kolnaar, & Ravensberg, 2019). The key advantage of this mode of action is that resistant strains in the pathogen population are more difficult to develop.

1.4.3 Induction of resistance

Induction of resistance to biotic or abiotic stresses involves accumulation of structural barriers and elicitation of many biochemical and molecular defense responses in the host, including mitogen activated protein kinase signaling (MAPK), reactive oxygen species generation (ROS), biosynthesis of terpenoid and phytoalexin via phenylpropanoid pathway, octadecanoic pathway, production of phytoalexins and PR-proteins, enhanced accumulation of phenolic compounds, lignification at the infection site and strengthening of host cell wall by formation of glycoproteins, lignin, callose, and other phenolic polymers (Lloyd et al., 2011; Shoresh, Harman, & Mastouri, 2010).

Treatment of peaches with yeast *C. laurentii* and methyl jasmonic acid (MeJA) stimulated the activities of enzymes chitinase, b-1, 3-glucanase, phenylalanine ammonia-lyase (PAL) and peroxidase (POD) in comparison to the application of yeast or MeJA alone (Yao & Tian, 2005) . The treatment reduced the diameter of disease lesions on fruit caused by *M. fructicola* and *P. expansum*. Onset of disease resistance against both pathogens paralleled closely with the increase in chitinase, b-1, 3-glucanase, PAL and POD activity. Extraneous application of microbial antagonist results in differential expression of genes and proteins levels both in host and in the antagonists. DNA microarray analysis of cherry tomato in response to antagonistic yeast *C. laurentii*

revealed the differential expression of genes levels in host tissue (jiang, Zheng, & Chen, 2009). The genes responsible for signal transduction, metabolism, and stress response were upregulated whereas the genes responsible for energy metabolism and photosynthesis were downregulated. Application of yeast *Pichia membranifaciens* on peaches stimulated cellular proteins and antioxidant enzymes activity (Chan et al., 2007). Higher levels of the enzymes, such as catalase (CAT), glutathione peroxidase, methionine sulfoxide reductase peroxiredoxin, and polyphenol oxidase (PPO), protect the host tissues against oxidative damage by *P. expansum* pathogen. Further, *P. membranifaciens* enhanced the activity of pathogenesis-related proteins (PR), such as PR-9, PR-10, GTP-binding and heat shock proteins. Application of antagonistic yeast *R. paludigenum* on mandarins at pre-harvest stage induced defense response by increasing production of defense-related enzymes, including b-1, 3-glucanase, PAL, POD and PPO (Lu et al., 2013; Spadaro et al., 2016). Although, correlation between induction of host defense and inhibition of pathogenic growth has not been completely established, molecular tools can be explored to identify different genes profile implicated in the antagonistic microbes–host–pathogen interactions in the induction of resistance in host.

1.5 Relevance of studying biocontrol mechanism

The management of the most important diseases caused by fungal pathogens is based mainly on the use of synthetic fungicides. Nowadays safer technologies for disease management stir up by the increasing public concerns about their toxic effects on human health and on the environment, are going to develop along with the progressive reduction of available pesticides (Damalas & Koutroubas, 2017; van Lenteren et al., 2018). Among them, biological control is a promising eco-friendly technology.

In practice, biological control is defined as the mitigation of pests and diseases through the use of antagonistic microorganisms named biocontrol agents (BCAs) (Eilenberg, Hajek, & Lomer, 2001). Nevertheless, the limited knowledge about the mechanisms of action of the BCA limiting their use in practical pest management programs. Understanding the molecular bases of biocontrol is a key requirement in order to maximize the efficacy of the BCA treatment. Different tools are available to achieve this knowledge such as genomic and transcriptomic approaches to fully exploit the potential of BCAs and to open new avenues for effective strategies in biocontrol of pathogens (Droby et al., 2016; Freimoser et al., 2019; Jia Liu et al., 2013).

1.6 NGS technique and new advances of fungal BCA study

Next generation sequencing (NGS) techniques can have huge impact on the biocontrol studies through the generation of whole genome sequencing and transcriptomics (RNA sequencing, RNAseq) data allowing comparative genome analysis and gene/protein expression analyses to identify molecular pathways and key genes potentially involved in biocontrol.

In biocontrol yeasts, Hershkovitz and colleagues applied RNAseq to study gene expression changes in the biocontrol agent *Metschnikowia fructicola* during interaction with grapefruit peel tissues and with the mycelium of the postharvest pathogen *Penicillium digitatum*. During interaction with the host, genes involved in oxidative stress, iron and zinc homeostasis, and lipid metabolism were induced, while during interaction with the pathogen genes involved in multidrug transport and amino acid metabolism were induced (Hershkovitz et al., 2013). In another study, Zhang and colleagues applied RNAseq to study the host response to the BCA *Yarrowia lipolytica*,

and found that this BCA induced host resistance through crosstalk between salicylic acid and ethylene/jasmonate pathways (H. Zhang et al., 2017). Rueda-Mejia et al. (2021) performed dual RNA-seq of *A. pullulans* NBB 7.2.1 during co-incubation with *F. oxysporum* NRRL 26381/CL57, and found that ~ 12% of all the *A. pullulans* genes were differentially expressed, with upregulated genes including secreted hydrolases such as glycosylases, esterases, and proteases, and genes encoding enzymes predicted to be involved in the synthesis of secondary metabolites. Conversely, only 80 genes were differentially expressed in *F. oxysporum*, with lipid and carbohydrate metabolism being the most represented Gene Ontology categories. Laur and colleagues performed a tree-way RNAseq during interaction of the BCA *Pseudozyma flocculosa* in the context of its biocontrol activity against *Blumeria graminis* f.sp. *hordei* as it parasitizes *Hordeum vulgare* (Laur et al., 2018). The authors found that *P. flocculosa* uses effectors to use nutrients extracted by *B. graminis* from barley leaves, hence indirectly parasitizes barley in a transient manner; the activity of these *P. flocculosa* effectors is synchronized with the activity of *B. graminis* austorial effectors, and a rapid decline of the photosynthetic machinery of barley. The authors named this mechanism hyperbiotrophy, because the ultimate host target of *P. flocculosa* is the plant, a parasitism that is achieved through the powdery mildew pathogen.

1.6.1 *Papliotrema terrestris* strain LS28

Papiliotrema terrestris is a basidiomycetous yeast belonging to the *Tremellaceae* in the *subphylum* Agaricomycotina. The genus *Papiliotrema* was first proposed in 2002 to when the new species *Papiliotrema bandonii* was added to the Tremellales (Sampaio, Weiß, Gadanho, & Bauer, 2002). *P. terrestris* was described by Crestani et al. (2009) as

ubiquitous in the soil (the name *terrestris*, which means “of the soil” in Latin). The microorganism studied in the present work is strain LS28 of *P. terrestris*, previously reported as *Cryptococcus laurentii* and reclassified by Miccoli et al. (2020); LS28 was isolated from the apple fruit epiphytic microbiota and selected for its ability to counteract fungal pathogens (Lima et al., 1998).

The biocontrol activity of *P. terrestris* LS28 on stored fruits is mainly preventive and is based on its rapid colonization of fruit wounds (wound competence) causing as consequence site exclusion for wound pathogens as *Penicillium* spp. and *Botrytis* spp. (Castoria et al., 2003; Castoria et al., 1997; Castoria et al., 2008; Droby et al., 2016; Lima et al., 1998; Lima et al., 2005). Wound colonization by LS28 is coupled with resistance to reactive oxygen species (ROS: superoxide anion [O2−·] and hydrogen peroxide [H_2O_2]) generated in wounded fruit tissues, as demonstrated through chemical and genetic approaches (Castoria et al., 2003; Castoria et al., 2021; Macarisin et al., 2010; Qin & Tian, 2005; Zhang et al., 2017).

Furthermore, the production of extracellular β-1-3-glucanase that degrade fungal cell wall could play a role in the induction of host defense contributing to its antagonistic activity (Castoria et al., 1997; Janisiewicz & Korsten, 2002)

In the last decade genomic data were for several BCAs, including *P. terrestris*LS28 (Palmieri et al., 2021).

1.6.2 *Penicillium expansum*

Within the genus *Penicillium* are included about 150 recognized species, of which 50 or more are of common occurrence (J. Pitt & Hocking, 2009), and include a group of

anamorphic fungi in the division Ascomycota, ubiquitous in warm and moderate climates. Some species with *Penicillium* as anamorph that produce the sexual stage are classified in the genus *Eupenicillium* and *Talaromyces*; however, dual nomenclature became obsolete. In the present study a transcriptomic approach was carried out including as postharvest pathogen *Penicillium expansum* able to infects a wide range of fruits, including pome and a variety of stone fruits (Jurick Ii et al., 2011).

Some species belonging to the *Penicillium* genera are of economic importance to the food industry because they are used in the production of cheese and meat products or because they are postharvest pathogens that cause food spoilage. One of the main features of the species that belong to the *Penicillium* genera is a strong producer of secondary metabolite (J. C. Frisvad & Filtenborg, 1983; Jens Christian Frisvad & Samson, 2004). In general, these compounds are not essential for the normal growth, development, and reproduction of the organism but may confer important ecological advantages, such as eliminating competing microbial species. Secondary metabolites can also be the source of important bio-active compounds. The antibiotic penicillin, derived from *P. chrysogenum*, is probably the widest used antibiotic produced by a fungus developed for large scale production.

The classification of *Penicillium* before the spreading of molecular barcoding technique (Schoch Conrad et al., 2012) was based on the microscopic morphology; in fact, the genus *Penicillium* is divided in subgenera based on the number and arrangement of phialides, metulae and rami originated by the same stip. Anyway, standard condition for *Penicillium* identification is obtained with growth on Czapek yeast extract agar (CYA) and on malt extract agar (MEA) at 25°C. Some specific microscopic traits (*e.g.* dimension and shape of conidia), and other diagnostic structures beside macroscopic

characters such as the diameter and color of the colony, allows the identification of *Penicillium* spp. in solid with a secondary metabolites profile (Pitt, 2014).

P. *expansum* is also of great concern to fruit processing industries (*e.g*., juicing, baby food, ready-to-eat salads), because is able to produce patulin, a mycotoxin that can contaminate infected fruits and their derived products (McCallum et al. 2002; Wouters and Speijers 1996).

Blue mold of apple fruit caused by *Penicillium expansum* Link. is considered the most important postharvest disease of apples worldwide (Cappellini, Ceponis, & Lightner, 1987). *P*. *expansum* infects apples through wounds, able to growth as a necrotrophic fungus (feeding on dead tissue); infected fruit tissues appear soft and watery, and in a few days a blue-white spore mass develops (Luciano-Rosario, Keller, & Jurick Ii, 2020). Furthermore, despite *P*. *expansum* was defined as wound pathogen may enter also by natural opening such as lenticels, open calyx/sinus, and stem pull areas.

Mature and overripe fruit are more susceptible to infection by *P*. *expansum* than immature fruit (Vilanova, Viñas, et al., 2014). Blue mold is typically controlled by treating harvested fruit with chemical fungicides prior to cold storage. Research efforts aimed to develop biocontrol strategies against blue mold were made during the last thirty year and many commercial products are available (Droby et al., 2016; Janisiewicz & Korsten, 2002; Wisniewski et al., 2016).

Domesticated apple (*Malus* × *domestica* Borkh.) cultivars vary in their quantitative susceptibility to blue mold, cultivars with high levels of resistance to *P*. *expansum* have not been identified (Konstantinou et al., 2011). *Malus sieversii* (Lebed.) M. Roem., a wild apple species native to Central Asia, is one of the ancestral progenitors of the domesticated apple; all the wild ancestors contain the whole genetic variability subjected

to the bottlenecks that result during the process of crop domestication (Spotts, Cervantes, & Mielke, 1999). *M. sieversii* exhibit several different characters respect to the wild apple species that are source of disease resistance in apple scion used in breeding, *i.e.* such as the occurrence of large and tasty fruit within the species (Forsline et al., 2003).

1.6.3 The host fruit: *Malus domestica*

The genus *Malus* belongs to the *Maleae* tribe of the *Amygdaloideae* subfamily of the *Rosaceae* family and comprises many interbreeding species with 25 to 40 taxa within the genus. Unlike other members of the *Rosaceae*, which have haploid chromosome numbers of 7, 8 or 9, members of the *Maleae* have a haploid chromosome number of 17, which appears to have been derived from a genome wide duplication of an ancient x = 9 ancestor and subsequent loss of a chromosome (Velasco et al., 2010). Genetically, *M.* × *domestica* is an allopolyploid exhibiting both monogenic and disomic inheritance from homoeologous chromosomes, but does not exhibit tetrasomic inheritance (Chevreau & Laurens, 1987; Norelli et al., 2017).

M. × *domestica* is derived from at least four progenitor species, including *M. sieversii*, *M. orientalis* Uglitzk., *M. sylvestris* (L.) Mill. and *M. prunifolia* (Wild.) Borkh. (Volk et al., 2015). Molecular analysis of 23 genes across 74 *Malus* sp. accessions identified *M. sieversii* as the primary progenitor of *M.* × *domestica* (Velasco et al., 2010). Analysis of chloroplast DNA, however, suggests that *M.* × *domestica* belongs intermix of species that includes species native to China and Western North America (Volk et al., 2015). Although these reports have established both the importance of *M. sieversii* as a major progenitor of *M.* × *domestica* and the importance of admixture from other species in the

domestication of apple, the ancient introgressions that led to the modern apple remain uncertain.

Being self-incompatible, *Malus* species are usually out-crossing, hence highly heterozygous. The *Malus* genome is known to vary in both size and structure. Among 100 accessions of Malus, which included both *M.* × *domestica* (59 accessions) and *M. sieversii* (14 accessions), genome size was found to vary by approximately 15% with 2C diploid values ranging from 1.44 to 1.72 pg (Korban et al., 2009). Copy-number variation, defined as deletions, duplications or insertions of DNA sequence fragments longer than 50 base pairs in length, have been found to be common in all 17 chromosomes of the *M.* × *domestica* genome (Boocock et al., 2015). Simple sequence repeats (SSRs) within DNA have historically played a major role in the genetic analysis of apple and remain useful because they are co-dominant, highly polymorphic, abundant and reliably reproducible (Liebhard et al., 2002). The development of a whole genome sequence for cv Golden Delicious apple in 2010, and the subsequent development of genotyping arrays based upon that sequence, have greatly aided and advanced genetic analysis of *M.* × *domestica* (Baumgartner et al., 2016; Bianco et al., 2014). Previous evaluation of *M.* × *domestica* derived single nucleotide polymorphism (SNP) markers suggested that such markers derived from the Golden Delicious genome would also be useful for the genetic analysis of *M. sieversii* (Kumar, Raulier, Chagné, & Whitworth, 2014).

In the past two decades, the use of transcriptomic and proteomic analyses of plants, in relation to growth, and development, stress-adaptation, and disease resistance, has increased exponentially (Simsek, Dönmez, & Kaçar, 2017; Tian et al., 2016). These approaches have become more readily available as the number of sequenced plant genomes has increased and sequencing technology has improved and become more cost-efficient. Microarray-based studies of resistance to *P. expansum* in apples have noted the

involvement of several defense-related genes in the resistance response, as well genes involved in detoxifying reactive oxygen species (ROS). These findings were revealed by comparing the response of apple fruit to a pathogen (*P. expansum*) and a non-pathogen (*P. digitatum*) (Vilanova et al., 2014) or by comparing the presence of polyphenolic compounds in "resistant" and "susceptible" commercial cultivars of apple (Ahmadi-Afzadi et al., 2015). The role of the oxi-proteome in response to wounding and infection by *P. expansum* or *P. digitatum* was further documented by Gemma Buron-Moles et al. (2015).

1.7 Functional genomics to identify fungal, yeasts, and bacterial genes important for biocontrol

Omics approach provides a "blast of knowledge" about molecular processes behind the biocontrol activity of BCAs against plant pathogens, giving also an opportunity to prioritize further experiments through the application of functional genetics approaches (*i.e.* targeted mutagenesis, or overexpression analyses) to confirm whether a certain gene/pathway is involved in the biocontrol.

In biocontrol agent yeasts only few functional genetics studies have been performed so far. Mutation and overexpression of the *C. oleophila* β-exoglucanase-encoding gene *EXG1* did not result in different biocontrol activity *in vitro* and *in vivo* against *Penicillium digitatum* compared to the wild type (WT) strain (Yehuda et al., 2003). A following study in *Pichia anomala* revealed that single or double mutants for the exo-β-1,3-glucanase-encoding genes *EXG1* and *EXG2* displayed some reduction in the antagonistic activity of *B. cinerea* on apples compared to the WT when applied at low cellular concentrations and on young apples (Grevesse, Lepoivre, & Jijakli, 2003). Overall, these studies

revealed that the production of exo-β-1,3-glucanases has a minor role in the biocontrol operated by Ascomycetes BCAs, and in certain condition, their contribution might be masked by more relevant modes of action, such as competition for nutrients and space.

Two other studies aimed at underlining the molecular bases of competition for nutrients. Fiori and colleagues reported that a leucine auxotrophic mutant of the biocontrol yeast *P. angusta* was unable to control brown rot lesion caused by *M. fructicola* compared to its parental WT strain; the addition and exogenous L-leucine to the infected wounds restored antagonistic activity in the leucine auxotrophic mutant, suggesting that amino acids utilization by the BCA might be important for nutrients competition (Fiori et al., 2008). In another study, a spontaneous colorless mutant of *Metschnikowia pulcherrima* with a premature stop codon in the transcriptional regulator gene *SNF2* was found to lack pulcherrimin exhibited reduced biocontrol activity against *B. caroliana in vitro* and *in vivo*. The reduced antifungal activity of the pigmentless *M. pulcherrima* cells supports a role for pulcherrimin in the antagonistic phenotype through an uncharacterized interaction with iron (Gore-Lloyd et al., 2019). Of note, pigmentless mutants only showed reduced antifungal activity and still strongly inhibited the growth of filamentous fungi, indicating that biocontrol is the result of a complex interaction that involves the coexistence of several different mechanisms.

There are two other studies that characterized the role of transcription factors in biocontrol activity through their involvement in resistance to abiotic stresses associated with antagonistic traits. Sui and colleagues mutated the transcription factor RML1 in *C. oleophila* and found that *rml1*Δ mutants displayed reduced resistance to heat stress (40°C), salt stress, and oxidative stress induced by hydrogen peroxide *in vitro*, and reduced ability of wound colonization and antagonistic activity against *B. cinerea in vivo* in kiwi fruit (Y. Sui et al., 2020). In another study, Castoria and colleagues mutated the

Papiliotrema terrestris transcription factors *RIM101* and *YAP1* and found that *in vitro*, *yap1*Δ mutant displayed increased sensitivity to oxidative, genotoxic and nitrosative stresses, while the *rim101*Δ mutant was unable to grow at alkaline pH and it was sensitive to cell wall-stressors. *In vivo*, both *yap1*Δ and *rim101*Δ mutants displayed a reduced ability of apple wound colonization, but only the *yap1*Δ displayed reduced antagonistic activity against *P. expansum* and *Monilinia fructigena* (Castoria et al., 2021). Both these studies demonstrated that resistance to abiotic stresses by the BCAs, in particular to oxidative stress, is important to outcompete the pathogen through the rapid and timely colonization of wounded fruit tissues (wound competence) that are characterized by high level of reactive oxygen species. These molecular studies confirmed previous biochemical and phenotypical studies (Castoria et al., 2003; Zhang et al., 2017).

2 Research objectives

- Analysis of the transcriptomic profile of *Papiliotrema terrestris* LS28, *Penicillium expansum* 7015 and *Malus domestica* cv Golden Delicious during their dual and tritrophic interaction.
- Understanding at molecular level the mechanisms of action underlying the biocontrol activity of *P. terrestris* LS28 through the generation of targeted mutants for genes that were highly expressed in the RNAseq analysis and extensive *in vitro* and *in vivo* phenotypic characterization.

3 Materials and methods

3.1 Microrganisms used in the present study and experimental set up for RNA sequencing

Papiliotrema terrestris LS28, was used as Biocontrol Agent (BCA). In all the biocontrol experiments, *P. terrestris* LS28 cells were prepared by growing the yeast overnight in 20 mL of YPD (Yeast extract 10 g L^{-1}; Peptone 20 g L^{-1}; Dextrose g L^{-1}) at 28°C 130 rpm, then the cells were collected, washed twice with sterilized distilled water, and adjusted at the required cellular concentration (as indicated in the relative paragraphs).

Penicillium expansum strain 7015 used in this study was isolated from the Plant Pathology Laboratory (Department of Agricultural, Environmental and Food Sciences, University of Molise, Campobasso). In all the biocontrol experiments, conidia were collected from a two-week-old PDA (Potatoes Dextrose Agar) plate, suspended in sterilized distilled water, and adjusted at the required concentration (as indicated in the relative paragraphs).

Initially, several biocontrol experiments were performed with the aim to identify the most suitable conditions for RNAseq analysis (*i.e.* number of *P. terrestris* cells, number of *P. expansum* conidia, and the time point for collecting samples for RNA sequencing).

To this aim, several *P. terrestris* cellular suspensions (5 x 10^6 CFU mL^{-1}, 1 x 10^7 CFU mL^{-1}, 1 x 10^8 CFU mL^{-1}) and *P. expansum* conidial suspensions (2 x 10^4 conidia mL^{-1}) were prepared.

Apples variety Golden Delicious were purchased from local market. The apples were washed with commercial sodium hypochlorite at 1% for 3 minutes, and rinsed two times distilled water. Once dried, on each apple 4 wounds of 0.2 mm of depth and 2 mm of diameter were made using a sterilized corkborer. For each wound, 30 μL of cellular

suspensions of the BCA prepared as above were inoculated; after about 30 minutes, 15 μL of conidial suspensions of *P. expansum* prepared as above were inoculated. Apples were incubated at 24°C, and subjected to microscopic observation every 6 hours.

3.2 Experimental settings

The biocontrol assays for RNA extraction and sequencing were carried out as reported above. The BCA concentration used was 1 x 10^7 CFU mL^{-1} while the pathogen concentration was 2 x 10^4 conidia mL^{-1} of suspension. Both the BCA and the pathogen were applied alone and in combination within artificial apple wounds, and samples for RNA extraction were collected after 36 h of incubation. Three apples each containing for wounds per each treatment were used. *P. terrestris* and *P. expansum* grown for 24 h *in vitro* in liquid YPD, and uninoculated apple wounds kept for 24 h, were used as controls. Collected sample were stored in -80° C until RNA extraction. A total of 16 samples were prepared, which included three biological replicates for each sample to be analyzed, with the exception of the uninoculated apple and the *P. terrestris* in liquid culture, which had two biological replicates.

3.3 Sequencing and bioinformatics analysis

3.3.1 RNA extraction and sequencing

Total RNA from controls and inoculated apple samples was isolated using a TRIzol protocol according to the manufacturer's instructions (Rio et al., 2010). RNA was treated with TURBO DNase according to manufacturer's instructions, and resuspended in 50 µl of nuclease free water. RNA quality was assessed spectrophotometrically using a Nanodrop. RNA sequencing was performed at Genomix4life (Salerno, Italy) using 150 bp paired-end sequencing with Illumina Hiseq 2500. RNA-seq libraries were prepared using the TruSeq mRNA PCR-free library kits following manufacturer's instructions.

3.3.2 RNAseq analysis

Initial data analysis was carried out by Sequentia Biotech SL (Barcelona). Reads obtained were subjected to quality control and bioinformatic analysis. The reads were trimmed using the software BBDuk v35.85 (http://jgi.doe.gov/data-and-tools/bb-tools/) and then were mapped against the genome assemblies with the program STAR (Dobin et al., 2013). The *P. terrestris* reference genome used was recently published by our group (Palmieri et al., 2021) (accession GCA_019857685.1). The *M. domestica* reference genome was the GDDH13v1.1 (Phytozome genome ID: 491; NCBI taxonomy ID: 3750) (Daccord et al., 2017). The *P. expansum* reference genome was that of the strain CMP-1 available on the Ensembl database (GCA_000769755). For the *P.*

expansum CMP-1 strain, the Pannzer2 pipeline was used to generate a functional Gene Ontology annotation because it was not available (Törönen, Medlar, & Holm, 2018).

After the mapping step gene expression values (counts) for each sample were obtained with FeatureCounts (Liao, Smyth, & Shi, 2014).

To identify differentially expressed genes (DEGs), the software HTSFilter (Rau et al., 2013) was first used to remove the lowly expressed genes. The quality of the biological replicates was analyzed by principal component analysis (PCA). Reads obtained from samples relative to the dual or tritrophic interaction were mapped against the respective control condition, for a total of 7 comparisons listed below. In particular, DEGs of *P. terrestris* LS28 were identified by comparing:

1) *Apple* + *P. terrestris* LS28 *versus P. terrestris* LS28 in YPD
2) *Apple* + *P. terrestris* LS28 + *P. expansum* 7015 *versus P. terrestris* LS28 in YPD

DEGs of *P. expansum* 7015 were identified by comparing:

3) *Apple* + *P. expansum* 7015 *versus P. expansum* 7015 in YPD
4) *Apple* + *P. terrestris* LS28 + *P. expansum* 7015 *versus P. expansum* 7015 in YPD

DEGs of the host *M. domestica* were identified by comparing:

5) Apple + *P. terretris* LS28 *versus* uninoculated apple
6) Apple + *P. expansum* 7015 *versus* uninoculated apple
7) Apple + *P. terrestris* LS28 + *P. expansum* 7015 *versus* uninoculated apple

The software EdgeR (Robinson, McCarthy, & Smyth, 2010) was used to identify DEGs, defined as genes/transcripts with a False Discovery Rate (FDR) < 0.05.

From the comparisons listed above, as a first step were selected all the DEGs (FDR <0.05) with log2FC (fold change) +/- 1. Subsequently, results within each reference organisms were compared with each other using the package VennDiagram (H. Chen & Boutros, 2011) resulting in additional datasets that includes: for *P. terrestris* LS28, genes in common in the comparison 1 and 2 reported above; for *P. expansum* 7015, genes in common in the comparison 3 and 4 reported above; for the host *M. domestica*, genes in common in the comparison 5, 6 and 7 reported above. As a last step, a further filtering step was carried out to select as DEGs genes having a log2FC +/- 2. DEGs of *P. terrestris* and *P. expansum* were subjected to BLASTp against *S. cerevisiae* to identify yeast ortholog genes and infer their function in the model yeast database (*Saccharomyces* Genome Database, SGD), while DEGs of *M. domestica* were subjected to BLASTp against *Arabidopsis thaliana*. Furthermore, all obtained *P. terrestris* and *P. expansum* DEGs were subjected to GO functional annotation using Pannzer2 pipeline (Törönen, Medlar, & Holm, 2018) to assign GO accessions and GO terms. For *M. domestica* the GO accessions of the DEGs were obtained from the Rosaceae Database Genome (GDR) database (Jung et al., 2019). GO accessions obtained were used for GO enrichment analysis using the topGO package pipeline according to the manual vignette (Alexa A. & Rahnenfuhrer J., 2016) and the p-value was converted into enrichment score applying the negative logarithm function. As further analysis, the GO accessions were also annotated using the KEGG orthology database (Kanehisa et al., 2016), with pathway enrichment analysis finalized using Clusterprofiler (Yu et al., 2012). Dataset manipulation and relative figures were created using Rstudio (version 3.6.2 and version 4); KEGG pathway visualization was carried out using the pathview package (Luo & Brouwer, 2013). The flow charts were created with BioRender.

3.4 Generation of deletion mutants

Among the most upregulated DEGs of the BCA *P. terrestris* LS28, eight highly expressed genes of the BCA *P. terrestris* LS28, four from the common dataset (Apple + *P. terrestris* LS28 versus *P. terrestris* LS28 in YPD, and Apple + *P. terrestris* LS28 + *P. expansum* 7015 versus *P. terrestris* LS28 in YPD), and five from the dataset "Apple + *P. terrestris* LS28 + *P. expansum* 7015 versus *P. terrestris* LS28 in YPD" were selected for targeted gene replacement using *Agrobacterium*-mediated transformation (AMT) and electroporation as previously reported (Castoria et al., 2021). The previously developed *P. terrestris* hygromycin (*HYG*) resistance cassette was used as a dominant marker (Castoria et al., 2021). Plasmids for targeted mutagenesis were obtained through recombination in *S. cerevisiae* using the plasmid pGI3 (Ianiri et al., 2016).

3.4.1 Screening of *S. cerevisiae* transformants – DNA extraction and primers used

Correct recombination in *S. cerevisiae* was confirmed with a colony PCR using primers JOHE 43279 coupled with JOHE46630 for the junction at the right border of the plasmid, and JOHE 43280 coupled with JOHE46631 for the junction at the left border, and positive strains with correctly recombined plasmids were processed for genomic DNA extraction.

Extracted DNA containing also recombining plasmids was electroporated into *A. tumefaciens* strain EHA105 (25 μF of capacity, 2.0 kV and 200 Ω resistance) for AMT transformation of LS28, or PCR amplification of the gene deletion constructs for

electroporation of LS28 following recently-developed protocol (Castoria et al., 2021). A scheme illustrated in figure 30 explains the procedure followed.

Electroporation of LS28 was carried out using a Bio-Rad Gene Pulser II Electroporator. An overnight culture of LS28 (at 30°C), was diluted with liquid YPD to an optical density (OD) of 0.2 and leave to grow for additional 4/5 hour until the OD reach 1. At this point the cells were collected by centrifugation (4000 rmp, for 5 min) and washed twice with EB buffer as reported by Castoria et al. (2021). After the washing steps 45 μL of cells suspension were aliquoted in pre-cooled cuvette (2 mm-gap) and electroporated with the appropriate quantity of DNA (2 μL at 370 ng μL^{-1}) with the following parameters: 0.45 kV, 125 μF capacity, and 600 Ω resistance. Transformed cells were suspended in 1 ml of YPD and recovered in shaking culture at 30°C for 4 h or ON before plating the appropriate volume on selective agar medium containing 200 mg/ml of Hygromycin.

The transformation of LS28 by AMT was obtained following the same procedure reported by Castoria et al. (2021).

Transformants of LS28 obtained were screened for correct event of homologous recombination by PCR using primers specific (see “Check the integration of the deletion cassette at target site in LS28” in table 20) for the *HYG* marker (figure 39, A, in red) in combination with external primers (figure 39, A, in blue) designed outside the region involved in the recombination event. A further PCR was carried out using external primers to assess the expected different size of the amplicons obtained in the WT strain and in the targeted mutants (figure 39, B). All the primers used in this study are reported in table 20.

3.4.2 Phenotypic characterization *in vitro*

Fresh cultures of the WT LS28 and the obtained deletion mutants maintained in rich media YPD were resuspended in sterile distilled water at 1 x 10^8 CFU mL^{-1}, subjected to ten-fold serial dilutions and spotted in a volume of 4 μL on agar plates containing different stressors. Stressors tested include NaCl (1,3 M), Congo red (1% w/v), lithium chloride (600 mM), sodium nitrite (250 mM), menadione (4,5 mM, dissolved first in DMSO), H_2O_2 (5 mM), cycloheximide (20 ppm), hydroxyurea (15 mM), dithiothreitol (20 mM).

Furthermore, the WT LS28 and the obtained deletion mutants were tested for their ability to grow on apple-mimicking media and minimal media (KH_2PO_4 1 g L^{-1}; KCl 0.5 g L^{-1}; $MgSO_4$ 0.5 g L^{-1}; D-glucose 15 g L^{-1}; 16 g L^{-1} of agar) in the absence and in the presence of 25 mM of inorganic nitrogen sources (Urea, ammonium sulphate, ammonium nitrate, and sodium nitrate), and four amino acids. The morphology of the colony was also examined (data not shown).

Last, RNAseq analysis revealed a predicted important role for response to and utilization of nitrogen sources for apple wounds colonization by *P. terrestris* LS28. Therefore, LS28 WT was tested for its ability to grow in minimal media (with D-glucose 1.5 % as carbon source) in presence of amino acids as organic nitrogen sources (final concentration 10 mM and 1.5 mM); inorganic nitrogen sources were also included, and minimal media without any source of nitrogen was used as control. The complete list of nitrogen compounds used is listed in table 22. The screen was carried out in 96-well plate (BRAND 96 U Bottom) and the growth kinetics recorded with an Infinite 200 PRO Tecan instrument, applying time point interval for measurements every 30 min until 48 hours, at 600 nm of wavelength, performing 800 sec of orbital shaking before the measurement.

The same parameters were used for monitoring the growth of the deletion mutants in the presence of amino acids as nitrogen source (1.5 mM, see table 22). The optical density (OD) was recorded at two time point, 24 and 48 hours post inoculation (data of 48 hours were not shown.

3.4.3 Phenotypic characterization *in vivo*

For in vivo phenotypic analysis biocontrol assays, apples (cv Golden Delicious) were purchased from the local market, then were washed with commercial sodium hypochlorite at 1% (w/v) for 3 minutes and rinsed two times with sterile distilled water for 3 minutes. On each apple, 4 wounds of 0.2 mm of depth and 2 mm of diameter were made with a sterile corkborer. Four apples, each representing a technical replicate, were used for each strain tested.

P. terrestris WT and deletion mutants were grown overnight in YPD, washed twice with water to remove residual nutrients, and cell suspensions were adjusted to 1 x 10^8 CFU mL^{-1}, 1 x 10^7 CFU mL^{-1} (data not showed), and 5 x 10^6 CFU mL^{-1}. For *P. expansum* strain 7015, conidia were collected from a of two-weeks-old PDA (Potatoes Dextrose Agar) plate and a suspended in sterile distilled water at a concentration of 2 x 10^4 conidia mL^{-1}.

In each wound were inoculated 30 μL of the yeasts cellular suspensions were inoculated, and after about 30 min/1 h, 15 μL of the *P. expansum* conidial suspension were added. At least three biological replicates were carried out. The percentage of infected wounds was recorded at 7, 9 and 12 days of incubation. The data of the biocontrol experiment were collected and analyzed for their representation and statistical meaning using

Graphpad prism version 8, applying one way ANOVA. Multiple comparison was carried out using Tuckey's test.

4 Results

4.1 Experimental set up pivotal for RNAseq analysis during dual and tritrophic interactions

As a first step, several pivotal biocontrol assays were carried out to determine the correct conditions for collecting samples for RNAseq analysis. Because in antagonistic assays usually one organism (either the BCA, or the fungus) rapidly outcompetes the other hindering its development, the rationale of these preliminary experiments was to find the conditions that allow the concurrent presence of the BCA *P. terrestris* and the phytopathogen *P. expansum* during their active interaction *in vivo* within artificial apple wounds. To this aim, different cellular concentrations of *P. terrestris* and *P. expansum* were inoculated within apple wounds, and their development monitored at a microscope during the first 3 days of incubation.

In the figure 2 are reported microphotographs of *P. expansum* inoculated alone and with the BCA *P. terrestris* LS28 at 6, 12, 18, 24 and 36 hours post inoculation (h.p.i.). At 6 and 12 h.p.i., both non-germinated conidia and yeast cells are clearly visible (figure 2, panels A – D). At 18 h.p.i., *P. expansum* conidia start germinating both in the presence and absence of the BCA *P. terrestris* LS28, although in this case their number was higher with a germ tube more developed (figure 2, panels I – L). At 24 h.p.i., while in the absence of the BCA there was a high number of *P. expansum* hyphae invading the apple tissues, in the presence of the BCA *P. terrestris* LS28, *P. expansum* hyphal growth was reduced (note a non-branched hypha in figure 2, O). At 36 h.p.i. branched *P. expansum* hyphae and the BCA *P. terrestris* LS28 coexisted within the apple tissue, furthermore the BCA at this time point colonize the niche competing for space with *Penicillium expansum* (figure 2, V). Of note, at 36 hpi *P. expansum* hyphae are less represented in the micro-niche when in the presence of the BCA (figure 2, U and V).

Based on this observations, 36 h of incubation was the optimal condition to collect samples for RNAseq analysis; the presence of the two microrganisms *P. terrestris* and *P. expansum* was also assessed by ITS sequencing (data not shown). At the same time, samples were collected also for *P. terrestris* and *P. expansum* inoculated alone in apple wounds. For *in vitro* liquid cultures, and for the uninoculated apple, sample for RNA extraction were collected at 24 h of incubation.

4.2 RNAseq analysis during dual and tritrophic interactions

For all the conditions three biological replicates were prepared, with the exception of uninoculated apple and *P. terrestris* grown *in vitro* that had two biological replicates. A total of 16 RNA samples were collected (figure 3). Reads were trimmed and mapped against the reference genomes using STAR (see Materials and Methods for details), and the results are displayed in table 1. The number of mapped RNA reads against the respective reference genomes in the dual (*i.e. P. terrestris* in wounded apple tissues, or *P. expansum* in wounded apple tissues) and tritrophic (*i.e. P. terrestris* and *P. expansum* in wounded apple tissues) interactions reflected the amount of each organism present in the withdrawn and processed samples that were used for RNA extraction; as expected, this value is about 70% or higher for *M. domestica*, and it is approximatively ~10% for both the biocontrol agent and the fungus in the dual interactions, and ~15% for the biocontrol agent and ~2% for the fungus in the tritrophic interaction. Only for the sample *P. expansum* grown *in vitro* (replicate #1) we found that only 50% of the reads mapped against the genome, likely indicating a contamination; this sample was then excluded.

After the mapping step, for all data FeatureCounts was used to quantify the mapped reads in order to obtain the gene expression values for each sample. Before statistical analysis to identify differentially expressed genes (DEGs), lowly expressed genes were removed with HTSFilter package and the Trimmed Means of M-values (TMM) normalization method. Subsequently, the quality of the processed samples was determined with the Principal Component Analysis, and statistical analysis to identify DEGs was carried out using the software EdgeR. For *P. terrestris* and *P. expansum*, DEGs with FDR <0.05 and log2FC +/-1 obtained from the first comparisons (*P. terrestris* or *P. expansum* inoculated alone in apple wounds versus *P. terrestris* or *P. expansum* grown *in vitro*; and *P. terrestris* co-inoculated with *P. expansum* in apple wounds *versus P. terrestris* or *P. expansum* grown in vitro) were further compared to each other to identify *P. terrestris* or *P. expansum* DEGs in common to both analyzed datasets (reported as common dataset), resulting in the 3 datasets for both upregulated and downregulated genes. A further filter was applied to select DEGs with log2FC +/- 2 (figure 4). Last, the function of the differentially-expressed genes identified was assigned through comparisons with the model organism *S. cerevisiae*, gene annotation, gene ontology (GO) and KEGG analysis as described in details in materials and methods. An illustration of the main steps common to all datasets is reported in figure 3. The procedures followed to analyze the data relative to the host will be discussed in more details in paragraph 4.5.

4.3 Analysis of *P. terrestris* transcriptomic changes during dual and tritrophic interaction

Principal Component Analysis (PCA) of *P. terrestris* LS28 datasets revealed three distinct groups according to the experimental design (figure 5). Complete lists of all DEGs of LS28 with log2FC +/-1 are reported in the supplemental material (File S1 and S2, upregulated and downregulated DEGs respectively) and a resuming graph bar is represented in figure 6. Overall, there were more upregulated DEGs compared to downregulated DEGs, with the highest number of DEGs found in the common condition between dual and tritrophic interactions.

Moreover, a further filtering for log2FC +/- 2 was applied, obtaining 1) 135 genes upregulated by *P. terrestris* LS28 when inoculated in artificial apple wounds alone; 2) 43 genes upregulated by *P. terrestris* LS28 in artificial apple wounds only in the presence of *P. expansum*; and 3) 624 genes commonly upregulated by *P. terrestris* LS28 in the two aforementioned conditions (figure 7, A). For the common dataset having two values of logFC, the sorting and cut-off for expression values was done using the dataset of the co-inoculated condition (LS28+ *P. expansum*).

With fold change cut off of log2FC +/- 2 downregulated DEGs includes: 1) 50 genes expressed only during the dual interaction with apple; 2) 16 genes during the interaction with the fungal pathogen and 3) 189 genes shared among the dual and tritrophic interaction (figure 7, C).

4.3.1 DEGs of *P. terrestris* during the interaction with the host (*M. domestica*)

The 135 upregulated DEGs represent genes expressed by *P. terrestris in vivo* without the pathogen (figure 7, A). GO analysis revealed that the most represented and enriched biological process terms are transmembrane transport, and carbohydrate metabolic process and transport, followed by metabolic processes; oxidation-reduction process includes a high number of genes, although it is characterized by a low enrichment score (figure 7, B). Molecular function annotation confirms that during the BCA colonization the metabolism of carbohydrate is significantly enriched (figure 8) by the highly represented membrane transporter encoding genes. At the same time genes that encodes for enzymes of tricarboxylic acid cycle were highlighted by cellular component enrichment, revealing that yeast requiring energy for growth in this phase (cellular component annotation and enrichment in figure 9).

The most expressed *P. terrestris* gene of this group is g5076.t1 (log2FC = 7.79), which is the *S. cerevisiae* ortholog *PHO89* that mediates acquisition of inorganic phosphate coupled with sodium ion transport (Pattison-Granberg & Persson, 2000). The second gene, g4427.t1, has no orthologs in *S. cerevisiae*, it is annotated as involved in carbohydrate metabolic process, and encodes for a glycoside hydrolase predicted to be secreted due to the presence of a signal peptide (the genes described in this condition are listed in table 2. The complete list is available in the supplemental file S1).

Other upregulated DEGs includes the permeases Seo1 and Tna1 responsible for allantoate transporter and nicotinic acid uptake, respectively, the phosphatidyl glycerol phospholipase C Pgc1, the transaldolase b Tal1 involved in the non-oxidative pentose

phosphate pathway, and the Sfc1 transporter involved in the carrier system of succinate-fumarate at mitochondrion level. Moreover, other upregulated DEGs include the ferric reductase Fre3 and the multicopper oxidase Fet3 involved in iron metabolism, the mitochondrial peroxiredoxin Prx1 involved in oxidative stress response and redox homeostasis, and several transporters of nutrients, including the glycerol proton symporter Stl1, the allontoate permease Dal5, and an uncharacterized zinc transporter. This group include only one transcription factor that is encoded by g7108.t1 (log2FC =2.99) and it is annotated as transcription factor RfeD with an unknown function. GO analysis count 47 DEGs involved in oxidation reduction process with many genes having an overlapping functional annotation, with other biological process already described. To be mention is g3163.t1 which had orthologs *PGC1* in *S. cerevisiae* that encodes for phosphatidyl glycerol phospholipase C that regulates phosphatidylglycerol accumulation.

KEGG analysis of the DEGs upregulated expressed only during apple colonization shows enrichment of the sulfur metabolism, amino acids (valine, leucine and isoleucine) degradation, propanoate metabolism, and biosynthesis of antibiotics. As note, the last is a general term automatically assigned by the program and the genes that were included in this group (table 7) are not involved in antibiotics biosynthesis (table 6).

50 downregulated DEGs were found for *P. terrestris* inoculated *in vivo* without the pathogen (figure 7, C). The more enriched GO group was inorganic ion transport, which includes 4 genes of which only 2 were annotated: g3413.t1 and g5416.t1 having *AMF1* ortholog in *S. cerevisisae* encoding for ammonium transporter. The most downregulated genes, g5931.t1 and g6425.t1, did not have an ortholog in *S. cerevisiae* neither

functionally annotated, and therefore their role is unknown. Of note, a large number of genes within this group did not have a *S. cerevisiae* ortholog.

4.3.2 Common DEGs of *P. terrestris* LS28 in dual and tritrophic interaction

Common DEGs analyzed includes 624 genes (figure 7, A and table 3) that are upregulated by *P. terrestris* LS28 in both conditions regardless the presence of the fungal pathogen, hence they are important for wounded apple tissues colonization and outcompete *P. expansum*. In this group the most represented and enriched GO biological process categories are transmembrane transport and oxidation-reduction process (figure. 7, B), as confirmed also by the molecular function and cellular component enrichment analysis (figure 8 and 9); in particular molecular function reveal as the most enriched terms genes involved in “vitamin B binding” and “pyridoxal phosphate binding” (figure 8). These molecular function terms include different class of genes that encodes for protein involved in aminoacidic metabolism, transcriptional regulators, cofactors etc.

The most expressed genes, g3033.t1 and g4389.t1, encode both for uncharacterized proteins with no ortholog in *S. cerevisiae* and without GO annotation. Further analyses revealed that g3033 has a NAD(P)-binding Rossmann-fold domain, while g4389 has several transmembrane domains. Notably, the common group includes also 213 upregulated genes encoding proteins of unknown function. GO analysis reveals that transmembrane transport is the most significant enriched process with 186 genes encoding for transporters of the MFS and ABC families. Highly expressed transporters include *S. cerevisiae* orthologs *OPT1* and *PUT4* encoding for oligopeptide transport and

proline permease, respectively. Note that two *PUT4* orthologs of *S. cerevisiae* were found as DEGs in LS28, g1105.t1 and g1990.t1, and their phylogenetic relation is reported in figure 48. Other highly expressed transporters belonging to the *permease* class are Thi73 involved carboxylic acid uptake, the maltose transporters Mal31 and Mal11, the uridine permease Fui1, *GAP1* regulated by nitrogen sources in *S. cerevisiae* and acting as general aminoacidic transporter, the ammonium transporters Amf1 (six orthologs detected as upregulated, g2090.t1, g840.t1, g7316.t1, g1871.t1, g3245.t1, and g4947.t1) and Mep2, *DIP5* mediating transport of L-glutamate and L-aspartate and also dicarboxylic acid, the ornithine transporter Ort1 involved in arginine biosynthesis, Ste6 that is required for the export of pheromone factor, and several others including a high number of transporters for allantoin (*DUR* and *DAL* genes), which can be used as an alternative source of carbon and nitrogen. The majority of the genes listed above are involved in nitrogen utilization in *S. cerevisiae* and are under the of the nitrogen catabolite repression (NCR) system.

The second one most enriched term "polyamine transport" includes 13 genes that corresponds to the *S. cerevisiae* orthologs *FLR1*, *AGP2*, *TPO3* and *DUR3*. The gene g1860.t1 is a orthologs of *FLR1*, a fluconazole resistance gene; in *S. cerevisiae* its expression is induced after mycotoxin patulin treatment, and in case of excess of amino acids this family of protein may regulate the efflux of amino acid across the membrane, avoiding toxic concentration (Iwahashi et al., 2006); g.5890.t1 is the *AGP2* ortholog in *S. cerevisiae* and it encodes for a polyamine transporter. *TPO3* is a polyamine transporter of the Drug:H+ Antiporter-1 (DHA1) family protein. The polyamine transporter *DUR3* is regulate by NCR and is induced by allophanate, the last intermediate product of the allantoin degradation; furthermore, the null mutant in *S. cerevisiae* is highly sensitive to

chemical cycloheximide and amitrole, unable in the uptake of urea and polyamines (spermidine, putrescine) as nitrogen sources.

Oxidation reduction process is a highly represented GO term with 182 DEGs, although it is not highly but enriched. This group include oxidoreductases and dehydrogenases that are predicted to play a role in stress response. In particular, we found g3330.t1 that encodes for a nucleoside di-phosphate epimerase, g.6692.t1 encoding for $NADP^+$-dependent serine dehydrogenase and carbonyl reductase, the bifunctional triacylglycerol lipase Ayr1 involved in the biosynthesis of phosphatidic acid (g5806.t1), the glyoxylate reductase Gor1 involved in metabolism of organic carbon, *ENV9* involved in vacuolar function, *GAL1* and *GAL7* involved in galactose metabolism, the amidase-encoding gene *AMD2*, g7494.t1 encoding a nitrite reductase, g4212.t1 encoding a putative zinc-binding oxidoreductase ToxD predicted to be involved in involved in copper metabolism, several uncharacterized short-chain dehydrogenases, the Major exo-1,3-beta-glucanase of the cell wall Exg1. Enzymes of the energetics metabolism are Icl1 (encoded by g137.t1) and Uga2 (encoded by g1107.t1) involved in the Krebs cycle.

Other genes with an important redox function according to GO analysis are *KIN3* (1870.t1) and *FOX2* (g6407.t1) involved in in beta oxidation of fatty acid, and *SER3* (g258.t1) involved in serine and glycine biosynthesis.

The enriched GO terms, such as “glutamine family amino acid metabolic process” includes orthologs of *S. cerevisiae* genes (*DIP5*, *UGA2*, *ASP3*-4, *FEN2*, *URA2*, *SOR1*, *OXP1* and *AGP2*) and ten that are uncharacterized. More in deep, among these uncharacterized g1179.t1 is a transcription factor and was the highest expressed; g2747.t1 encodes for a proline dehydrogenase that is the ortholog of CNAG_02048 in *Cryptococcus neoformans* var. grubii strain H99; g3457.t1 corresponds to *C. neoformans*

CNAG_07736 and it encodes for endo-1,3-alpha-glucosidase; g4161.t1 is a hypothetical protein, and g6404.t1 is likely a glutamine synthetase.

KEGG analysis revealed that the most enriched metabolic processes are “phenylalanine metabolism”, “arginine and proline metabolism”, “styrene degradation” and “glycine serine and threonine metabolism” (figure 11).

Downregulated DEGs of the common dataset includes 189 genes, with the most enriched GO processes being “response to abiotic stimulus”, which includes 13 annotated genes (*CPR6*, *MAD2*, *SGT2*, *YDJ1*, *MIC19*, *GLO1*, *SOD1*, *SBA1*, *RAS1*, *OPI10*, *CAB3*, *THI4*, *CDC37*) and 2 uncharacterized, and “protein targeting”, which includes 11 annotated genes (*SGT2*, *YDJ1*, *HSP10*, *SAM50*, *SBA1*, *MGE1*, *RAS1*, *PAM17*, *PDX1*, *LAT1* and *SPC3*) and 3 genes uncharacterized. The most downregulated genes of this group are g6702.t1 (log2FC = -9 in both datasets), which is the Thi4 ortholog involved in the thiamine biosynthesis, a uncharacterized multicopper oxidase type 1 (log2FC = -4 and -8). Following the sorting of the most repressed genes there are two uncharacterized genes (g1838.t1 and g6821.t1). Within the most downregulated DEGs there is g3806.t1 ortholog of *URE2* in *S. cerevisiae*, that is characterized by the Glutathione S-transferase domain, and involved in nitrogen regulation. The gene g1285.t1 ortholog of *RGD1* of *S. cerevisiae* is a GTPase activating protein involved in the polarization of the microtubules at cytoskeleton level in yeast. The complete lists of downregulated genes are available as supplemental file (see file S2).

4.3.3 DEGs uniquely expressed by *P. terrestris* LS28 when inoculated with *P. expansum* in apple wounds

The last group includes 43 upregulated DEGs expressed by *P. terrestris in vivo* in the presence of the pathogen *P. expansum* (figure 7, B). Intriguingly, the majority of genes belonging to this group had no orthologs genes in *S. cerevisiae*, annotation and GO classification. The most expressed genes, g3655.t1 and g2837.t1, encode for uncharacterized proteins: The gene g3655.t1 has a N-terminal signal peptide and hence is predicted to be secreted, while g2837.t1 contains a HYR domain, which generally is involved in cell adhesion (Callebaut et al., 2000). The third most expressed genes, g142.t1, it was annotated as a glutathione S-transferase family, while g1861.t1 is predicted to have a F-box domain.

GO analysis found “chemotaxis” as the most enriched group. This group includes four genes, the serine/threonine protein kinase Kin3, the kinesin-related motor protein Kin2 involved in mitotic spindle positioning and in cell cycle, an uncharacterized ras guanine nucleotide exchange factor (g2995.t1), and an unknown gene (file S1). These genes are in common to several other identified GO group reported in figure 8 (file S1). This gene according to the GO seems to help the perceive signal came from the fungal pathogen, indeed another DEGs (not listed in the table 5 extract) encode for a Ras protein that are involved in signal transduction and microtubule organization.

KEGG analysis in this case did not provide significant results due to the low number of genes.

Downregulated DEGs include only 16 genes. As expected, GO analysis did not identified highly enriched group, with “regulation of protein phosphorylation” being the most

represented term with 2 genes, the ortholog of chitin deacetylase Cda1, and an uncharacterized beta-glucosidase. The most downregulated gene of this group is an ortholog of the *ATO2* gene, which encodes a putative transmembrane protein involved in export of ammonia.

4.4 Analysis of *Penicillium expansum* transciptomic changes during dual and tritrophic interaction

Principal Component Analysis (figure 12) revealed three distinct groups according to the experimental design. DEGs were obtained as illustrated in figure 4 and described in paragraph 3.3.2.

Similar to *P. terrestris*, also for *P. expansum* there were more upregulated DEGs compared to downregulated DEGs, with the highest number of DEGs found in the common condition between dual and tritrophic interactions. An overview about the starting dataset (with log2FC +/-1 cut-off, supplemental file S3 for upregulated and S4 for downregulated) used to get DEGs that are the starting point of the analysis even for *P expansum* are illustrated in figure 13).

Then refining the filter for log2FC (+/- 2) for the final analysis, were obtained 1) 60 genes upregulated by *P. expansum* when inoculated in artificial apple wounds alone; 2) 141 genes upregulated by *P. expansum* in artificial apple wounds only in the presence of *P. terrestris*; and 3) 816 genes commonly upregulated by *P. expansum* in the two aforementioned conditions. For the common dataset having two values of logFC, sorting for expression values was done using the dataset *P. expansum* + LS28.

At the same time, among downregulated genes there are 1) 180 DEGs during the apple colonization without the BCA; 2) 88 DEGs during the interaction with LS28 and 3) 1068 DEGs commonly expressed in the dual and tritrophic interaction already described.

4.4.1 DEGs of *Penicillium expansum* during interaction with the host

The 60 upregulated DEGs represent genes expressed by *P. expansum* without the biocontrol agent in wounded apple tissues. GO analysis shows that the most enriched term is oxidation-reduction process (11 DEGs), followed by GO groups including low number of genes (< 4) that are involved in fatty acid biosynthesis, response to toxic substance, cell wall rearrangement and carbohydrate catabolic process (represented by the graph in figure 14, C).

Among the annotated (see table 8 for the table format of this paragraph. The complete lists are available in the supplemental file S3 and S4 for *P. expansum*), the most expressed gene is PEX1_050910 which encodes for a protein with a HMG high mobility group domain, and with GO molecular function as DNA binding; most of these proteins work as gene expression regulators. The second one PEX1_003930 is annotated as polyamine transporter I ortholog of *HNM1*. The following gene PEX1_005170 is member of the patulin biosynthetic gene cluster (Tannous et al., 2014); in particular, the oxidoreductase patO is responsible for conversion of gentisyl alcohol to isoepoxydon in the vacuole (B. Li et al., 2019). The gene PEX1_005150 is involved in transmembrane transport and is part of the patulin cluster, with a role in the secretion of E-ascladiol that was converted to patulin by patE, as revealed by the comparison with the better characterized *P. expansum* strain MD-8 (White, O'Callaghan, & Dobson, 2006). The protein encodes

PEX1_002430, also is part of the patulin gene cluster and it corresponds to 6-methylsalicylic acid synthase involved in the first step of patulin synthesis via condensation of acetate and malonate units. The last gene reported in the selection of DEGs is PEX1_037860 that encodes for a cytochrome P450.

The gene PEX1_038390, ortholog of the *S. cerevisiae* gene *SNQ2*, encodes a transmembrane transporter protein belonging to the ATP-binding cassette (ABC transporter superfamily) involved in drug resistance (Rogers et al., 2001). The gene PEX1_045200 is ortholog of the *S. cerevisiae FAT1* gene that encodes for fatty acyl CoA-synthetase activator of imported long fatty acid (Zou et al., 2002).

During apple colonization by *P. expansum*, KEGG analysis performed on DEGs is in agreement with the GO enrichment analysis, revealing that metabolic pathway of fatty acid metabolism is significantly enriched followed by pathway related to mycotoxin biosynthesis (table 11).

Furthermore, in the same condition there are 180 downregulated DEGs with the most enriched biological process being carbohydrates transport (PEX1_028210, PEX1_082380, PEX1_064670, PEX1_025550, PEX1_059220 and PEX1_059120) that includes *RGT2*, *HXT10*, *SNF3* and *MAL13*. The most significant GO downregulated include also transmembrane transport (28 genes were assigned to this function involved in glucose, polyamine, allantoate and nicotinic acid transport). The molecular function enrichment analysis (figure 15) confirms the biological process activity and reveal transcriptional regulator activity as one of the most enriched among downregulated, including several genes as *CST6*, *PHR1*, *GAL4*, *ASG1* and *CST6*. And last cellular component enrichment (figure 16), in agreement with the whole GO enrichment analysis

confirm that genes encoding for plasma membrane constituent are more enriched within downregulated DEGs for this condition.

4.4.2 Common DEGs of *Penicillium expansum* in dual and tritrophic interaction

In the common dataset 816 DEGs are highly expressed (figure 14, A). GO analysis revealed that oxidation-reduction process is the most enriched biological process (figure 14 B) with 177 DEGs, followed by peptide biosynthetic process and translation, ribosome biogenesis, Transmembrane transport includes a high number of genes but it is not highly enriched. Molecular function analysis confirm that genes involved in structural constituent of ribosome are significantly enriched, in parallel with redox activity (figure 15) (see the table 9 for the table format of this paragraph).

The most expressed gene (taking apart those uncharacterized, complete lists supplemental file S3 and S4) of *P. expansum* in the common dataset is PEX1_083840 that encodes for a class of enzymes that catalyze the addiction of the glucosyl group from UTP-sugar to a small hydrophobic molecule (Burchell et al., 1991). PEX1_058740 is an MFS transmembrane transporter. PEX1_085620 encodes for a virulence factor enzyme with a pectolytic activity on pectins, one of the main components of the fruit cell wall (Marín-Rodríguez, Orchard, & Seymour, 2002). PEX1_052260 is annotated as a formate or nitrite transporter. The gene PEX1_023520 is involved in metabolic process and oxidize the aromatic ring into a non-aromatic one by di-oxygenase activity at membrane level (Neidle et al., 1991). PEX1_019820 encodes for a protein involved oxidation of pyrimidine (Kim et al., 2010). PEX1_053980 is an MSF involved in sugar transport.

PEX1_088550 has a glycoside hydrolase domain and has been classified as virulence factor by Levin et al. (2019). The gene PEX1_010530 encodes a cytochrome p450 known for its function in substrate oxidation and also drug metabolization and detoxification (Lah et al., 2011). PEX1_058130 encodes for MSF transporter family ortholog of the *S. cerevisiae* allantoate transporter Dal5 (Rai et al., 1987). PEX1_025200 encodes for a protein involved in pantothenate biosynthetic process.

In agreement with Levin et al. (2019), PEX1_083670 encodes a virulence factor produced by *P. expansum*. The gene PEX1_049460 corresponds to *S. cerevisiae DUR1,2* and it encodes for a protein involved in the breakdown of the urea to carbon dioxide and ammonium (T. G. Cooper, Lam, & Turoscy, 1980). PEX1_059870 includes NAD-dependent formate dehydrogenase domain. PEX1_045780 encodes for a protein involved in the degradation of carbohydrates with a protein domain belonging to glycoside hydrolase (family 28). PEX1_058750 is a transmembrane transporter belonging to the multi antimicrobial extrusion (MATE) family, which might be involved efflux protein involved in the extrusion of xenobiotic and cationic drugs compound across the membrane (J. Liu et al., 2015). PEX1_096480 has no orthologs in *S. cerevisiae* and it is an MSF involved in transmembrane transport. PEX1_080170 also has no ortholog in *S. cerevisiae* and domain analysis revealed the presence of aminoglycoside phosphotransferase function usually involved in phosphorylation process (Trower & Clark, 1990).

In agreement with GO the KEGG analysis (figure 17, table 11), the metabolic pathways involved in peptides biosynthesis such as phenylalanine, alanine, proline and other amino acid are enriched. Other metabolic pathways related to biosynthesis of tropane piperidine and alkaloid are enriched (table 11).

The common group includes 1068 DEGs (figure 14, C). The most significantly enriched and highly represented GO term is oxidation-reduction process, followed by lipid biosynthesis, isoprenoid biosynthesis and response to osmotic stress (figure 14, D). Among the downregulates DEGs, the most significantly enriched molecular function is ATPase regulatory activity that includes several genes as *AHA1*, *FES1*, *INH1*, *GDE1*, *DMC1* and *INH1*. While GO enrichment of the cellular component category, highlighted the most significant intrinsic components of the membrane (figure 15 and 16, respectively molecular function and cellular component).

4.4.3 DEGs uniquely expressed by *P. expansum* when inoculated with *P. terrestris* LS28 in apple wounds

141 DEGs were upregulated by *P. expansum* only in presence of LS28 during interaction in apples (figure 14, A). GO annotation revealed that amino sugar catabolic process was the most enriched process with five genes (PEX1_019210, PEX1_022400, PEX1_010940, PEX1_093060, PEX1_093090), two of which containing a glycoside hydrolase domain. Transmembrane transport activity is the most enriched term among molecular function category (33 DEGs) the majority of which belonging to MSF transporter superfamily associated to different substrates, mainly sugar and amino acids. Included in molecular function, metal ion binding is one of the most enriched followed by transcription factor activity by RNA polymerase II (15 DEGs are assigned to this function) as *STB5* involved in oxidative stress response (Larochelle et al., 2006).

Oxidation-reduction process overlaps between the three datasets analyzed and includes 34 DEGs enriched in the present dataset with wide range of molecular function spanning

from NADH or FAD oxidoreductases-dependent (PEX1_088250, PEX1_049140, PEX1_083830, PEX1_010380, PEX1_072790, PEX1_049310, (PEX1_088900, PEX1_040120, PEX1_055860, PEX1_061500, PEX1_010380), to cytochrome P450 (PEX1_038280, PEX1_063200).

The most expressed gene of this group is PEX1_022400 encoding a glycoside hydrolase (family 2), in particular a exo-beta-D-glusosaminidase classified in CaZymes database database as GH2 (Lombard et al., 2014). This protein contains also an immunoglobulin-like domain and a sugar binding domain typical for GH2 members (Côté et al., 2006). The second highly upregulated gene is PEX1_012350 encoding for an exo-alpha-sialidase which is also a member of the glycoside hydrolase (family 33). Sialidase enzymes are able to catalyze hydrolysis of glycosidic linkage of terminal sialic acid residues in oligosaccharides, glycoprotein, glycolipids, colominic acid and synthetic substrates (Rothe et al., 1991).

PEX1_027760 encodes a chitosanase that catalyzed the degradation of chitin, also associated with the presence of a signal peptide. The gene PEX1_023120 is the *HNM1 S. cerevisiae* ortholog involved in choline and amino acids transport (Kiewietdejonge et al., 2006; Z. Li, Haase, & Brendel, 1991). PEX1_010930 encodes a MFS that is the and *TPO2 S. cerevisiae* ortholog involved in the polyamine transport (Tomitori et al., 2001). PEX1_056040 encodes for a protein with a chitosanase activity. PEX1_012360 belongs to DHDPS-like or Dihydrodipicolinate synthetase family, member of the class I aldolases. PEX1_065360 is uncharacterized and it contains a domain a jelly roll-like topology belonging to RmlC-cupin like domain superfamily, mainly involved in the synthesis of L-rhamnose (Giraud et al., 2000). PEX1_088900 encodes for an alcohol dehydrogenase, zinc type enzyme; the domain analysis highlights also a GroEs-like fold

at N-terminal and C-terminal domain having a classical Rossman fold, therefore its wide function spread among the most important biological phases (Medvedev et al., 2019). The gene PEX1_019210 is a glycoside hydrolase involved in carbohydrate metabolic process. PEX1_005750 has a domain ThiJ/PfpI is the ortholog of the *S. cerevisiae* gene *HSP31* encoding a methylglyoxalase involved in oxidative stress resistance (Skoneczna, Miciałkiewicz, & Skoneczny, 2007). PEX1_044380 is a predicted acetoacetate decarboxylase that in some pathogenic fungi is required for polyketide toxin production (Rose et al., 2002). The gene PEX1_071780 encodes for an aminotransferase class IV. PEX1_103690 encodes a fungal transcriptional activator of the citrinin biosynthesis that contains a cysteine-rich region that binds DNA in a zinc-dependent fashion. PEX1_072210 is a aquaglyceroporin like protein that allow the passive transport of water or glycerol selectively (Sui et al., 2001).

PEX1_049140 has a “TIM barrel" domain typical of aldolase enzymes (class I), and it is involved in oxidation-reduction process. PEX1_093060 encodes for a protein predicted to be involved in N-acetylglucosamine metabolic process. PEX1_004560 is involved in tryptophan biosynthesis (Braus, 1991). PEX1_063240 encodes for MSF protein, a proton-coupled transporters that facilitated the transport of monocarboxylate as lactate and pyruvate. PEX1_010380 is a aldehyde dehydrogenases, while the last gene is PEX1_038110, which encodes a hypothetical protein (Kulkarni, Kelkar, & Dean, 2003).

KEGG analysis revealed that amino sugar and nucleotide sugar metabolism is the most enriched pathway, and it includes proteins involved in amino sugar degradation, some of which included in CaZymes database (Lombard et al., 2014). Other enriched pathways are phenylalanine metabolism, isoquinoline and alkaloid biosynthesis, phenylpropanoid biosynthesis, tyrosine metabolism, and other glycan degradation (figure 18).

The interaction between *P. expansum – P. terrestris* LS28 in apple includes also 88 downregulated DEGs (figure 14 C). GO analysis revealed that "methylation" is the most enriched biological process among the downregulated, furthermore all the genes assigned to this term are uncharacterized. Molecular function enrichment confirm that methylation activity is important among downregulated. Whereas, the most enriched molecular function activity is "heme binding" and includes four genes that encode for *ERG5*, one gene that encodes for *BNA2*, and two genes assigned to this function but uncharacterized (figure 15).

4.4.4 Comparison with RNAseq dataset previously published

In total for *Penicillium expansum* 201 DEGs identified in this study are in common with the DEGs obtained in the study of Ballester et al. (2014). In particular, 62 genes differentially expressed in our study during apple colonization without the BCA are in common with DEGs identified by Ballester and colleagues at 48 h post inoculation and significantly enriched for translation and ribosome biogenesis, in according with our study. Sixty-seven DEGs are in common with DEGs identified by Ballester and colleagues at both 48 h and 72 h post inoculation, and they are enriched for ATP binding, zinc ion binding, arginine and proline metabolic process.

In agreement with DEGs identified by Ballester and colleagues, the remaining 55 DEGs at 72 h post inoculation that overlap with the "common genes" dataset of *P. expansum* of the present are mostly enriched for catabolic process of amino acid and DNA transcription (figure 19).

4.5 Analysis of *Malus domestica* transcriptomic changes during the interaction with *P terrestris* LS28 and *P. expansum*

The principal component analysis (figure 20) represents the statistical variation of the RNAseq experiment obtained for *Malus domestica.* While the different biological replicates of each treatment are grouped according to the experimental design, sample of *M. domestica* during interaction with *P. expansum* showed higher variation compared to the other treatment.

All the samples analyzed are distributed in clusters corresponding to the treatments, as shows by the PCA in figure 20.

The figure 21 reports the distribution of the upregulated and downregulated genes present in the dataset filtered for log2FC +/-1.

Malus domestica datasets with DEGs listed based on log2FC +/- 1, available in the supplemental file S5 and S6. After the comparison and further filtering for fold change (log2FC +/- 2) the genes were grouped in 4 datasets following the flow chart of figure 22. Finally, DEGs were compared to *A. thaliana* database, and analyzed through GO and KEGG.

4.5.1 DEGs of *Malus domestica* during the interaction with *Papiliotrema terrestris* in wound

DEGs of *Malus domestica* in response to *P. terrestris* LS28 include 47 upregulated genes (30 + 17 common with tritrophic condition 23, A), with the most expressed listed in table 13 (for the complete lists of DEGs see supplemental file S5 and S6). The most enriched

GO biological process terms are carbohydrate metabolism and oxidation-reduction process. Among biological process we found also genes that encode for protein involved in cytoskeleton organization (uncharacterized), potassium transport (*POT5*) and hydrogen peroxide catabolic process (*PER52*). Furthermore, molecular function enrichment (figure 24) confirm that hydrolase activity is the most important activity in apple during the interaction with LS28. Interestingly, the enrichment of cellular components shows that during the interaction with the BCA LS28 the most enriched cell compartment is the ribosome (figure 25).

MD13G1133200 is the most expressed *M. domestica* gene and it encodes for a protein involved in potassium transport. The second gene is MD07G1254600 ortholog of *SKU5* in *A. thaliana* that encodes for a protein of unknown function with its expression was detected in expanding tissues (Zhou, 2019). MD06G1147000 encodes a β-glucosidase that could play a role in a plant defense activation (Morant et al., 2008). MD13G1148400 is the *GA2OX6* ortholog of *A. thaliana* that allows plants to adapt and tolerate stress conditions (C. Li et al., 2019).

KEGG analysis shows enrichment of the carbohydrate and starch biosynthetic pathway; furthermore, adds other clues such as the presence of diterpenoid pathway and cyanaminoacid metabolism that have been significantly activated in the host in response to the yeast (table 17).

Furthermore, this dataset includes 171 DEGs that are downregulated in *M. domestica* during interaction with *P. terrestris*. The most enriched process is cell wall modification and it includes 5 genes which all encode for pectin-esterase enzymes (*PME3*, *PME7*, *PME12* and *PME17*). Furthermore, the following genes assigned to the terms "pollination" (*RKS1* and *B120*), "pollen-pistil interaction" and "multicellular organism

process" are involved in signal recognition and have a less significative enrichment value. Molecular function enrichment (see figure 24) reveals among the less enriched there are genes involved in glutamate decarboxylase activity (*GAD4*) and also genes that encode for receptor proteins as *WALK9* and involved in carbohydrate binding *LECRK91*, *LECRK32*, *BGAL8*, *BGAL8* and *AtGH9C2*.

4.5.2 Common DEGs of *Malus domestica*

M. domestica DEGs commonly identified during interaction with both the pathogen and the biocontrol agents includes 694 + 35 upregulated genes (figure 22), and 458 + 11 downregulated genes (figure 23).

GO analysis revealed that the most enriched term is oxidation-reduction process with 110 genes. Metabolic process is the most represented GO term but it has low enrichment score, and it includes metabolic process of phenylpropanoid, lignin, and aromatic compounds that are highly enriched and characterized by a lower number of genes (less than 20). The molecular function annotation confirms the enrichment of oxidoreductase activity (figure 24). Furthermore, cellular component analysis (figure 25) shows enrichment of the extracellular region.

The most expressed genes of this condition are listed in table 14 (the complete list of genes is available in supplemental files S5 and S6). MD03G1142500 encodes a NAD(+) ADP-ribosyl-transferase enzyme that is able to interact with a wide range of substrates from DNA to proteins and it is involved in modification of macromolecules (Aravind et al., 2015).

MD12G1211300 encodes for *β*-glucosidase ortholog of *A. thaliana* AtBGLU17, a GH1 in CAZy database, known for its hydrolytic activity on non-reducing terminal beta-D-glucosyl residues. The genes MD14G1140400 and MD14G1141000 are ortholog of *A. thaliana* DMR6-LIKE OXYGENASE 2 that catalyzes the conversion of salicylic acid (SA) to 2,3-dihydroxybenzoic acid (2,3-DHBA), hence acts as suppressor of immunity; furthermore, their overexpression conferred susceptibility to downy mildew (Zeilmaker et al., 2015). The gene MD05G1285000 encodes for cytochrome P450 that in *Arabidopsis* is involved in biosynthetic pathway of sterol. The genes MD12G1211500, MD00G1145300 and MD03G1204700 encode for proteins with a glycoside hydrolase domain involved in carbohydrate metabolic process. MD16G1215200 encodes for 1-aminocyclopropane-1-carboxylate oxidase homolog1, (table 14) dependent on Fe^{2+} and 2-oxoglutarate (2OG), which typically catalyzes the oxidation of an organic substrate using iron as cofactor; this class of enzymes in plants is involved in the biosynthesis of plant hormones, such as ethylene, gibberellins, anthocyanidins and pigments such as flavones (Rudus, Sasiak, & Kępczyński, 2013).

MD10G1155000 is the *CAD7* ortholog of *Arabidopsis* and it converts (Z)-3-hexenal to (Z)-3-hexen-1-ol during green leaf volatiles bursts (GLVs), including six-carbon (C6) aldehydes which are aroused by the partial wounding of *Arabidopsis* leaves (Tanaka et al., 2018). MD05G1287200 encodes the *AGL62* ortholog of encoding for a transcriptional regulator (Pellegrini, Tan, & Richmond, 1995). MD16G1278900 is responsible of xyloglucan endotransglucosylase/hydrolase protein synthesis; this enzyme is involved in the ripening process and it is able to modify the assembling of the cell wall polymers, also influencing the susceptibility to postharvest pathogens (Atkinson et al., 2009).

MD06G1043900 is the *A. thaliana* ortholog *BAM3* encoding for beta-amylase 3 involved in polysaccharide metabolic process, in particular starch degradation (Xiao et al., 2018). *CSLB3* ortholog of *A. thaliana* and it is involved in beta-glycan synthase that polymerized the backbones of non-cellulosic polysaccharides (hemicelluloses) of plant cell wall. MD11G1146000 and MD10G1196700 are cysteine-rich RLK (receptor-like protein kinases) 8 that transfer of gamma-phosphoryl group from ATP to serine/threonine residues on protein substrates, usually at cytoplasmic level. Cysteine-rich RLKs are associated with the perception of ROS during plant-pathogen interaction (Bourdais et al., 2015). The MD05G1287100 is ortholog of the *A. thaliana* transcription factor AGL29 involved in several processes as pollen development and growth (Pina et al., 2005).

The MD12G1228300, MD12G1227900 and MD12G1228200 genes encodes a copine family protein, and its orthologs gene in *Arabidopsis* is *BON3*. The copine family is involved in the regulation of programmed cell death, and is activated during the biotic attack but need to be repressed in absence of biotic stress because their expression negatively influenced the development of the plant (Lee & McNellis, 2009; Y. Li, Pennington, & Hua, 2009; Yang & Hua, 2004). The gene MD17G1141300 is a transcriptional activator with MADS box domain and a keratin-like (K-box) domain, which promotes protein dimerization. MD05G1320800 and MD05G1320500 encode for a polyphenol oxidase (PPO) a group of Cu-containing enzymes normally located in the chloroplast, which if lose the sub compartmentalization as consequence of injury, senescence or biotic factors, came in contact with vacuolar phenolic substrates producing browning (Taranto et al., 2017). MD16G1212000 and MD02G1095700 encode for cellulose synthase-like protein G2 that in *A. thaliana* (AtCslG2) is involved in the biogenesis of the cell wall located at Golgi compartment level. Several expressed genes

encode for laccase enzymes (MD04G1142600, MD12G1157100, MD04G1142900, MD00G1014900, MD07G1307400) that have oxidoreductase activity and are associated with lignin metabolism. Biochemically, laccase enzymes degrade polyphenol and their activity could be related to the phenol oxidase reaction (PPO) (Mayer & Staples, 2002; J. Wang et al., 2015). MD02G1076800 encodes for cytidine deaminase protein, *CDA1* ortholog of *A. thaliana*; the main feature of this enzymes was capability of acting on nucleic acids by promoting a second level of regulation beyond that encodes in the genome (MacElrevey & Wedekind, 2008). This class of enzymes is used in genome editing, associated with double strand-breaks mediated by Cas9 protein reported for several organisms (Huang et al, 2019; Yu Wang et al.; Zong et al., 2017).

Last gene is MD02G1295200 that encodes for a transcriptional regulator belongs to the family of mitochondrial transcription termination factors (MTERF), a novel type of nuclear-encoded protein. The proposed function for these regulators is intriguing, it was found that the expression of this genes at mitochondrial level is to regulate the expression of nuclear genes in response to abiotic stress (Quesada, 2016).

GO analysis is consistent with KEGG orthology of common genes, in which the redox process is represented by different pathways (platinum drug resistance and cytochrome P450) and also in this analysis the phenylpropanoid biosynthetic pathway is significantly represented (figure 26, and table 17).

For downregulated DEGs, GO analysis revealed that response to auxin is the most enriched biological process. The second most enriched term in this condition is “plant-type cell wall organization” that includes genes as *EXPA* associated with the loosening and relaxation of the cell wall by microfibrils and glucan bonds modification at cell wall level; other downregulated process involves the transport process. Indeed, molecular

function enrichment (figure 24), confirms as the most enriched terms transporter activity that includes gene as *ZIP11*, *GLTP1*, *SLAH3*, *PLT6*, *PIP2-8*, *APE2*, *NPF5*.1, *POT2*, *AKT2*, *TMT2* and *STP1*. Cellular component analysis reveals a role of Expansin encoding genes (assigned to the terms "extracellular region", figure 25).

After comparing the datasets 17 DEGs upregulated (figure 23, A listed in table 16) and 5 DEGs downregulated of *M. domestica* (see supplemental file S6) were found exclusively expressed by the apple inoculated with both with BCA and *P. expansum* and not included in the common genes described above. However, GO and KEGG analysis did not show enrichment of any process and pathways (figure 23 B and D; figure 24 and 25).

4.5.3 DEGs of *M. domestica* during the interaction with *P. expansum*

When the apple was inoculated with *P. expansum* the highest amount of DEGs were obtained. In particular, we found 684 + 1448 upregulated DEGs (figures 22, 23) with the most significant DEGs listed in table 15 (supplemental file S5).

GO analysis revealed that "protein phosphorylation", metabolic process of phosphate-containing compound, and protein modification are the most enriched biological process, including genes that encoding for leucine-rich repeat transmembrane protein (LRR); this class of protein are mainly involved in signal transduction, with the majority of them encoding for receptor like kinase family proteins (RLK) that promote protein phosphorylation and macromolecule modification process.

The most expressed gene MD05G1275700 encodes for the subtilisin-like protease *SBT1*.7 involved in many biological processes such as peptide hormone synthesis, viral

protein processing and receptor maturation (Bergeron, Leduc, & Day, 2000). The gene MD10G1248500 is ortholog of *WAK2* in *A. thaliana* and it encodes for Wall-associated receptor kinase 2. *WAK2* is a class of protein able to mediate a linkage between the extracellular membrane (by the N-terminus region tidy associated with the cell wall) along with the cytoplasm (by a cytoplasmatic C-terminus region with serine/threonine protein kinase domain) (Wagner & Kohorn, 2001).

The ortholog of the gene MD16G1010800 is *GAD4* in *A. thaliana* involved in the production of γ-aminobutyric acid (GABA); this enzyme is crucial in the response to biotic and abiotic stresses in plants has been controlled by Ca2+/CaM, (Reddy, Ali, & Reddy, 2002). The gene MD16G1065700 is the ortholog of *A. thaliana CM3*, which encodes for chorismate mutase required for biosynthesis of the aromatic amino acid phenylalanine and tyrosine (Schnappauf, Krappmann, & Braus, 1998). MD06G1232800 is the *A. thaliana* ortholog of the U-box domain-containing protein *PUB21* involved in protein ubiquitination (Azevedo, Santos-Rosa, & Shirasu, 2001). MD11G1254600 is ortholog of *A. thaliana LECRK91* encoding for a potential immune receptor protein characterized by the presence of two domains, a non-cytoplasmatic legume lectin domain and a cytoplasmic protein kinase domain. This class of protein (*LECRK*) are associated with pathogen recognition and transduction of the signal (Yan Wang et al., 2015).

The gene MD05G1144900 encodes for a protein with a kinase domain. MD09G1098900 encodes for a G-type Serine Receptor-like protein kinases involved in cell recognition and signal transduction. The gene MD01G1027900 is ortholog of *CRK26* in *A. thaliana* and it encodes for a protein involved in pathogen recognition and hypersensitive response induced by hydrogen peroxide and salicylic acid (K. Chen, Fan, Du, & Chen, 2004). MD03G1134800 encodes for arogenate dehydratase in *A. thaliana* involved in the first

step of the sub-pathway that synthesizes L-phenylalanine from L-arogenate (Cho et al., 2007).

MD13G1066600 encodes for the chorismite mutase *CM3*. MD01G1033900 has no ortholog in *A. thaliana* and is involved in the synthesis of chorismate from D-erythrose 4-phosphate and phosphoenolpyruvate. MD15G1076500 is a G-type lectin S-receptor-like serine/threonine-protein kinase, main determinant of self-incompatibility of pollination in many species and probably involved also in both defense and development (Hatakeyama et al., 1998). MD02G1047500 is also involved in pollen recognition. MD07G1157600 is the *KCS1* ortholog of *A. thaliana* and it is involved in fatty acid biosynthesis. MD15G1391000 and MD05G1039400 encode for G-type lectin S-receptor-like serine/threonine-protein kinase involved in cell recognition and signal transduction (Sun et al., 2020).

KEGG analysis revealed that “plant pathogen interaction”, “MAPK signaling pathway” and “biosynthesis of amino acid” are the most enriched pathways (see *Malus domestica* + *P. expansum* in table 17 and figure 27). Most enriched defense gene expressed by the apple are represented in the KEGG pathway of figure 28 with a focus on “plant pathogen interaction”.

Downregulated genes of this condition were obtained by the sum of genes specifically expressed by the apple during the interaction with *P. expansum* plus those shared with the tritrophic condition (921 + 1478 DEGs, explained in figure 22) and displayed in the Venn diagram, figure 23, (C).

The GO analysis of downregulated genes shows as the most enriched biological process is photosynthesis (figure 23 D) with 62 gene assigned to this term and the most downregulated is *LHCA2*. The molecular function enrichment (figure 24) reveal that genes involved in the calcium ion binding are the most enriched; anyway, the molecular function is also associated with metal ion binding and with photosynthetic process (*e.g. CPK13*, *PSBO2*). Cellular component in figure 25, highlight the most enriched genes are those that encodes for protein located in the thylakoid part, in agreement with biological process described in this paragraph (For the complete list of genes of this condition see the condition *Malus domestica* + *P. expansum* in the supplemental file S6).

In the figure 29 describes the most expressed protein kinases during the interaction with the BCA, the fungal pathogen and both.

4.6 Selection of genes for target mutagenesis transcriptomic-based in LS28

Based on transcriptomic data obtained, a targeted mutagenesis approach was applied to study the function of selected upregulated genes. For mutagenesis, some of the most expressed DEGs belong to the common and " *P. terrestris* + *P. expansum*" datasets were selected and analyzed (table 18). Information on the related proteins can be found in table 19. For mutagenesis we selected genes predicted to be involved in the most represented GO terms, transport as *PUT4* (g1105.t1), oligopeptide transporter *OPT1* (g5541.t1) and oxidative stress response *LOT6* (g960.t1); moreover, several genes of unknown function (see table 18).

The most expressed genes during the interaction with *P expansum* was g.2837.t1 included in the list for mutagenesis encodes for an uncharacterized protein and after the screening, the phenotypic characterization doesn't show any differences in terms of growth on rich media or impairment respect to the wild type. The gene g142.t1 encodes for a protein that contain a glutathione S-transferase (GSH) domain, and the relative phenotypic characterization is ongoing. This gene is involved in several process, able to act as mediator of oxidation/reduction reaction and as scavenger of reactive species as radicals and known for its ability to protect cells against toxic chemicals. More important the synthesis of glutathione in the cell was coupled with the consumption of cysteine and its degradation give rise to cysteine, hence provide to the reservoir of sulfur compound in the cell (Miyake et al., 2002; Mountainet al., 1991).

The mutant for the ortholog of the *S. cerevisiae LOT6* gene (g960.t1) did not display any sensitivity to the stressors used, including the oxidative stress inducing agent menadione (table 21). This is surprising because the Lot6 protein is a quinone reductase expected to be required in oxidative stress resistance (Sollner et al., 2007).

Other uncharacterized genes selected for target mutagenesis were g3033.t1 (for which a deletion mutant was still not obtained), g1861.t1 and g4389.t1 characterized both *in vitro* and *in vivo.* The gene g4389.t1 encodes for a polypeptide of 127 amino acid that seems to have a transmembrane domain. The gene g5541.t1 encode for an oligopeptide transporter (*OPT1*); in *S. cerevisiae OPT1* is involved in the glutathione transport and localized mainly at the plasma membrane level (Elbaz-Alon et al., 2014); experiments are ongoing for the confirmation and characterization of the deletion mutant strain in LS28. The last gene selected g1105.t1 encoding for Put4 protein composed by 546 amino acid; in LS28 we found also another gene that through blast against *S. cerevisiae* was

annotated as a proline transporter (g1990.t1, see table 3). Put4 in *S. cerevisiae* was described as highly specific amino acid permeases (Wiame, Grenson, & Ars, 1985).

The proline permease Put4, encoded by the gene *PrnB*, was characterized in *A. nidulans* (Gournas et al., 2015); in this study was also demonstrated that some residues along the protein are crucial for the interaction with the substrate, indeed their substitution mimicking-Put4 broadened the specificity of PrnB. Anyway, the uptake of proline was mediated also by the low affinity transporter Gap1 in *S. cerevisiae*. Proline uptake otherwise confers several advantages to the yeast indeed is reported to give resistance to herbicide and hydrogen peroxide acting as cryoprotectant (C. Chen & Dickman Martin, 2005). To study the effective involvement of the transport of proline mediated also by Gap1 Poole and colleagues use radiolabeled proline and most important the concentration used exceed the K_m of put4 (0.025 mM) and is lower respect to the K_m value of Gap1 (13 mM).

4.7 Deletion mutants' generation and confirmation

All the constructs for targeted gene deletion in *P. terrestris* were successfully assembled in *S. cerevisiae* using the binary vector pGI3, (Ianiri et al., 2016) confirmed by colony PCR, and electroporated in *A. tumefaciens* strain EHA105 following the diagram reported in figure 30. The complete maps of the vectors generated are represented in the figures 31-38. The LS28 deletion mutants were obtained through electroporation using the deletion constructs reported in figures 31-38 with the exception of a *put4*Δ mutant obtained by AMT (*put4*Δ::*HYG*-2#3). All *P. terrestris* deletion mutants were confirmed

by PCR as described in materials and methods, with one representative example reported in figure 39. A summary of the results is reported in the table 18.

4.8 Screening *in vitro*

The *in vitro* screening showed that all the deletion mutants tested did not display different phenotypic traits compared to the WT strain when exposed to oxidative, genotoxic and nitrosative stress. Conversely, on lithium chloride all the deletion mutants are more resistant to the cell wall stress respect to the wild type. The same occur on cycloheximide, with the exception of mutant *lot6*Δ::*HYG*-1#2 (figure 40). Also, at macroscopic level no difference in the colony morphology is evident between deletion mutant and WT LS28.

4.9 Growth kinetics in media supplemented with amino acids and other nitrogen sources

The growth kinetic of *P. terrestris* WT was tested on different nitrogen sources, including the 20 amino acids (see table 22). Glucose was used as carbon source. The results represented as heatmap in figure 41, clearly show three main groups of nitrogen source utilization, with the preferred ones being asparagine (Asn), aspartic acid (Asp), ammonium, glutamic acid (Glu), di-aminopentanoic acid (Diapa) and apple mimicking media. The second group includes the largest number of tested compounds, ranging from proline, glycine, urea and GABA that support a better growth of *P. terrestris* compared to methionine, lysine and isoleucine; the less preferred nitrogen sources are cysteine,

leucine, and threonine, while there was no growth on tyrosine, nitrate, histidine and uridine.

The deletion mutants also were tested for their ability to grow in minimal media in the presence of amino acids (1.5 mM) as listed in table 22. In particular all the deletion mutants tested have a defective growth respect to the wild type in all the main amino acids present in apple except the strain g4389Δ::*HYG*-1#6 having the same pattern of the wild type (figure 42).

4.10 Antagonistic activity of generated deletion mutants

Mutants generated were tested in biocontrol assays against *P. expansum* to determine the involvement of the deleted genes in the antagonistic activity of *P. terrestris*. In the first experiment carried out two different cell concentrations were used, and only *put4*Δ::*HYG*-2#3 and *lot6*Δ::*HYG*-1#1 showed a significant reduction of their biocontrol activity when used at 5 x 10^6 CFU mL^{-1} (figure 43 and 45). Experiments were repeated using only for *put4*Δ::*HYG*-2#3 and *lot6*Δ::*HYG*-1#1 in the same condition (with the BCAs at 5 x 10^6 CFU mL^{-1}, figure 44 and 46).

Confirming previous results, there was a significantly higher biocontrol activity of the WT compared to *put4*Δ::*HYG*-2#3 (α = 99,9 %, $P < 0.001$. table 24 and figure 11) and *lot6*Δ::*HYG*-1#1 ($P < 0.005$ significant per $1 - \alpha$ = 99,5 %).

5 Discussion

5.1 *Papiliotrema terrestris* LS28

In this study we performed the analysis of the transcriptomic profiles of the BCA *P. terrestris* LS28, *P. expansum,* and host *M. domestica* during their dual and tritrophic interaction.

Focusing on *P. terrestris* LS28, data analysis revealed that most of the DEGs belong to the "common genes" dataset, suggesting a significant transcriptional rewiring by the BCA to colonize apple wounds regardless the presence of the fungal pathogen. DEGs belonging to the common group are mainly classified under the GO terms transport and oxidation-reduction process. In particular, a large number of genes encoding transmembrane transporters (permeases) are highly expressed (table 2-4 and figure 7 and 10). Compared to the ascomycetes *S. cerevisiae* and *C. albicans* we noticed an expansion of the permease protein family.

Among the permease, nitrogen transporters are highly represented, suggesting a role of nitrogen metabolism in the early phase of the tritrophic interaction in wounded apple. In our result represented among significantly enriched genes polyamine transporter encoding genes (*TPO*), are mainly expressed in the common dataset. Several studies report that polyamines are produced in the fruit and/or in plants during the pathogen attack (Valdés et al., 2012); moreover, seems that in yeast treated citrus fruit the level of polyamine is lower if compared to the non-treated citrus fruits (Liu et al., 2016). Furthermore, additional genes that encode for polyamine transport (*TPO1* and *DUR3*), vacuolar amino acid uptake (*VBA5*), allantoate permease (*SEO1* and *DAL5*) are exclusively expressed during the dual interaction BCA-apple. The most abundant nitrogen transporters are *PUT4*, *FUI1*, *VBA5*, *AGP2*, *DUR3*, *DAL4*, *SEO1*, *DIP5* and

DAL5 mainly represented in the common dataset (figure 10). Genes that encode for allantoin permease seems to be crucial in this analysis (*DAL4* orthologs are g7895.t1; g5778.t1; g5132.t1; g2532.t1; g7922.t1) indeed, ortholog of *DAL1* (g1181.t1) is also upregulated and is involved in allantoin degradation into glyoxylate and CO_2 (Wong & Wolfe, 2005).

On the other hand, less amount of nitrogen transporters genes resulted differentially expressed during the interaction with *P*. *expansum*, not referring to the common gene set.

What is intriguing is that, besides few exceptions (*e.g*. *ARG4*), genes involved in biosynthetic process of amino acids and other nitrogen sources were not upregulated. This indicates that the BCA *P. terrestris* LS28 does not need to synthetize these essential nutrients for its growth, but rather it activates permeases-encoding genes to readily utilize nutrients provided by the apple.

Although several studies are available for aminoacidic composition of grape juice there is limited information for apple juice and apple yeast assimilable nitrogen (YAN) concentration and composition; Mangas and colleagues reported that amino acids tend to decrease during fruit ripening (Mangas et al, 1998). In another study was reported that the levels of YAN of 12 cultivar harvested and analyzed for two year was 59 ± 3 mg N L^{-1}, where the ammonium reach the 8 ± 1 mg L^{-1} (Boudreau et al., 2017).

As reported by Ma et al. (2018) aspartic acid, asparagine and glutamic acid are the most abundant amino acids in apple juice. In a different study Ma and colleagues reported and quantified the amino acid composition of 13 apple cultivar grown in Virginia (USA); asparagine result as the most abundant amino acid, followed by phenylalanine, aspartic acid and glutamic acid. By the way seems that the aminoacidic composition may be

greatly subjected to a variation between the cultivar and other agronomic factors (Ma et al., 2018).

In Ascomycetes fungi, such as *S. cerevisiae* and *C. albicans*, amino acid uptake is mediated by the SPS complex (not directly involved in the transport), which is located on the plasma membrane and it is formed by the proteins Ssy1, Ptr3 (involved in the signal transduction from Ssy1 to Sss5) and Ssy5 (Silao & Ljungdahl, 2022). In *S. cerevisiae* amino acid signaling is mediated also by Gap1, that works as transceptor (activating the protein kinase A) and Mep2, involved in nitrogen sensing and transport both essential in pseudohyphal growth (Van Nuland et al., 2006). Surprisingly, blast analysis of the key component Ssy1 against the *P. terrestris* genome finds a gene that correspond to *S. cerevisiae DIP5*. Similarly, blast against the basidiomycete *C. neoformans* identify the permease CNAG_07902, hence suggesting that this amino acid sensor machinery is not conserved in the Tremellomycetes.

Nitrogen in general is an essential nutrient for all the form of life, and for yeasts too, indeed, yeasts are able to use almost 30 nitrogen-containing compound that include amino acids, urea, ammonium, nitrogen bases, and purine derivatives (Godard et al., 2007). In general, the utilization of nitrogen sources by the yeasts follows a hierarchical preference among the different nitrogen sources available. A conspicuous body of literature confirm that in presence of the preferred nitrogen sources yeasts repress several genes involved in the utilization of a non-preferred one (ter Schure et al., 1998).

Moreover, we noticed that the majority of the upregulated DEGs involved in nitrogen metabolism have *S. cerevisiae* orthologs that are involved in nitrogen catabolite repression (NCR).

In *S. cerevisiae*, NCR is regulated by four GATA family zinc finger transcription factors, two transcriptional activators, Gln3 and Gat1, and two repressors, Dal80 and Gzf3 (Deh1 and Nil2) (Cooper, 2002). Gat1 and Gln3 are both required for activation of NCR-sensitive genes, but the degree to which they influence the expression of a given gene is dependent on the individual gene as well as the nature and quality of the nitrogen source available in the media. When cells are grown on nitrogen-poor media, *GAT1* transcription is upregulated by Gln3 (Coffman et al., 1996). Under nitrogen-rich conditions, downregulation of *GAT1* transcription is mediated by the repressor Dal80. When optimal nitrogen sources are available, pre-existing Gat1 is phosphorylated by the TOR kinases Tor1 and Tor2. The phosphorylated form of Gat1 is bound by the regulatory protein Ure2, which sequesters it in the cytosol and doesn't allow the entrance into the nucleus, preventing transcription of NCR-sensitive genes. Therefore, when optimal sources of nitrogen are available, Ure2 acts as a transcriptional corepressor. (Coschigano & Magasanik, 1991).

In contrast, when nitrogen is limiting or chemically induced with rapamycin, Gat1 is dephosphorylated by the phosphatase Sit4, subsequently enters the nucleus, and interacts with the transcriptional co-activator Hfi1 to upregulate the expression of NCR-sensitive genes (Beck & Hall, 1999; Cooper, 2002; Daugherty et al., 1993).

The transcriptional regulation of *S. cerevisiae* NCR appears to be partially conserved among fungi, as both in *C. albicans* and *C. neoformans GAT1* is required for NCR (Kmetzsch et al., 2011; Limjindaporn, Khalaf, & Fonzi, 2003) and also for filamentous fungi as *Aspergillus* spp. (Arst & Cove, 1973).

Importantly, *P. terrestris* genome search revealed the presence of only one NCR activator, *GAT1* (g1827.t1), and one NCR repressor, *GZF3* (figure 47). Surprisingly,

these two crucial genes for NCR were not highly expressed or repressed in our dataset, with log2FC lower than 0.5. Conversely, in our dataset one ortholog of *URE2* (g3806.t1) is strongly downregulated (log2FC <4), which would be consistent with a grown in a poor nitrogen condition. However, the growth of *P. terrestris* LS28 in apple media follow the kinetics of growth in favorite nitrogen sources, as it clusters together with aspartic acid, asparagine, glutamic acid, ammonium, etc. (figure 41).

It might be reasonable that in our condition of analysis, at 36 hours post inoculation, the BCA shifted from preferred nitrogen sources to non-preferred one, hence explaining the upregulation of *GAT1* and the downregulation of *URE2*. This hypothesis is corroborated by KEGG analysis, which showed enrichment of glycine, serine, threonine, arginine, and proline metabolism (figure 11 and table 6).

Analysis of DEGs reveals also that oxidation-reduction processes are important in the tritrophic interaction. Oxidation-reduction processes involved mainly genes involved in oxidative stress response, corroborating previous phenotypic and genetic studies that demonstrated that resistance to oxidative stress is a crucial feature of *P. terrestris* LS28 for a timely wound colonization, with key role is played by the transcription factor Yap1 as demonstrated with functional genetics by Castoria et al., (2021). Although in the present study *YAP1* is not a DEGs, several other genes involved in the redox balance are differentially expressed as resulted by the bioinformatic analysis.

Molecular function enrichment reveals as nicotinic acid transporters encoded by *TNA1* promote the availability of NAD that is required as acceptor of reducing equivalents. Hence, part of the “oxidation-reduction process” annotated-genes are linked to the active metabolism during the early growth phase due to sugar metabolism through the glycolysis. In agreement with this is reported that in *Saccharomyces cerevisiae* is know

that *TNA1* contribute to increase NAD+/NADH levels, and seems that the expression level was high during the log phase growth (James Theoga Raj et al., 2019).

When the expression profile of LS28 is analyzed during dual interaction with the apple wound, redox processes are most linked with ATPase activity coupled with transmembrane transport. The permease activity significantly highlights the expression of genes encoding for iron and ion transporters (figure 10) as *FRE3* and *FET3* and involved in iron uptake. Although is known the role of iron as cofactor in many cellular processes as mitochondrial respiration hence energetic metabolism, several works remark the key role of iron as crucial for the yeast growth and competitive fitness as well as biocontrol (Ramos-Alonso et al., 2020; Spadaro & Droby, 2016).

Moreover, as illustrated by the GO enrichment of cellular component (figure 9), the tricarboxylic acid (TCA) pathway was the third process most enriched during BCA-apple interaction, including genes that encodes for pyruvate dehydrogenase (*PDB1*, *PDA1*) that catalyze the first step of the TCA and probably the most important involved in the regulation of glycolysis, and ketoglutarate dehydrogenase (*KGD2*) (Pronk et al., 1990).

Last, during the interaction with postharvest fungal pathogen oligopeptide transporters are significantly expressed (*OPT2*) such as *YHK8* member of the DHA family, involved in the extrusion of cytotoxic compound from the cell. Becerra-Rodríguez and colleagues report that the expression of *OPT1* seems to be induced by non-preferred nitrogen sources, while, *OPT2* is not affected by nitrogen sources as *OPT1* (Becerra-Rodríguez, Marsit, & Galeote, 2020), included in our list of genes subjected to target mutagenesis (the experiments are ongoing). n literature was reported that in *S cerevisiae OPT1* is localized at plasma membrane level while *OPT2* was predicted to be in the peroxisome

compartment; furthermore, *OPT2* deletion affect glutathione homeostasis and resistance to oxidative stress (Elbaz-Alon et al., 2014).

These data explain as competition for nutrients, mainly nitrogen and carbon-based compound greatly affect the biocontrol ability of the BCA. Furthermore, is clear that during the interaction with pathogen differentially expressed genes are mainly involved cytotoxic compound extrusion and genes for nutrient metabolism are less differentially represented.

By our functional study is confirmed that redox process and nutrient competition are the main process that drive the biocontrol properties of LS28. The *in vitro* characterization of *LOT6* deletion mutant doesn't show any difference respect to the wild type. Moreover, *in vivo* characterization shows a significant level of impairment ($P < 0.005$) in biocontrol ability of *lot6*Δ compared to the wild type.

The same circumstance occurs for the proline transporter *PUT4*; the *in vitro* characterization doesn't show particular impairment in response to chemicals stressors applied in the spotting assay, although in the biocontrol ability was strongly defective if compared to LS28 wild type strain.

The key reason that justifies this "gap" between *in vitro* and *in vivo* results may be object of discussion. Gene function is not ever linked directly with one single function as we expected to. More often a gene deletion that could affect the phenotype is evident when a sum of limiting factors occur, as *in vivo* condition is instead. This reflects the complexity of the gene expression regulation in processes such as biocontrol; indeed, with this approach we focused only on one gene, but biocontrol may depend by reciprocal regulation of multiple genes or also post transcriptional regulation process may have a key role (Massart & Jijakli, 2007).

5.2 *Penicillium expansum*

The transcriptomic profile of the postharvest fungal pathogen *P. expansum* was analyzed during interaction with the wounded apple tissues alone or during co-incubation with the BCA *P. terrestris* LS28. At 36 hours post inoculation the analysis of DEGs upregulated shows that oxidation-reduction process is one of the most enriched and most important terms, regardless the presence of the BCA. Notably, in the common dataset this process includes catalases and peroxidases. The oxidation-reduction terms, as already discussed for the LS28 GO analysis, includes more broadened processes as several NADPH dependent reactions that may have a widespread function in the fungal cell, *e.g*. ROS generation (X. Zhang et al., 2021).

ROS species are inter-kingdom signal molecules that at interface of host-pathogen may drive the outcome of the infection process. Some necrotrophic pathogens are known to start the infection process when an elicitor-mediated oxidative burst has been triggered in the host. The first step is mediated by pathogen recognition receptor (PRR), causing hypersensitive response in the host cell (Lu & Tsuda, 2020). At the same time ROS production may occur in the fruit tissues also when occasionally wounds may be caused by handling process (Buron-Moles et al., 2015). Furthermore, necrotrophic-pathogens have several strategies to overcome the basal or a-specific level of resistance in the host: like the production of several ROS-detoxifying enzymes (SOD, APX and POD) (Qin et al., 2007; Y. Wang et al., 2019) or through the production of effectors proteins.

Fungal pathogens recognition by the host occurs because of the conserved structure that characterize fungal cell wall; *e.g*. chitin and glucans that are the “skeleton” of the fungal cell wall may be hydrolyzed by chitinase enzymes; the product represented by the chitin

oligomers can be defined as microbial associated molecular pattern (MAMPS). Some pathogen to overcome the recognition are able to secrete effector that interfere/compete with the chitin recognition system of the host plant, hence, affecting the plant immune response. This is the case of the fungal Lysin motif (LysM), one of the most studied effectors; anyway, protein having this type of domain are widespread among eucaryotes organism and may differ about the number Lys domain residue (de Jonge & Thomma, 2009).

Several studies aimed to characterize the role of LysM effectors in *P. expansum* reveal that if subjected to gene deletion the single mutation of the gene encoding for the effector did not affect the pathogenicity in apple, and was hypothesized that they work in a synergistic manner (Levin et al., 2017).

In our study we identify three genes included in the DEGs, PEX1_026840 and PEX1_011780 and PEX1_027760, were describe under the control of the effector protein PeLysM15 in the study conducted by Chen et al. (2020). The gene PEX1_027760 was classified as degrading enzyme in the study carried out by Levin et al. (2019) and for the latter we describe the presence of a signal peptide, furthermore, by Chen and colleagues was included among the effectors (Chen et al., 2020). The upregulated gene PEX1_032640 present in our common dataset is a chitin binding effector protein that bind oligomers of its own cell wall preventing the defense response triggered by chitin in plant hosts (Chen et al., 2020) as described above. In this regard, the same mechanism allows the pathogen to escape from the recognition receptor and may secure fungal cell wall–derived chitin fragments so that chitin cannot stimulate an immune response; this type of effector was found also in *Cladosporium fulvum* able to secrete the protein Ecp6

containing 3 LysM motifs, specifically expressed during host colonization on tomato plant (de Jonge et al., 2010; de Jonge & Thomma, 2009).

P. expansum is able to produce several secondary metabolites most of it are still not yet studied in detail. Patulin is the most common secondary metabolite produced by *Penicillium* spp. and the production levels may vary within different strains; however, patulin seems to be not involved in the virulence or pathogenicity of the producer strain (Ballester et al., 2014; Sommer, Buchanan, & Fortlage, 1974). In the present study, we found that genes of the patulin cluster were DEGs; it should be emphasized that in the absence of the BCA part of the patulin gene cluster appears to be upregulated. , while in the presence of the BCA LS28 one gene encodes for a transcription factor responsible for citrinin biosynthesis is highly expressed (PEX1_103690, logFC 4.44). Furthermore, mycotoxigenic activity appear among the most enriched pathway, annotated by KEGG as “aflatoxin biosynthesis” (table 11).

During the interaction with the BCA the most enriched biological process includes the production of cell wall degrading enzymes as PEX1_088550 (homolog of PEX2_031340) and PEX1_083670 (homolog of PEX2_107230) which were found upregulated also in the work of (Levin et al., 2019) and classified among pathogenicity factor secreted during the decay of infected fruit caused by *P. expansum*.

In general, nutritional requirement seems to have a key role in the present study, indeed the role of nitrogen has to be discussed also for the postharvest pathogen. KEGG enrichment analysis highlighted the genes involved in metabolism of arginine and proline as well as the phenylalanine was significantly expressed (figure 17; table 11 see “common genes” and “*P. expansum* + *P. terrestris*”); at this regard these amino acids seem to be the most represented in the apple. In similar interaction was underlined that

nitrogen could be a limiting factor after 24 hours of growth in apple wound for the postharvest fungal pathogen as well (Vero et al., 2002). In our study specifically during the tritrophic interaction the growth seems supported by the availability of sugar as was highlighted by the GO (figure 14), but at the same time this growth contributes to nitrogen depletion. The transcriptomic evidence was supported by the in vivo experiment that demonstrate as during the biocontrol experiment *Penicillium expansum* was able to infect the 100% of the wounds treated with *P. terrestris PUT4* deletion mutant (Figure 43 A). Anyway, we cannot exclude that some permease could be involved in the secretion of some effectors or pathogenicity factors (Baev et al., 2004).

5.3 *Malus domestica*

In the present study the host response *M. domestica* during both dual and tritrophic interaction with the BCA LS28 and/or *P. expansum* was analyzed by studying its transcriptomic profile.

Overall, there are less *M. domestica* DEGs during the interaction with *P. terrestris* LS28 than during interaction with *P. expansum*, indicating a more robust transcriptional activation by the apple seems in response to the infection of the wounded fruit tissues operated by fungal pathogen. This likely reflects the different trophic relations established by this microorganism with the host. *P. expansum* being a necrotrophic pathogen that aims to kill the host tissues with invading hyphae and with the production an arsenal of pathogenicity factors (*e.g.* effectors, cell wall degrading enzymes, etc.), while *P. terrestris* establishing a non-pathogenic relation.

During the interaction with the BCA, the most enriched biological process in the host involves the metabolism of carbohydrates, with several beta-glucosidases that may work as potential activator of plants defense response when the integrity of the cell is compromised (Minic, 2007; Morant et al., 2008; Zhang et al., 2020). The *β*-glucosidase expression was also observed in other non-pathogenic relation as reported by Zhang et al. (2020) for apple-*Wickerhamomyces anomalus* interaction. Moreover, several studies report that this enzymes is involved in the production of toxic compound from an inactive glycosylate precursor, mainly being a key factor in the resistance against fungal pathogens (Sánchez-Torres & González-Candelas, 2003; Simmons et al., 2001).

In the dual interaction apple-BCA we didn't find a high expression level of genes involved in pathogen trigger immunity and effector trigger immunity (PTI and ETI). Receptor like protein and receptor like kinase (RLP and RLK) are known to play a crucial role in plant immunity, especially for the activation of the basal level of resistance, hence for the recognition of conserved patterns among pathogenic microorganisms (PRR or more specifically PAMPS, Pathogen-associated molecular patterns). RLP during the dual interaction apple-BCA are mainly downregulated except two upregulated genes, as shown in table 26.

The ortholog of *A. thaliana GLIP1* gene (MD17G1151700) was expressed during the interaction with the BCA and it seems to be related to the induction of resistance in the host (Oh et al., 2005).

Plant hormone signaling pathway may have a role also in the interaction with biocontrol agent; furthermore, the cross-talk between growth/defense is shifted from the growth side. Indeed, neither of those genes involved in the induction of the systemic acquire resistance (SAR) were found. Conversely, genes related to growth and development are

differentially expressed such as the gene MD16G1148400 that encodes for *GA2OX6* a gibberellin 2-oxidase (Han & Kahmann, 2019; Leone, Keller, Cerrudo, & Ballaré, 2014).

Is interesting that among downregulated genes by *M. domestica* induced by the BCA, there are some pectin methyl-esterase inhibitor (*PMI*) encoding genes that are usually upregulated in response to pathogen as reviewed by Wormit and colleagues; this downregulation provides to lowering the degree of methylation in pectin, that in turns allows cell wall loosening; this fact hypothetically can promote the interaction with the BCA and nutrients exchange. Moreover, in agreement with our transcriptomic data, the largest amount of *PMI* expressed gene is among upregulated DEGs during the interaction with the postharvest fungal pathogen (Lionetti et al., 2017; Wormit & Usadel, 2018). These differences in the host response to the BCA respect to the fungal pathogen may have a role in the molecular mechanism that drive the biocontrol properties of LS28.

Conversely, during the interaction with the fungal pathogen, several proteins involved in PTI and ETI were highly expressed (table 24 and 25). Indeed, cell wall-associated protein kinases (*WAK*) exhibit a higher level of expression in response to the pathogen respect to the BCA-treated apple. *WAK* and *WAKL* are widely represented; in particular seven *WAK2* encoding genes are upregulated during the interaction with pathogen. In *Arabidopsis WAK1* and *WAK2* and are involved in the perception of oligogalacturonic acid, having as main function the monitoring of the degrading status of the cell wall targeted by fungal pathogen degrading enzymes (CWDE) (Andersen et al., 2018; Mengiste, 2011; Yuan et al., 2021).

Involved in PRR perception *CERK* genes are also highly expressed in presence of *P. expansum*; *CERK* is essential for chitin-mediate recognition and defenses in *Arabidopsis* as described by Petutschnig and colleagues (2010).

After the recognition mediated by RLK and RLP during the interaction with *Penicillium expansum* an oxidative burst is mediated by *RHBDO* that was able to modulate calcium dependent kinase protein (*CDPK*); this pathway was represented as whole in response to the fungal pathogen (for the list of genes expressed see table 24). Furthermore, several resistance proteins were found and included in the effector trigger immunity pathway (ETI) (table 25).

The main difference between PTI and ETI is that the first start from a surface recognition of PAMPs and is non-specific, while ETI is triggered intracellularly by NBS-LRR type-receptors and is more durable and targeted; anyway, is important to point out that these two pathways are correlated and interconnected (Yuan et al., 2021).

In general response to necrotrophs is mediated by Jasmonate-Ethylene signaling pathway that is known to have an antagonistic regulation respect to SA mediated defense response. Furthermore, during the interaction with *P. expansum* the jasmonate-mediated signaling was detected in relation to the presence of *JAZ10* encoding genes (MD13G1127100 and MD16G1127400) and *TFY* genes that encode for transcription factors family identified in apple (Li et al., 2014). Accordingly, in *Arabidopsis* JAZs repressor protein was a central regulator of the jasmonate signaling pathway; indeed, upon perception of bioactive Jas, JAZ proteins are rapidly degraded via the ubiquitin/26S proteasome-dependent pathway (Corn et al., 2014).

Furthermore, JAZs were able to interact with *MYC* transcription factor, another essential protein in the activation JA-responsive genes (Fernández-Calvo et al., 2011) encoded by MD06G1120000 (orthologs of *MYC4*) in *P. expansum* treated apple. Ethylene responsive factors (*ERF*) and genes involved in the ethylene biosynthesis (*ACO4*) were upregulated in response to the fungal pathogen as well as *MYB* transcription factors confirming the

fact that JA and ethylene defense response was active when the integrity of the cell was lost (Ballester et al., 2017).

At molecular level was found that *WRKY* that have a key role in the regulation of systemic acquire resistance in plant. In *A. thaliana* more than 72 genes encoding Wrky transcription factors (Tfs) that are induced by salicylic acid (SA) (Dong, Chen, & Chen, 2003). As reported by Wang and colleagues Wrky transcription factor is able to bind a consensus sequence of several genes as NPR1, important key regulator, that in turn mediate the expression of pathogenesis related genes (PR genes) interacting with TGA transcription factors, as reported by (Fan & Dong, 2002; Wang, Amornsiripanitch, & Dong, 2006).

In *Malus domestica* we found that several class of *WRKY* encoding genes are differentially expressed during the interaction with the fungal pathogen; furthermore, in this condition also *NPR1* encoding gene is differentially expressed (MD05G1256300). *WRKY* are upregulated in presence of calcium signal modulator (through calmodulin encoding genes *EDA39*, upregulated DEGs in *Penicillium*-treated apple) and activated by elicitors molecules as lipopolysaccharides, flagellin or chitin oligomers that collectively are described as pathogen/microbes associated molecular patterns (PAMP), (Phukan, Jeena, & Shukla, 2016) (see figure 28).

Moreover, in the apple treated with LS28 we found one *WRKY* encoding gene, *WRKY48* (MD16G1151000) differentially expressed; surprisingly Xing and colleagues remarks that *WRKY48* was the only *WRKY* gene not induced by salicylic acid; indeed, as reported by Xing et al. (2008) *wrky48* deletion mutant showed high level of PR1, PR2, and PR5 on the contrary, the constitutive expression of *wrky48* block the expression of the pathogenesis related protein in *Arabidopsis* concluding that *WRKY48* was a negative

regulator of plant basal defense. Several evidences seems to corroborate the fact that some specific members of WRKY superfamily of transcription factors as *WRKY7*, *WRKY11*, and *WRKY17* are negative regulator of plant basal defense (Chen, Zhang, & Yu, 2010), none of these was present in our study during the interaction with the postharvest fungal pathogen.

6 Conclusions

Through the application of RNAseq performed in the present study we attempt to elucidate the biological processes that act at the same time in the biocontrol agent *P. terrestris*, in the pathogenic fungus *P. expansum*, and in the host M. domestica during their tritrophic interaction. Our data confirms that competition for nutrients, mainly nitrogen and carbon deprivation mediated by several permeases, in conjunction with resistance to oxidative stress is critical for the BCA to outcompete the pathogen.

About fungal pathogen, our study corroborates previous transcriptomic studies of *P. expansum* during interaction with the host, with the activation of virulence genes and pathogenicity factors to infect and invade the apple tissues. In the presence of the BCA, *P. expansum* activate genes important to directly outcompete the biocontrol yeast, suggesting a substantial nutritional rewiring of the pathogen. Last, analysis of the transcriptomic changes of the host *M. domestica* revealed overexpression of genes involved in host defense signaling pathways in the presence of both the BCA and the pathogen, and a prevalence of pathogen triggered immunity (PTI) and effector trigger immunity (ETI) host genes overexpressed only during interaction with *P. expansum*. This comprehensive analysis contributed to advance the knowledge on the molecular mechanisms that underlie biocontrol activity and the tritrophic interaction of the BCA with the pathogen and the host.

7 Figures

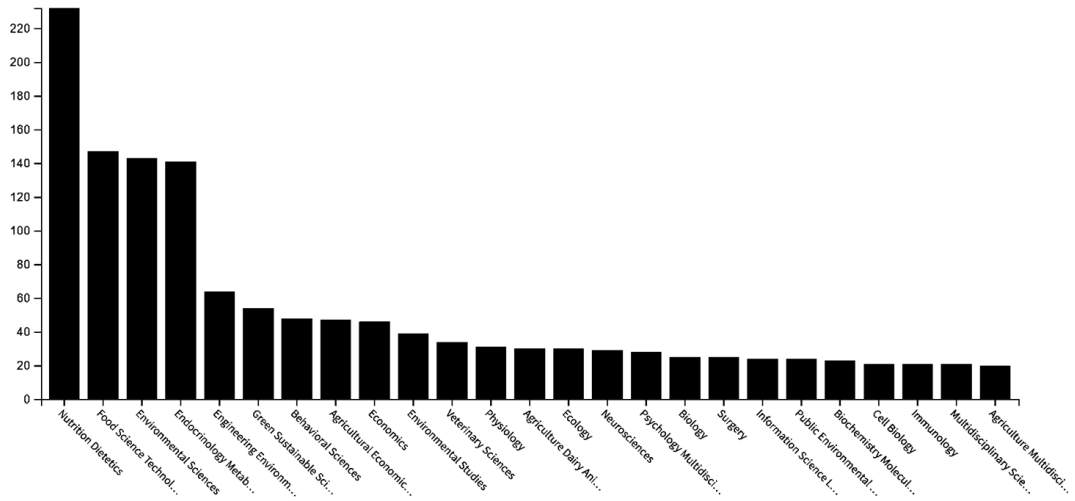

Figure 1. Graphical output obtained by Web of Science by typing “food losses” as a keyword; about 1.150 publications were distributed among many different scientific disciplines (disciplines x *axis*).

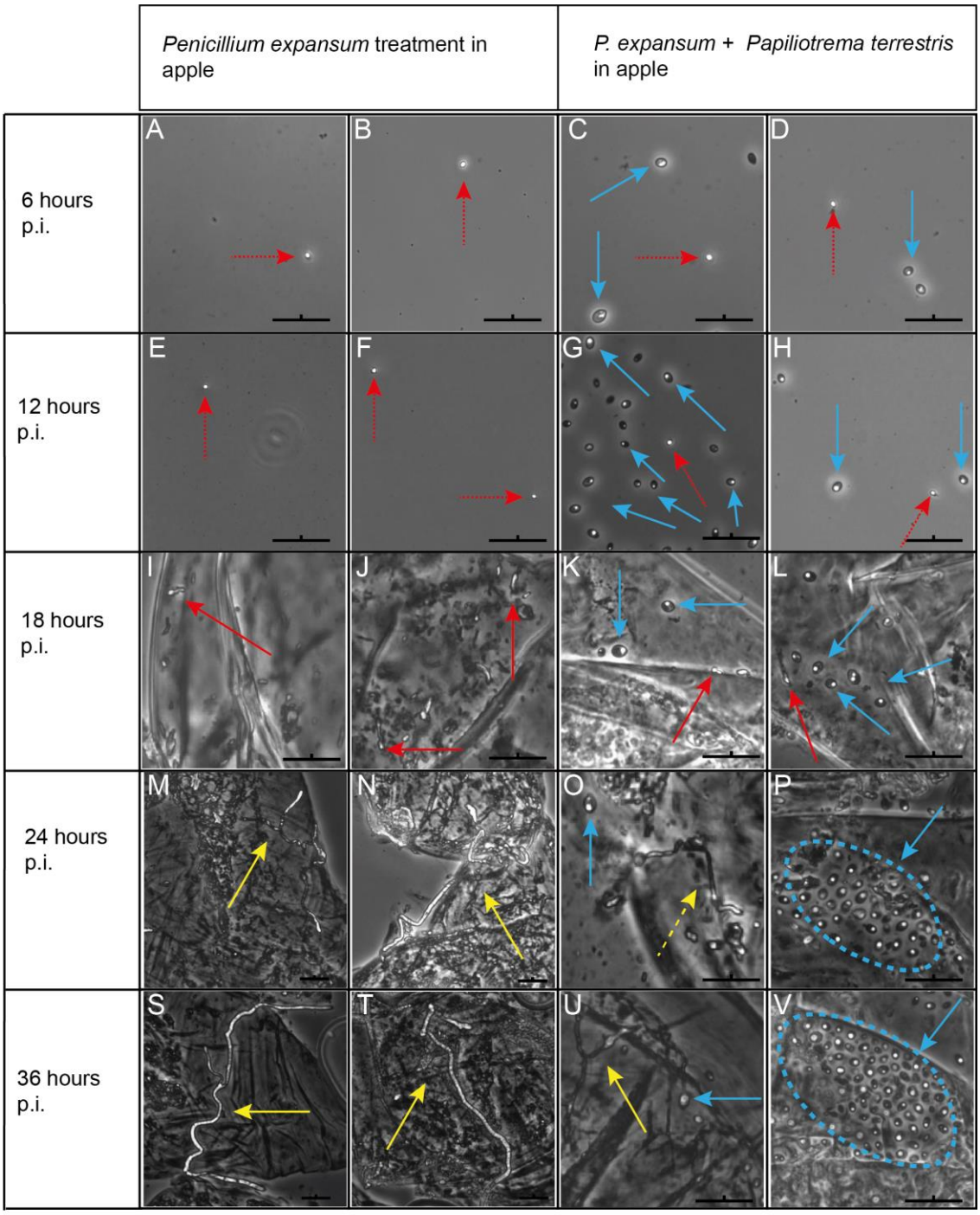

Figure 2. Tritrophic interaction at microscopic level. The reference scale at right corner of each image is referred to 25 µm in all the cases. Blue solid arrows indicate yeast cell of *Papiliotrema terrestris* LS28; red dashed arrows indicate non-germinated *P. expansum* conidia; red solid arrows indicate *P. expansum* germinated conidia; yellow dashed arrows indicate non-branched hyphae, and yellow solid arrows indicate branched

P. expansum hyphae. In the blue dashed-circle (P and V) is highlighted the colonization of the yeast that compete for space in the micro-niche at 24 and 36 h.p.i.

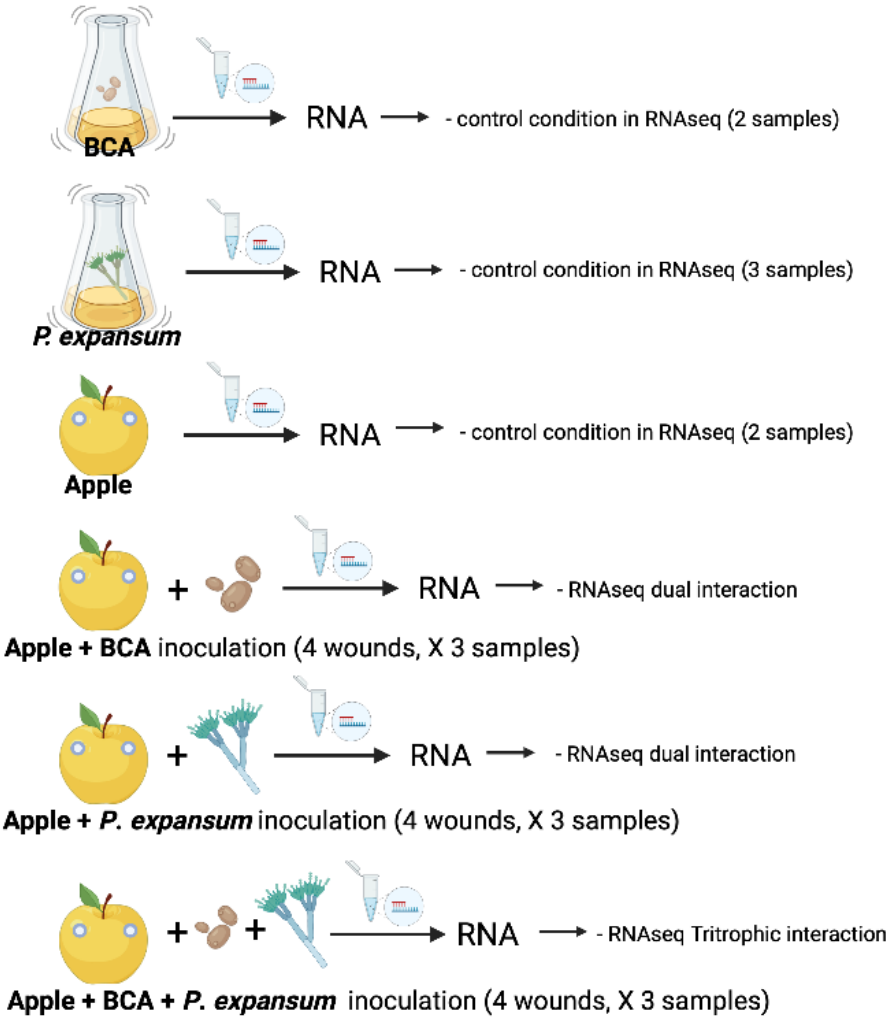

Figure 3. Illustration of the experimental design used to perform the RNAseq analysis.

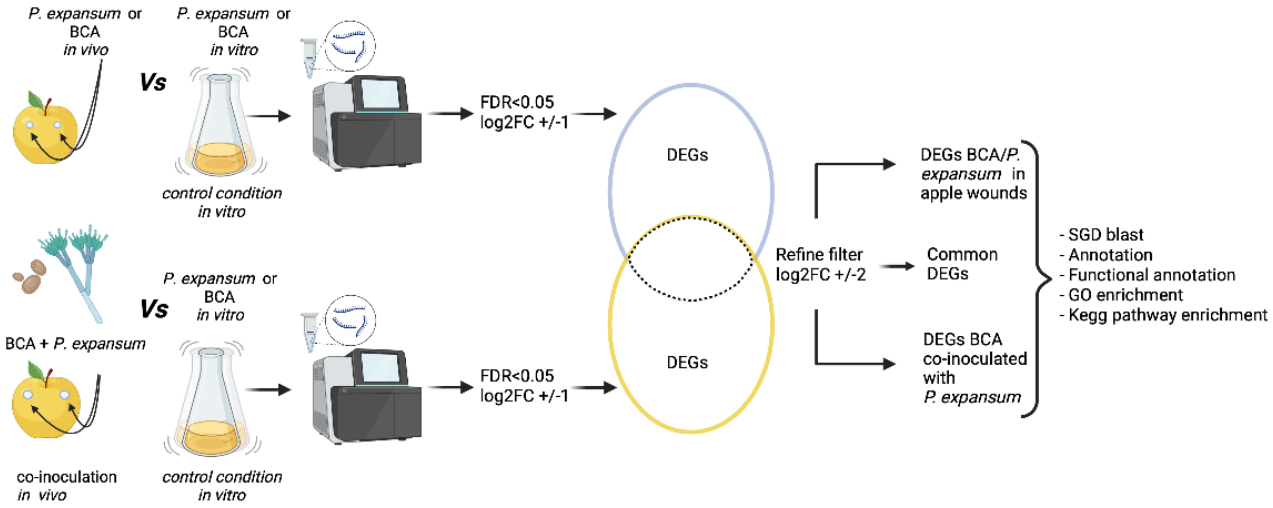

Figure 4. Flow chart description of the pipeline applied to get DEGs for LS28 and *P. expansum*.

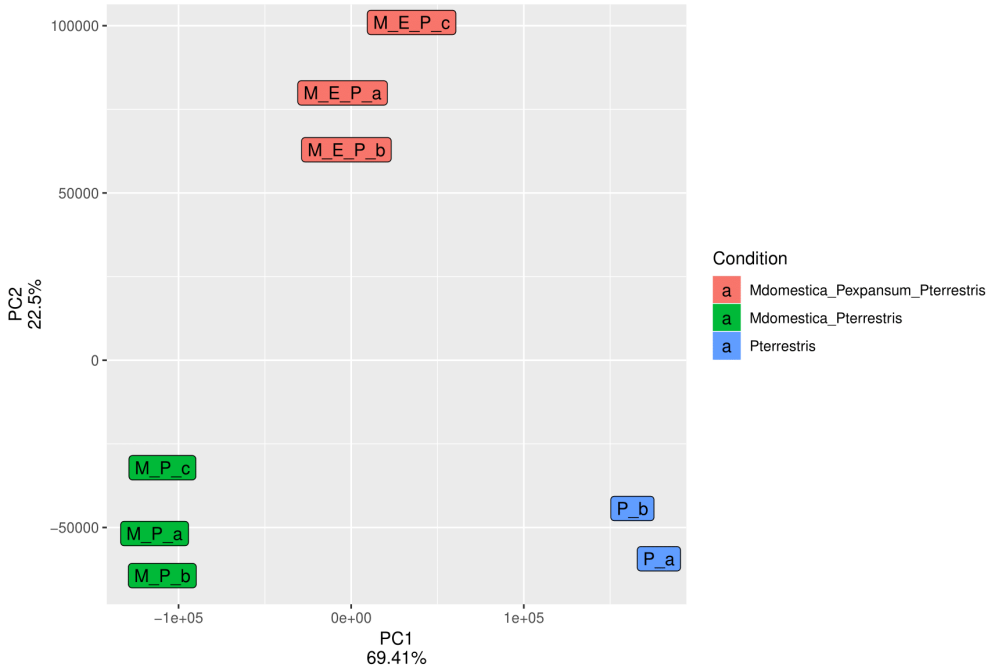

Figure 5. Principal Component Analysis relative to *P. terrestris* samples.

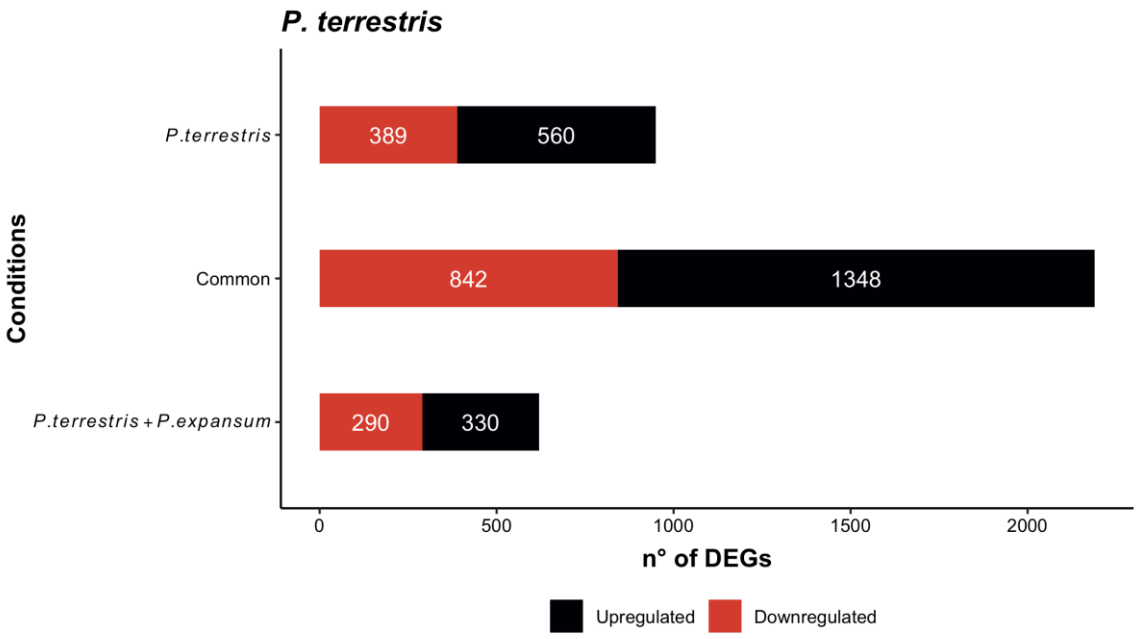

Figure 6. Representation of the amount of DEGs with upregulate and downregulated fold change (applying cut off FDR < 0.05; log2FC +/-1) according to the respective condition, based on the starting datasets available in the supplemental material S1 and S2, illustrated in the figure 4 (third arrow).

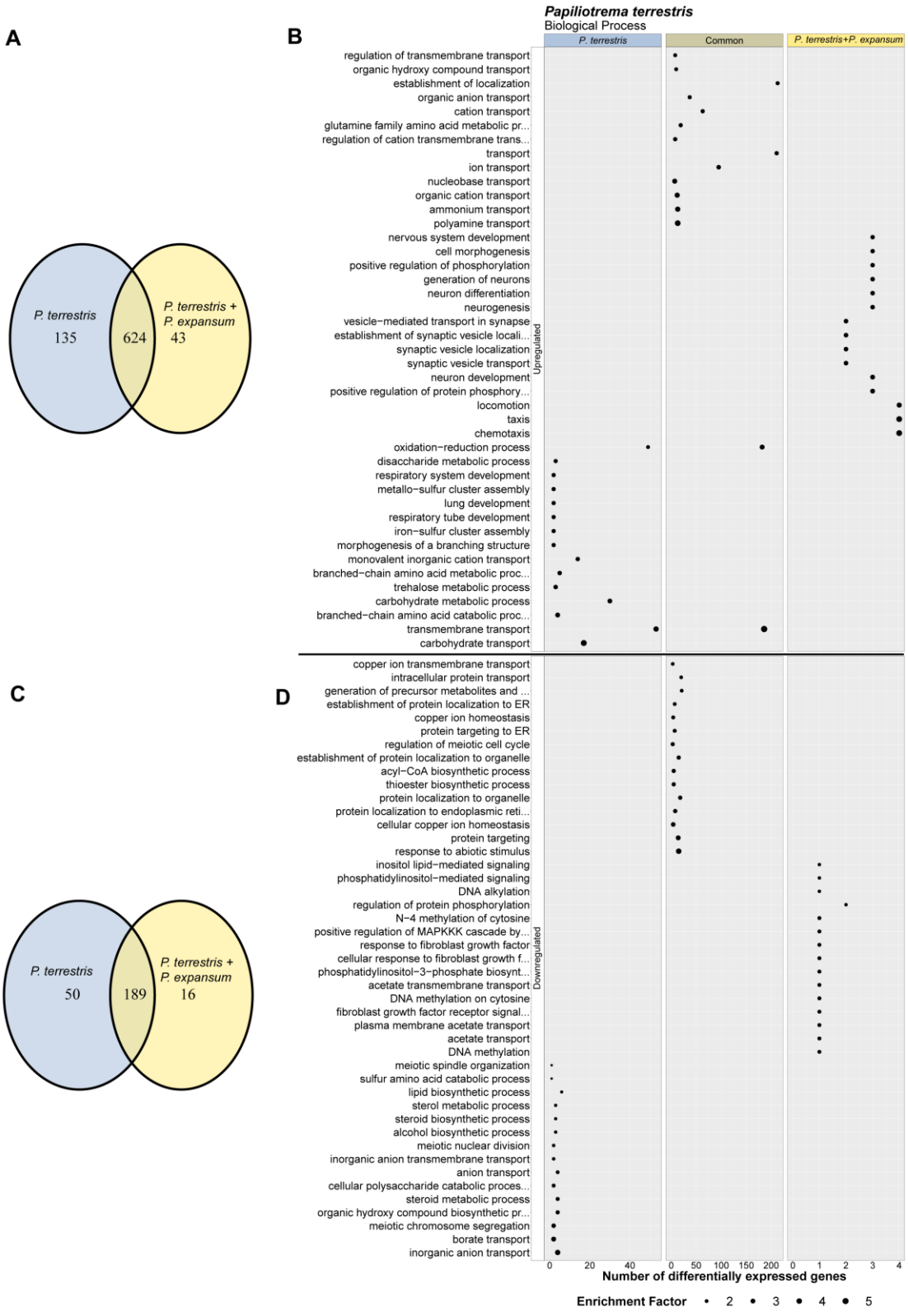

Figure 7. A and C, Venn Diagram of upregulated and downregulated DEGs obtained applying logFC +/-2 as cut-off, respectively; in blue were represented DEGs expressed by LS28 in apple *Vs*. DEGs of LS28 + *P*. *expansum* in apple (in yellow). B and D, GO

enrichment analysis of upregulated and downregulated DEGs, described by dot dimension, referred to the enrichment factor (bigger dots mean the process is more significant is in terms of statistics).

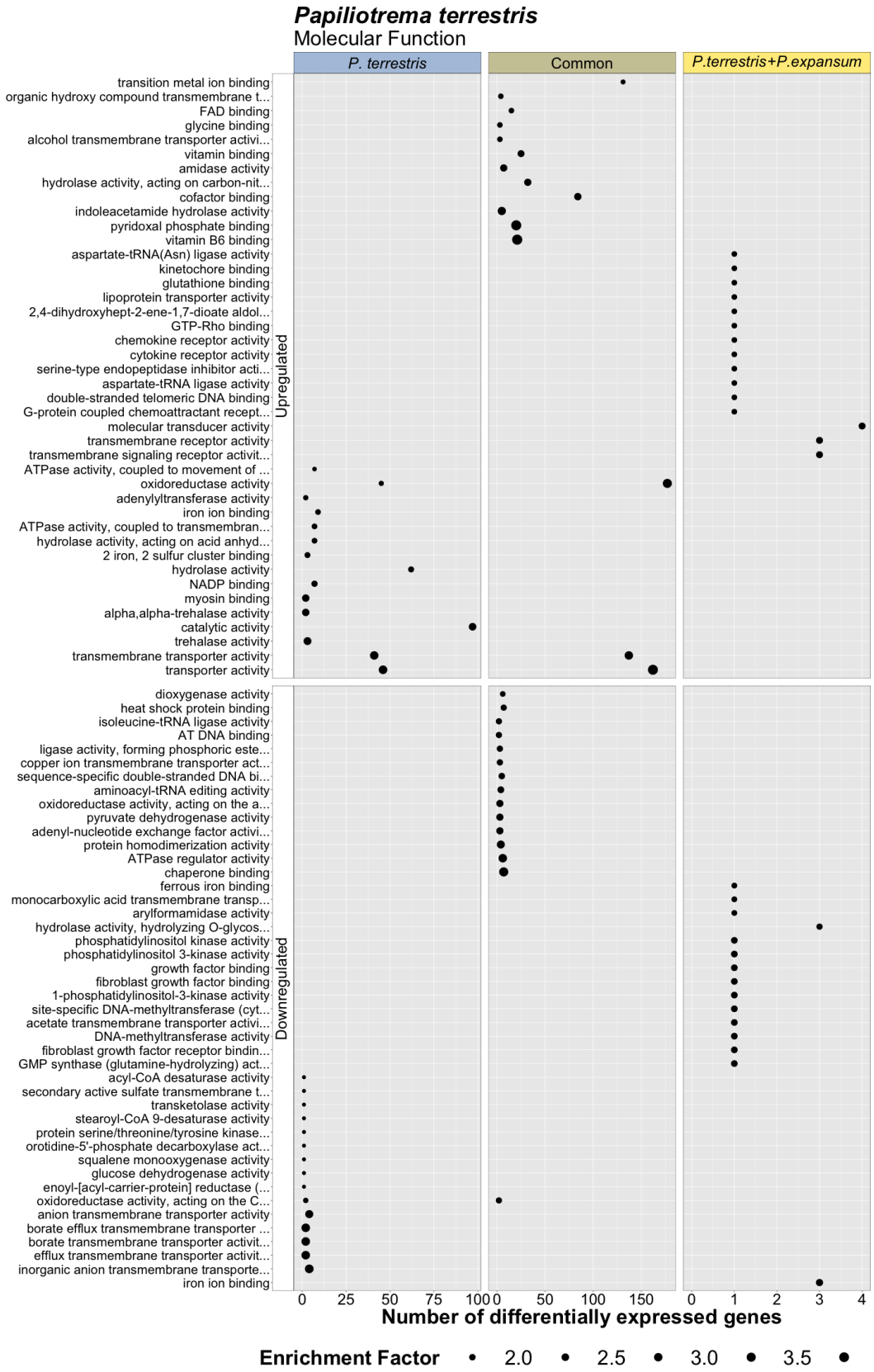

Figure 8. Most enriched Molecular Function of *Papiliotrema terrestris* LS28 *versus P. terrestris* + *P. expansum* both in apple. Transporter activity is the most enriched terms

during apple colonization by the BCA (blue panel). In the common dataset redox activity and transporter activity are still the most significant. Molecular transducer activity is the most enriched terms in the interaction with postharvest fungal pathogen (yellow panel). Enrichment factor is calculated by the algorithm and is represented by dots dimension (see material and methods).

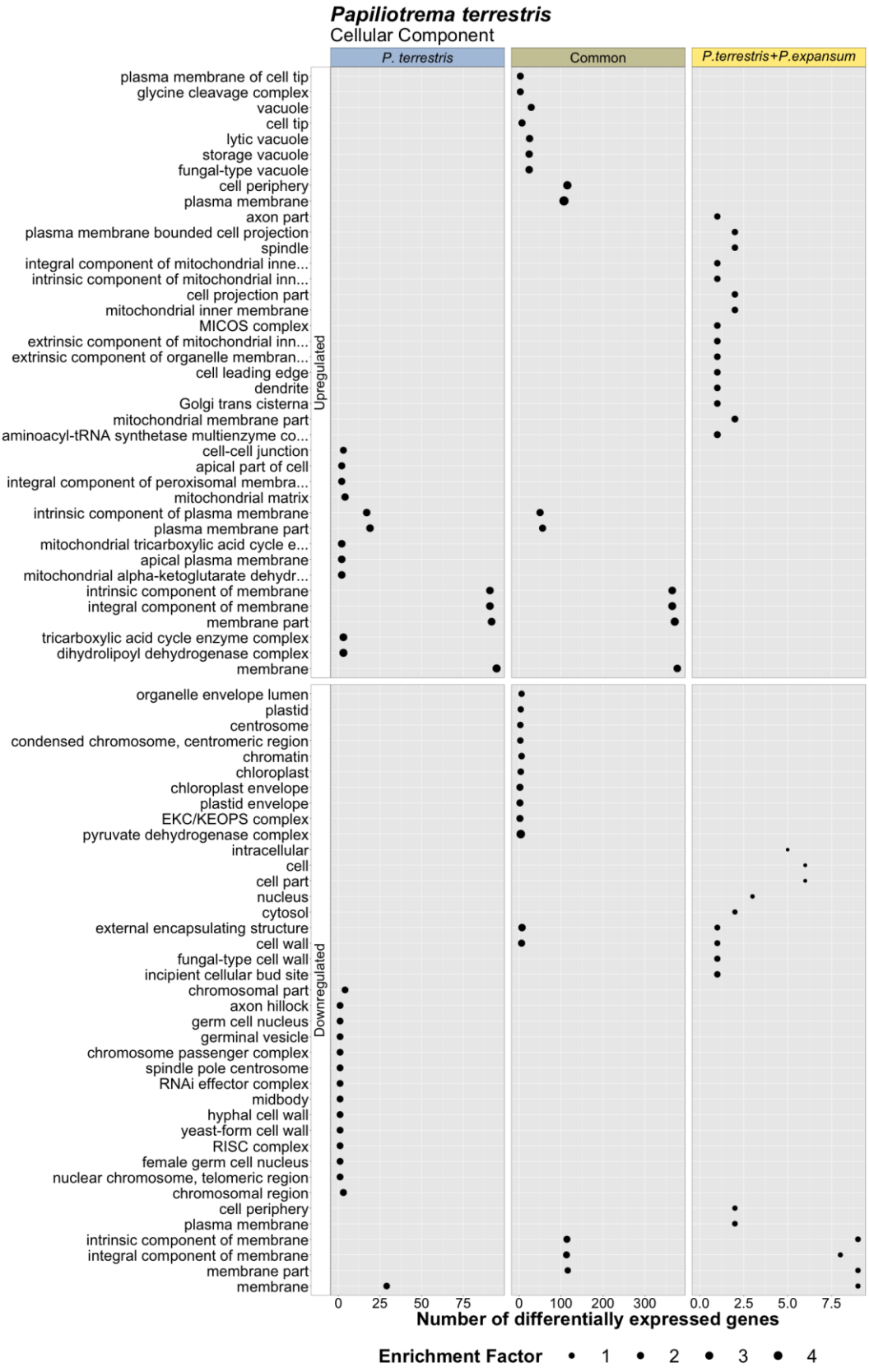

Figure 9. Cellular component enrichment by GO for *Papiliotrema terrestris* LS28 *versus P. terrestris* LS28 + *P. expansum* both in apple.

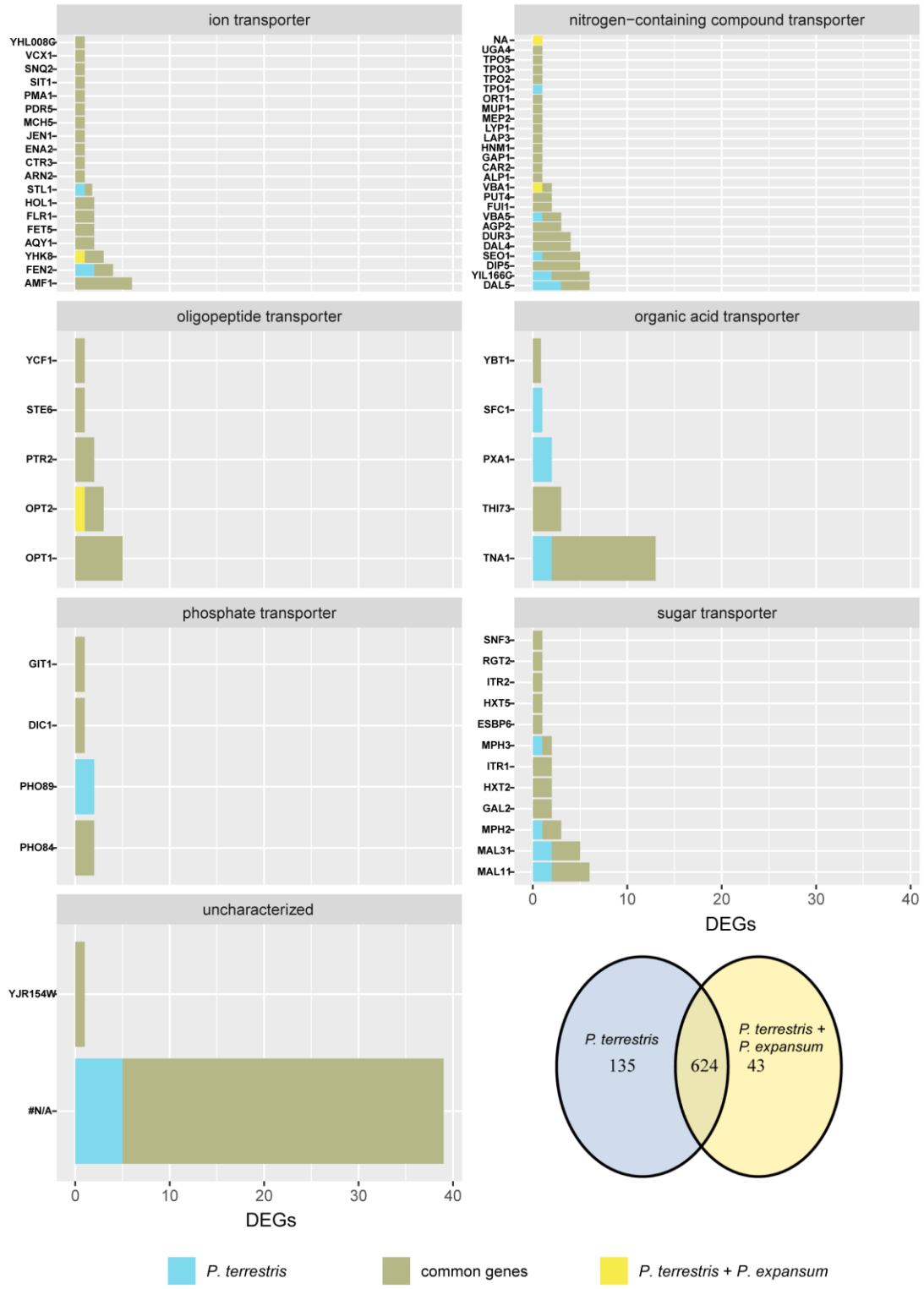

Figure 10. Transmembrane transporter annotated by the GO and described according to their main function in each condition.

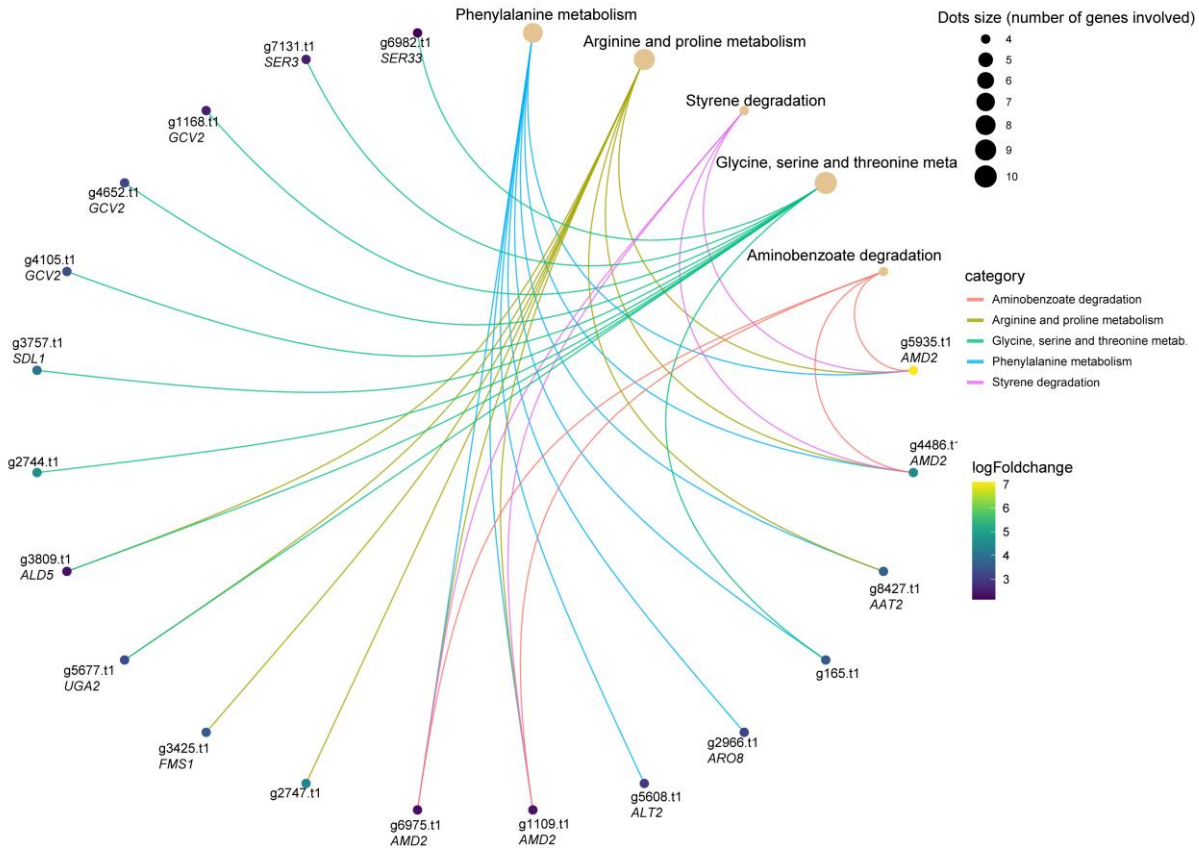

Figure 11. KEGG enrichment analysis of the most significant “common genes” dataset of *P. terrestris* LS28, listed in table 3.

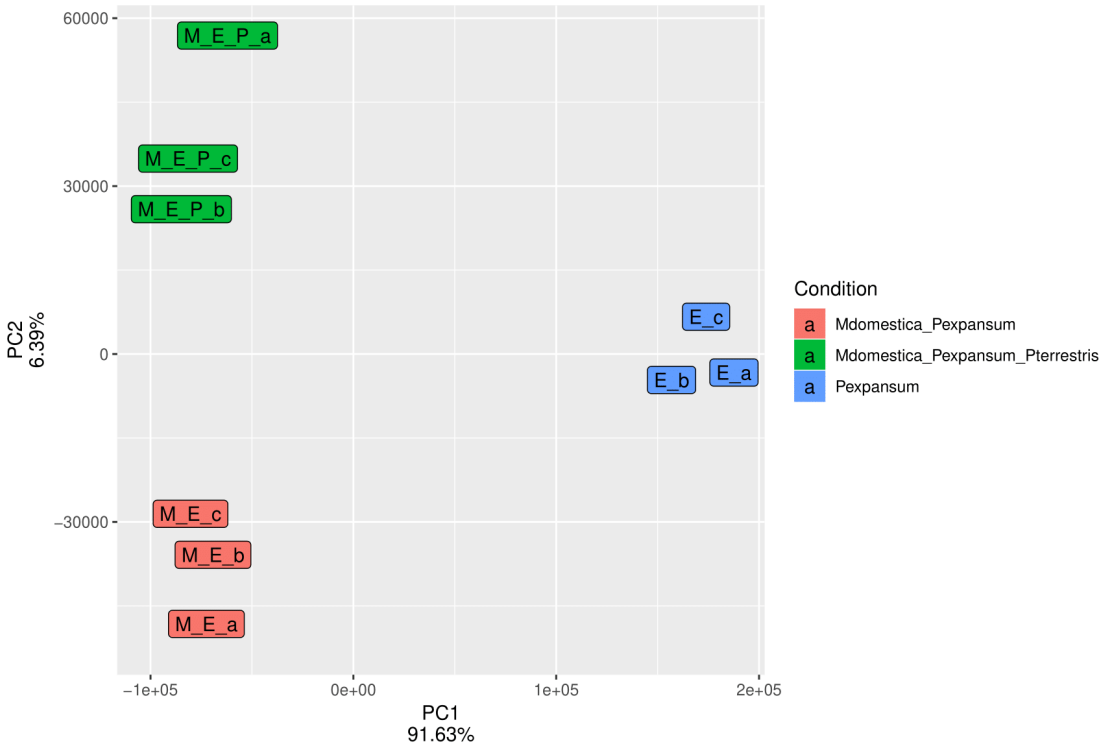

Figure 12. Principal Component analysis based on *Penicillium expansum* samples.

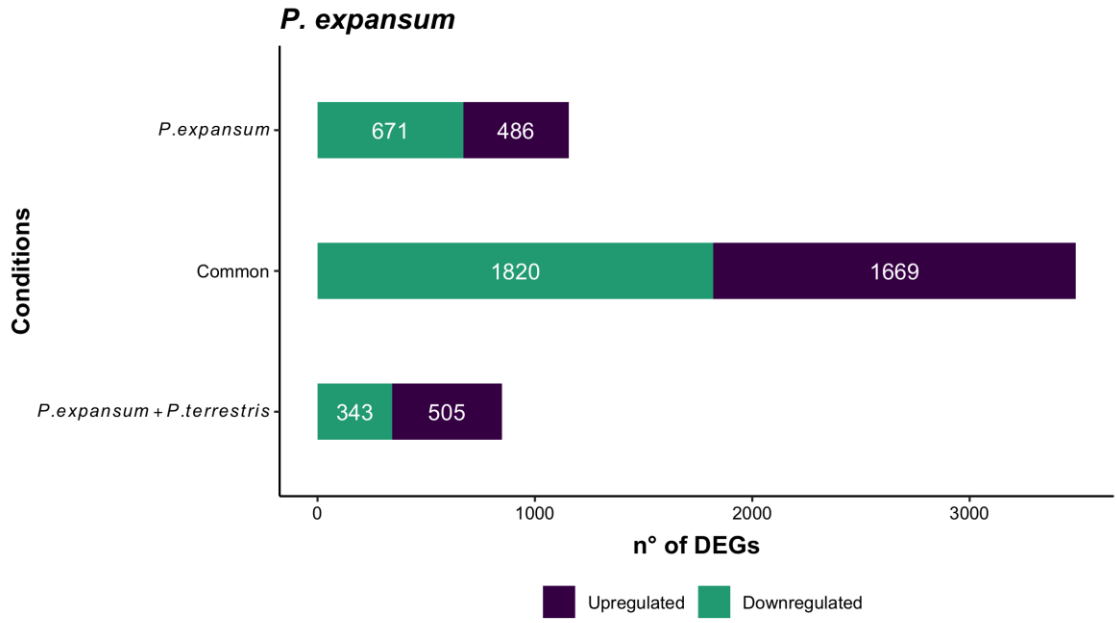

Figure 13. Comparison within DEGs upregulated and downregulated of *Penicillium expansum* (FDR < 0.05; log2FC +/-1) referred to the list present in the supplemental file S3 and S4.

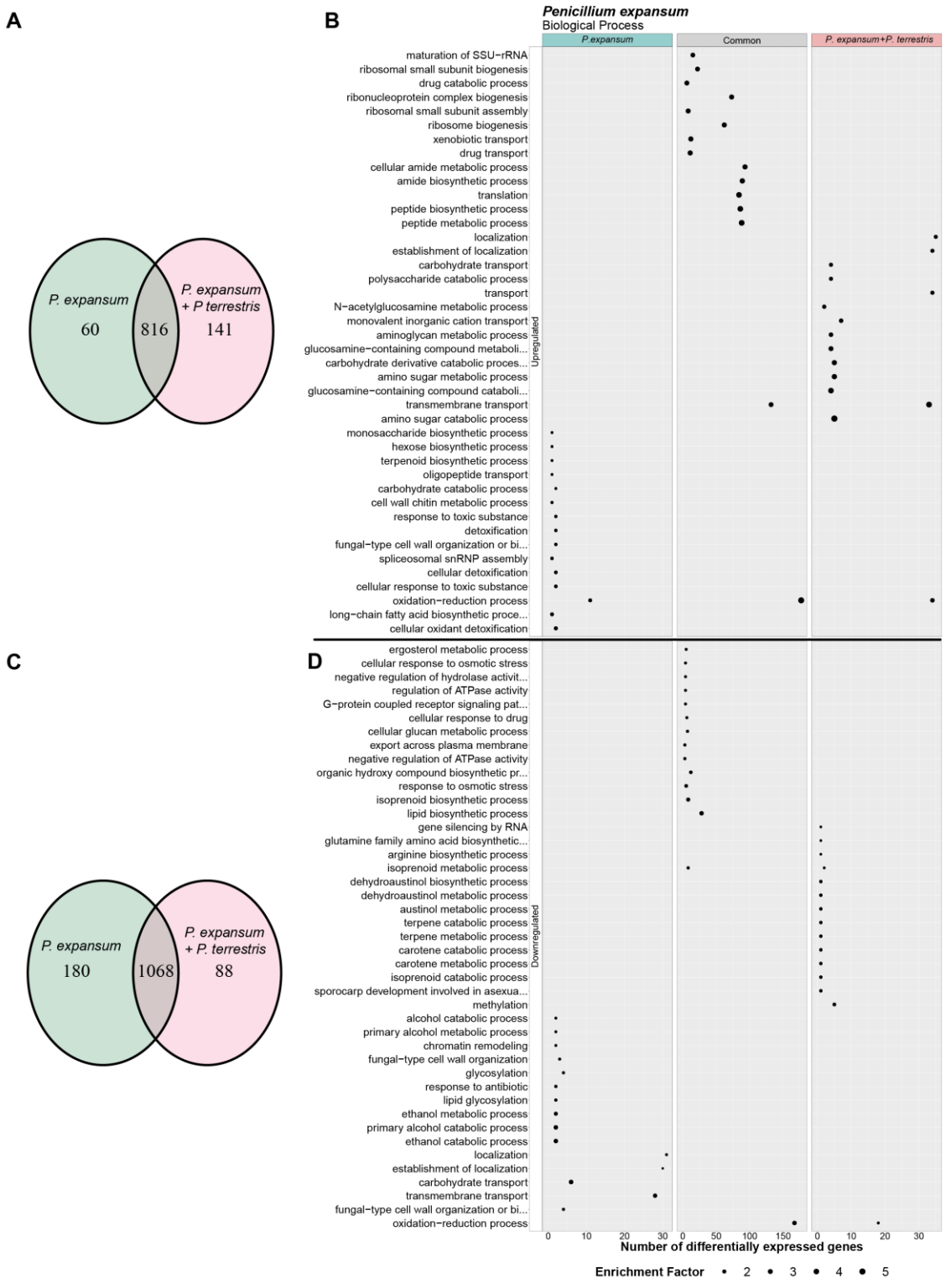

Figure 14. A and C, Venn Diagram of upregulated and downregulated DEGs of Penicillium expansum (fold change +/-2), versus P. expansum + P. terrestris. B and D, most enriched of Biological Process of upregulated and downregulated DEGs.

Figure 15. GO enrichment analysis of Molecular function category for *Penicillium expansum* DEGs.

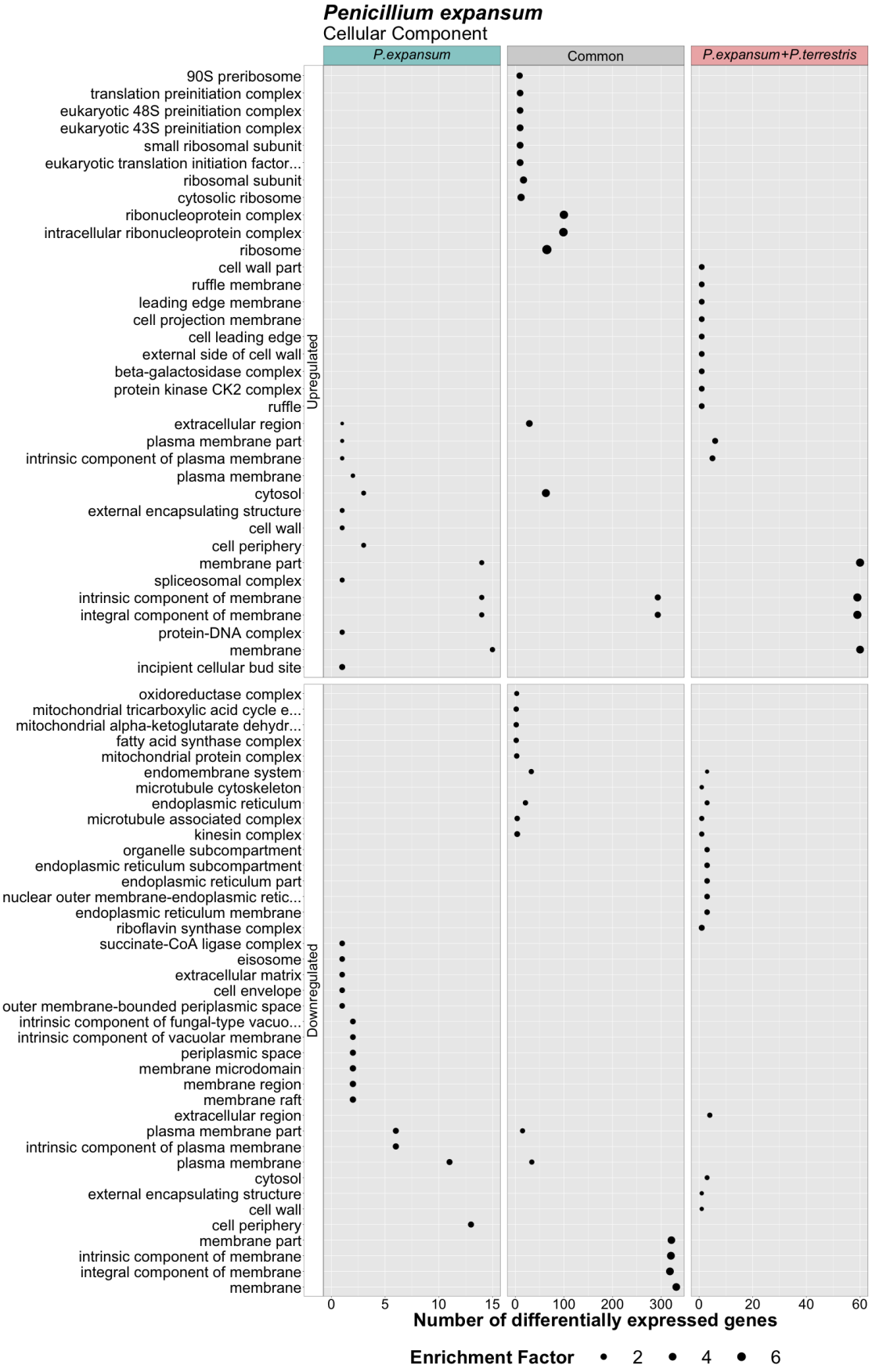

Figure 16. GO enrichment analysis of Cellular component category for *Penicillium expansum* DEGs.

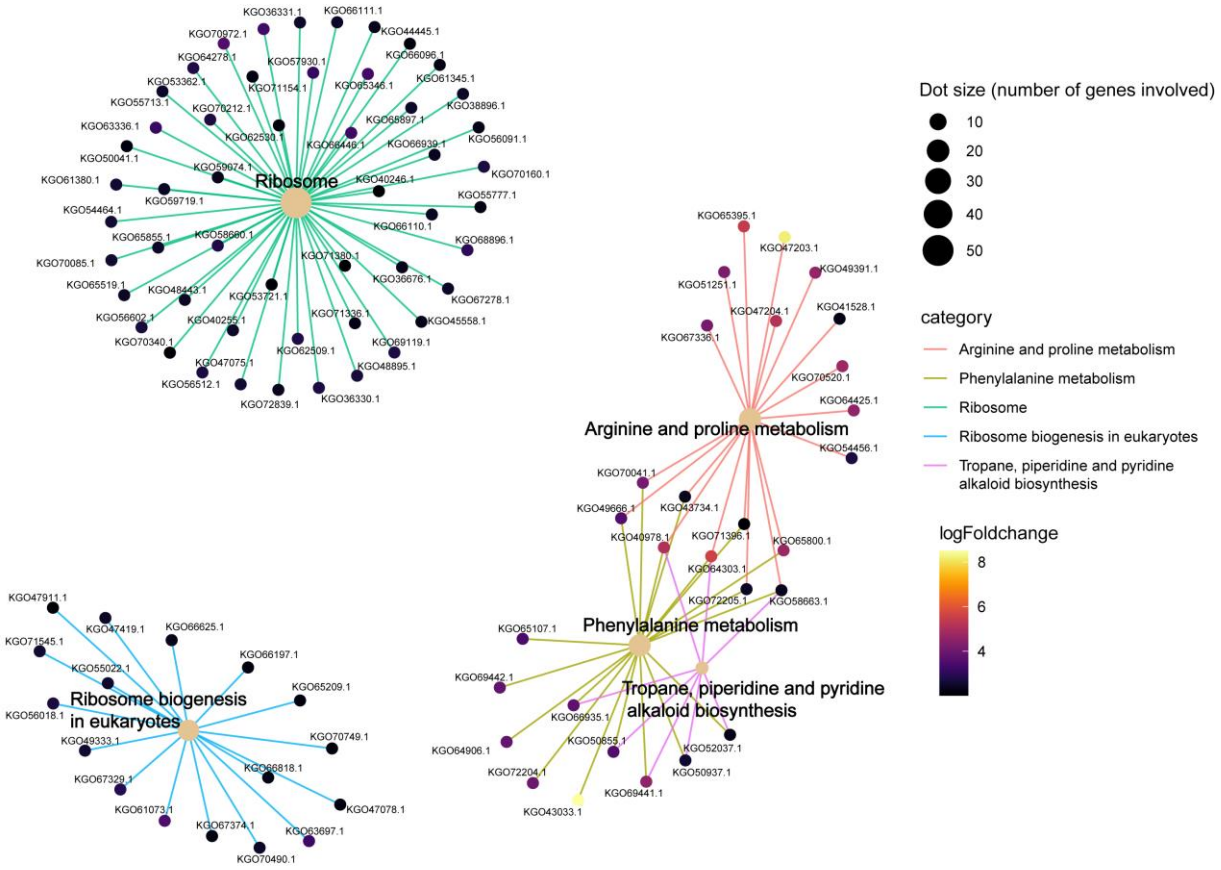

Figure 17. KEGG graphical representation of “common genes” dataset of *Penicillium expansum*, and related protein involved in the most significantly enriched pathway.

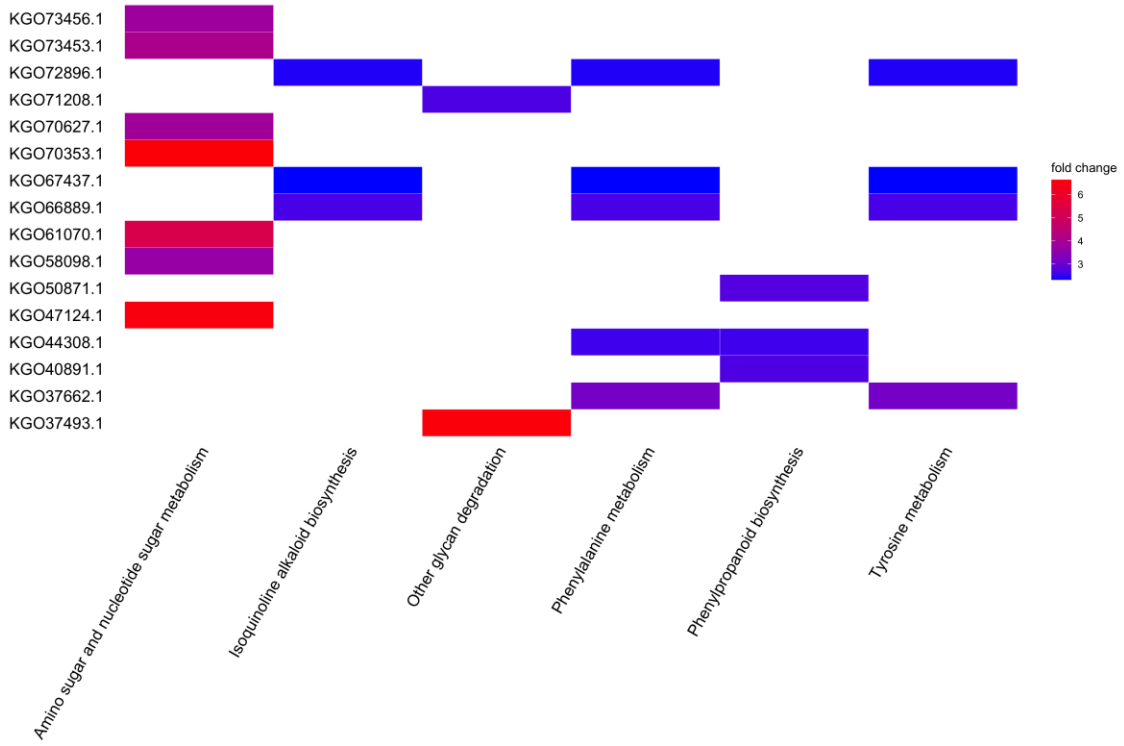

Figure 18. Most enriched KEGG pathway in the interaction with LS28, represented by heatmap. The y axis report the protein ID of *P. expansum* related to each patway (on the x).

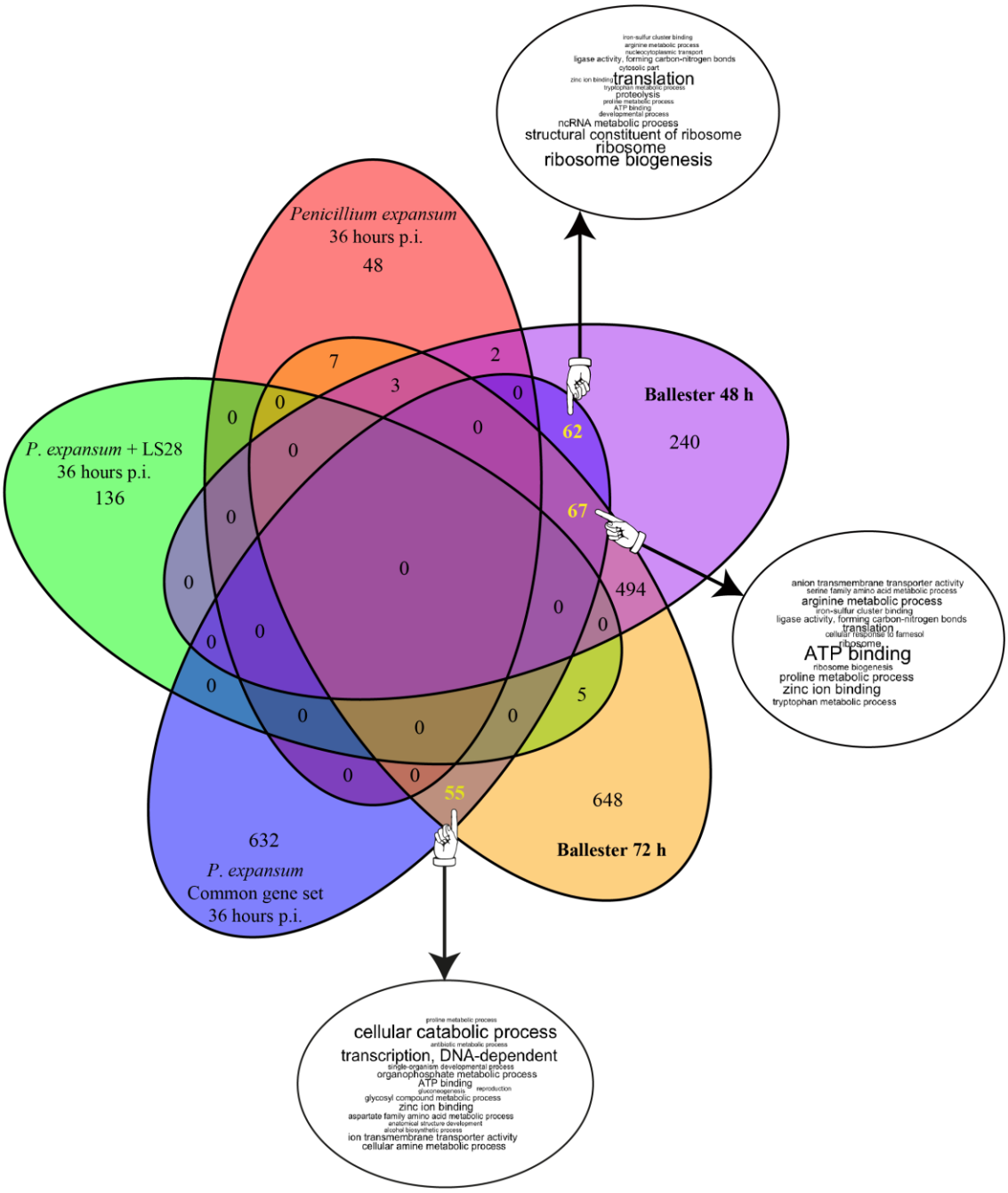

Figure 19. Venn Diagram allowing the comparisong between the most enriched DEGs obtained from the GO described in the RNAseq of Ballester et al. (2014) and the DEGs of the present study.

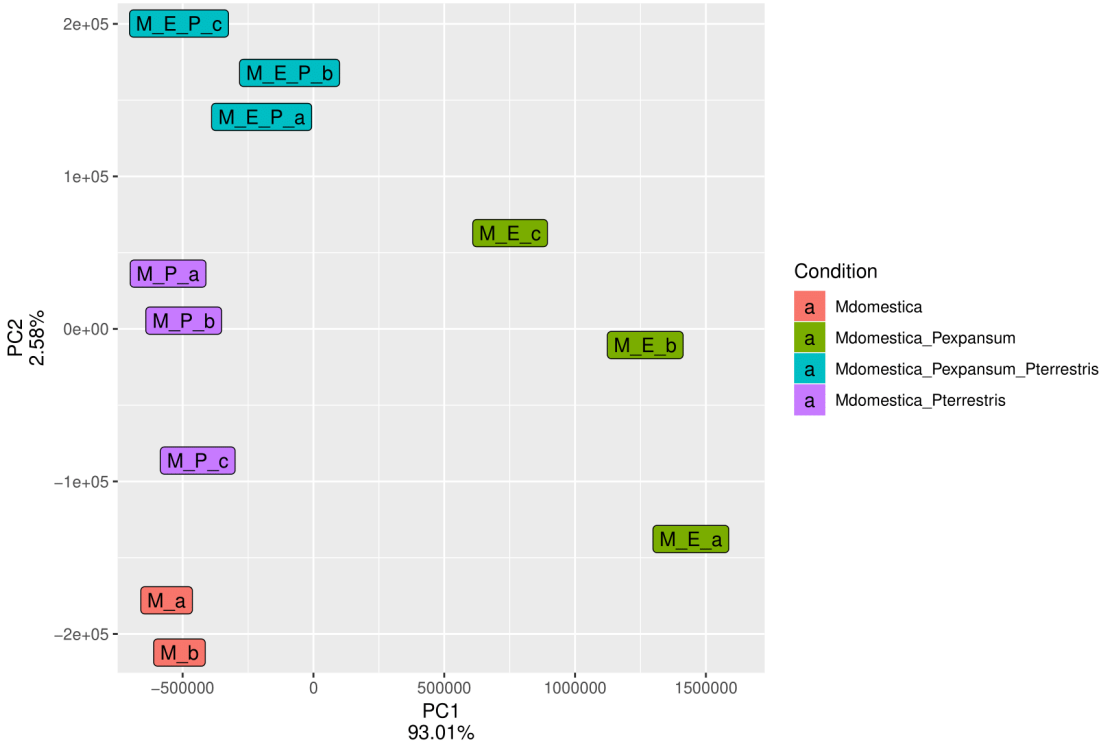

Figure 20. Principal Component Analysis of *Malus domestica* samples.

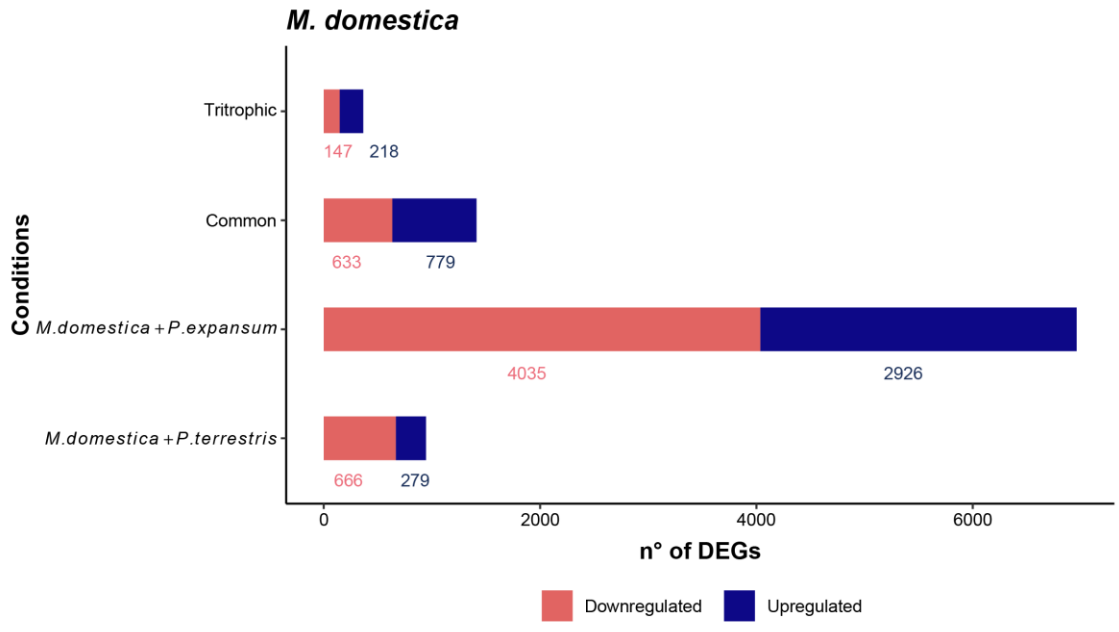

Figure 21. Illustration of the starting datasets of DEGs obtained from *Malus domestica*, (applying cut off FDR < 0.05; log2FC +/-1 as filter) available in supplemental material, file S5 and S6.

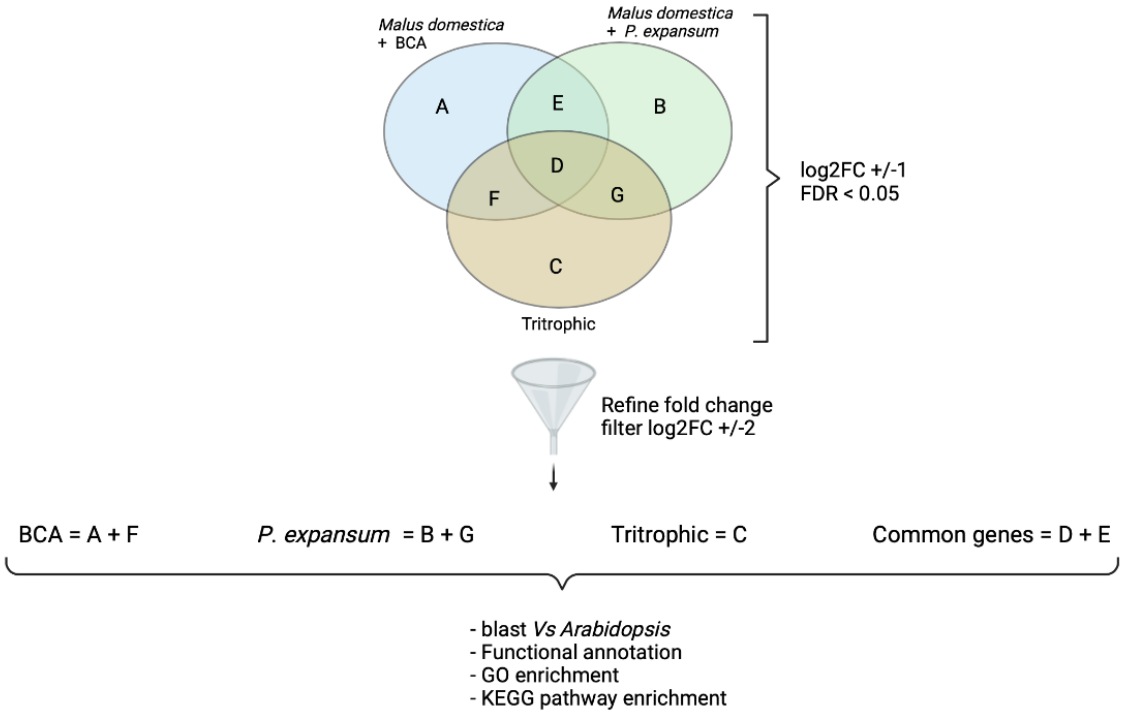

Figure 22. Flow chart illustration (starting from the datasets obtained applying log2FC +/- 1 as filter) of the pipeline applied to get DEGs in *M. domestica* and grouped lists (log2FC +/-2) on which GO enrichment and KEGG enrichment are based.

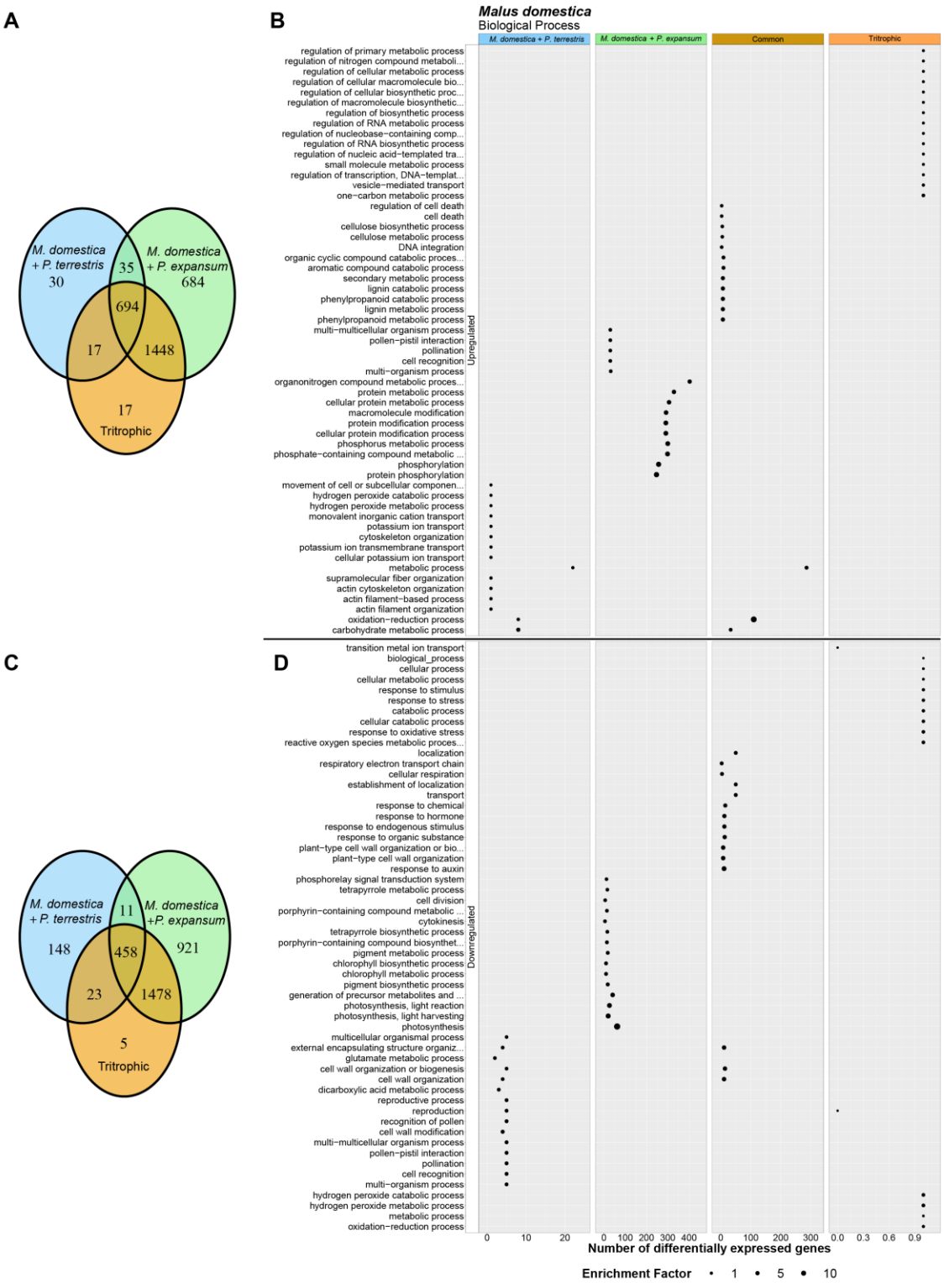

Figure 23. Venn Diagram of DEGs datasets upregulated (A), and downregulated (C) of *Malus domestica*. GO enrichment graph of biological process of *Malus domestica* (B and D).

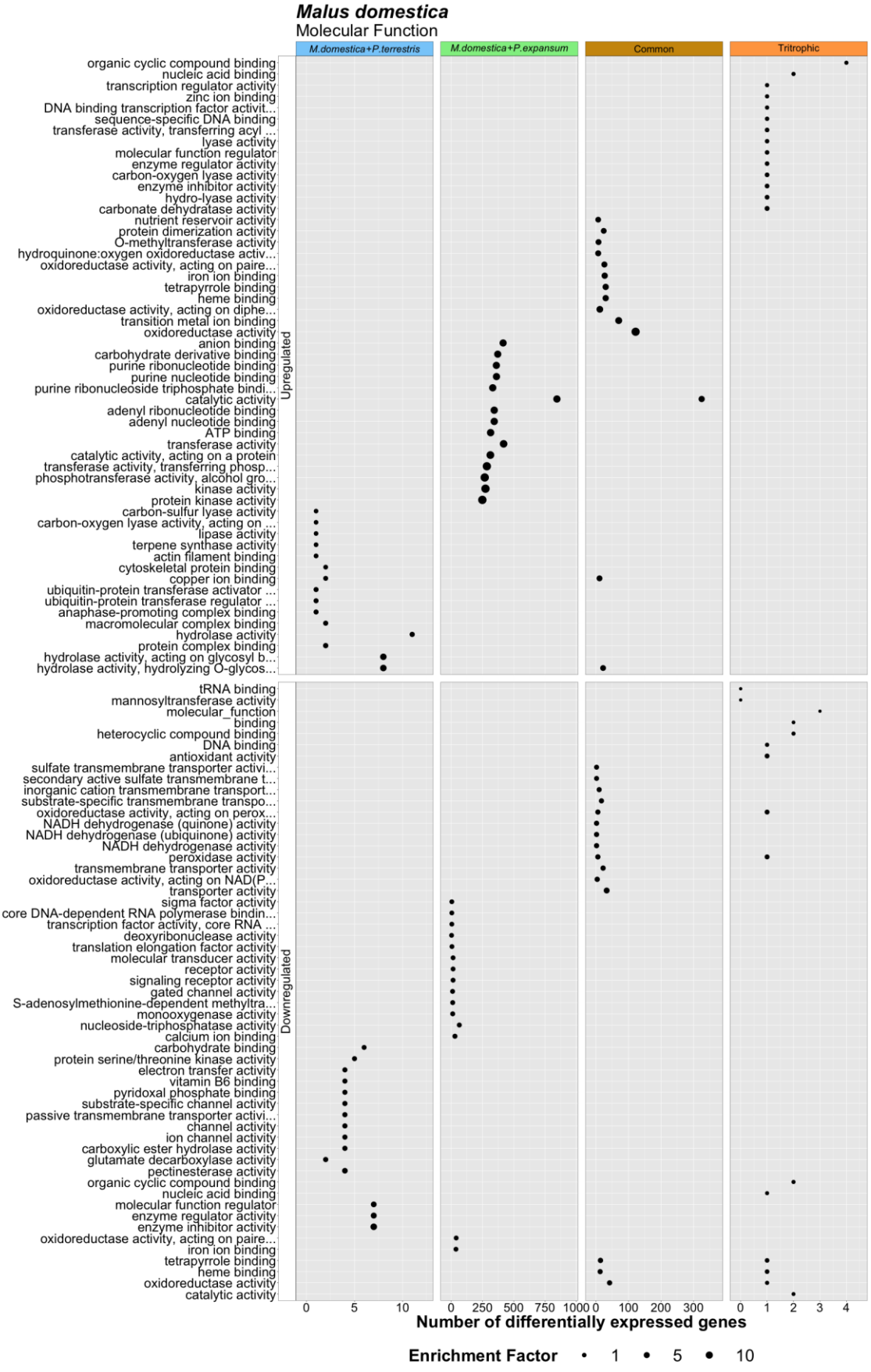

Figure 24. GO enrichment analysis of Molecular function category for DEGs of *Malus domestica*.

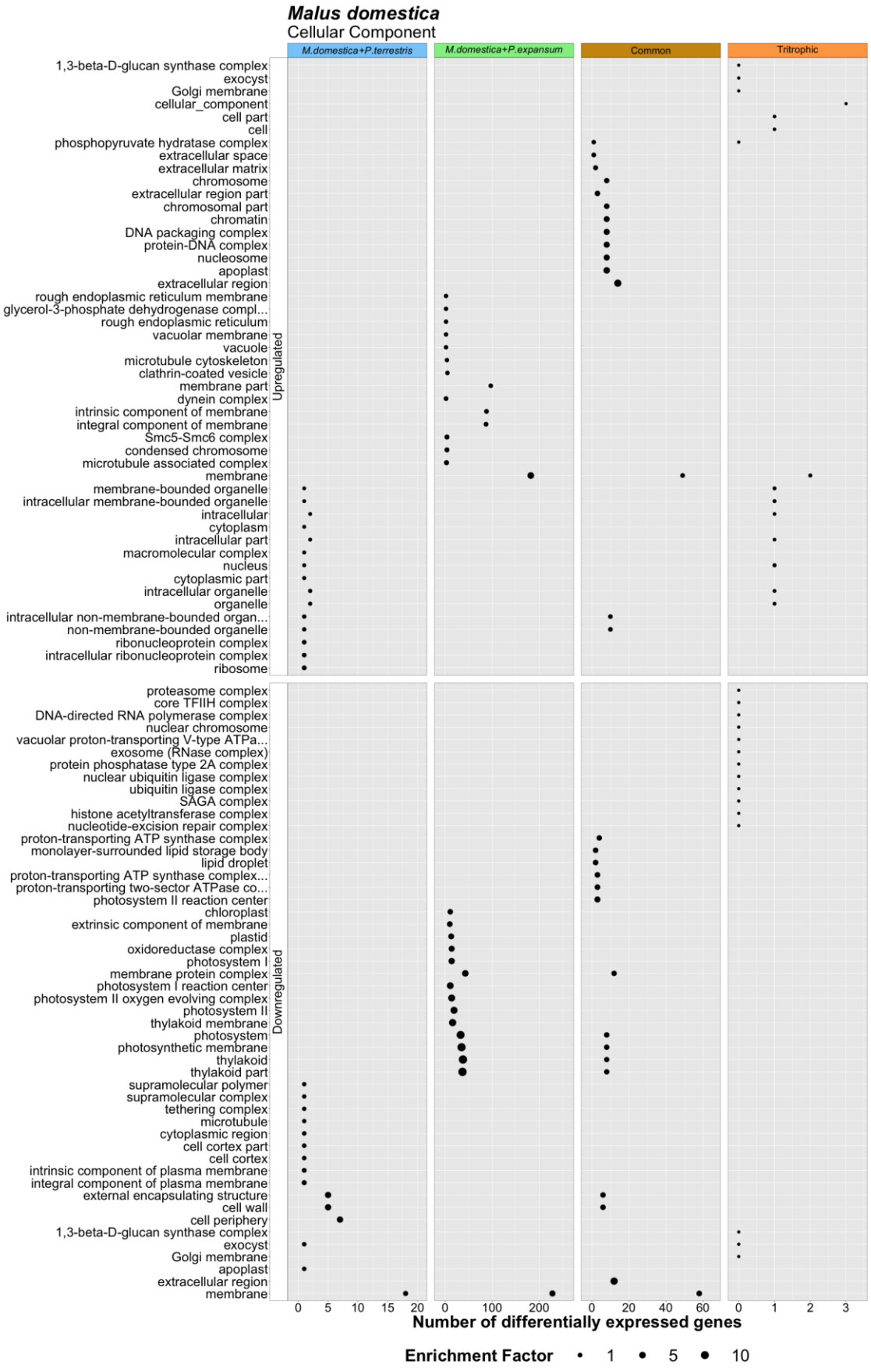

Figure 25. GO enrichment analysis of Cellular component category for DEGs of *Malus domestica*.

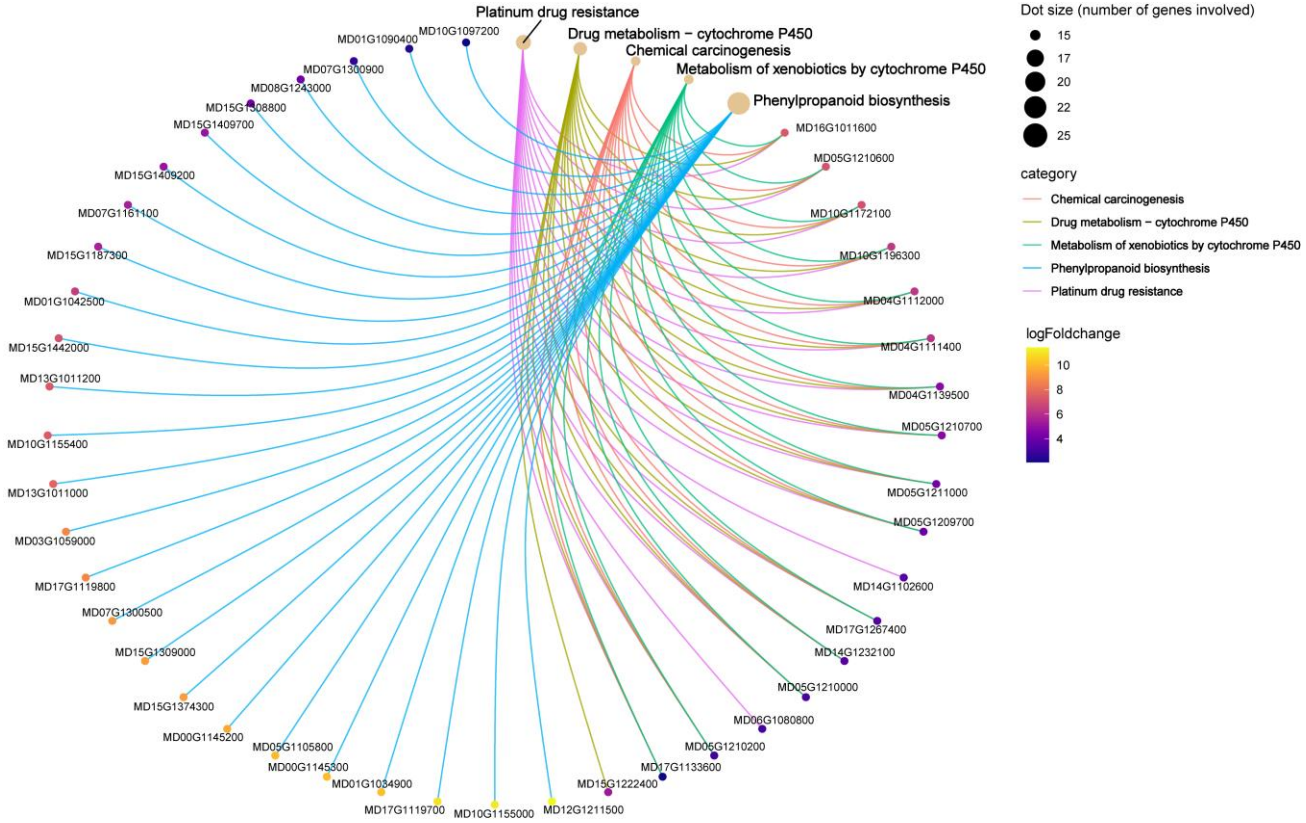

Figure 26. Circular plot of the most enriched pathway of *Malus domestica* belong to the "common genes" dataset.

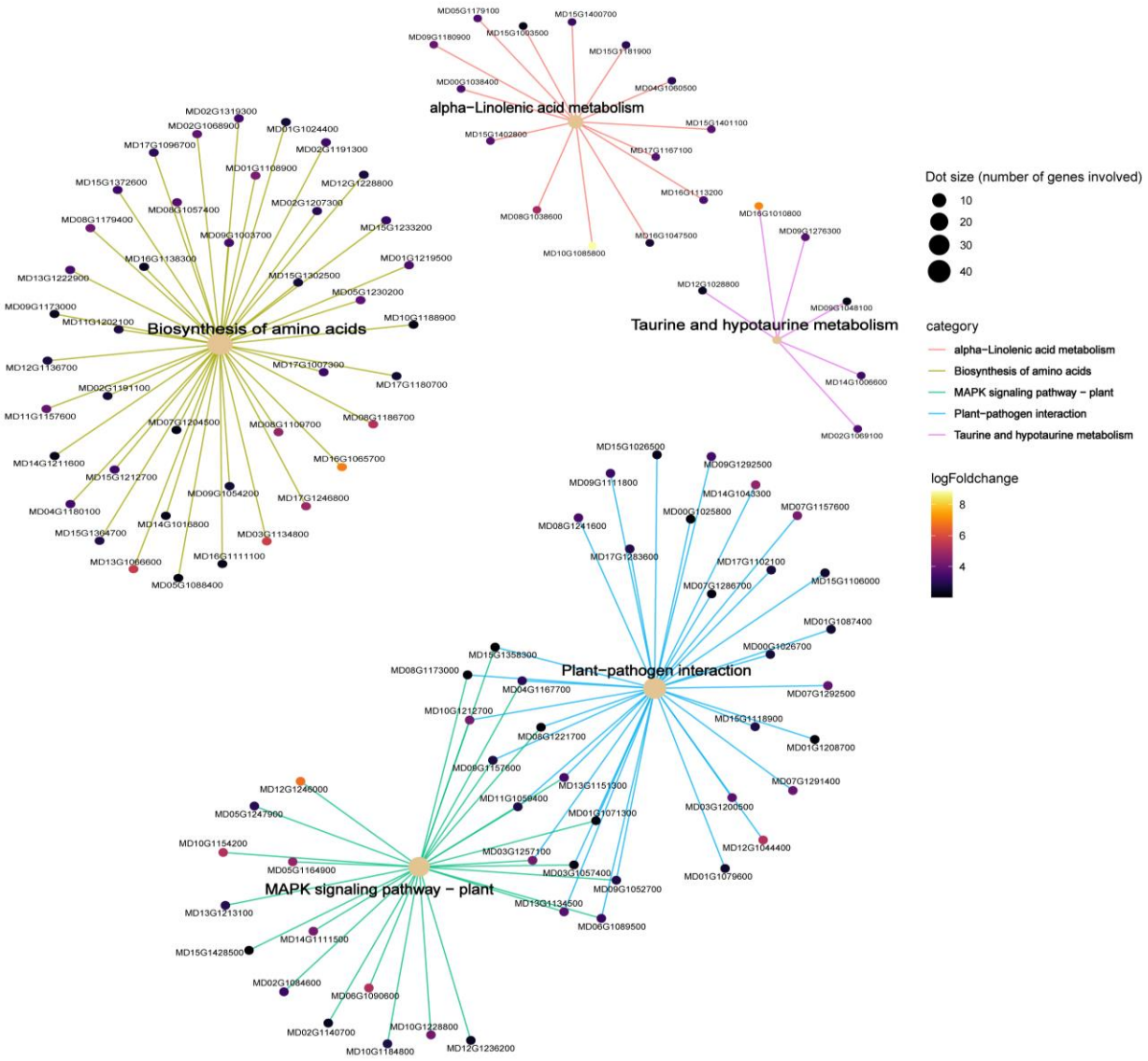

Figure 27. Most enriched KEGG pathway of *Malus domestica* when inoculated with the postharvest fungal pathogen.

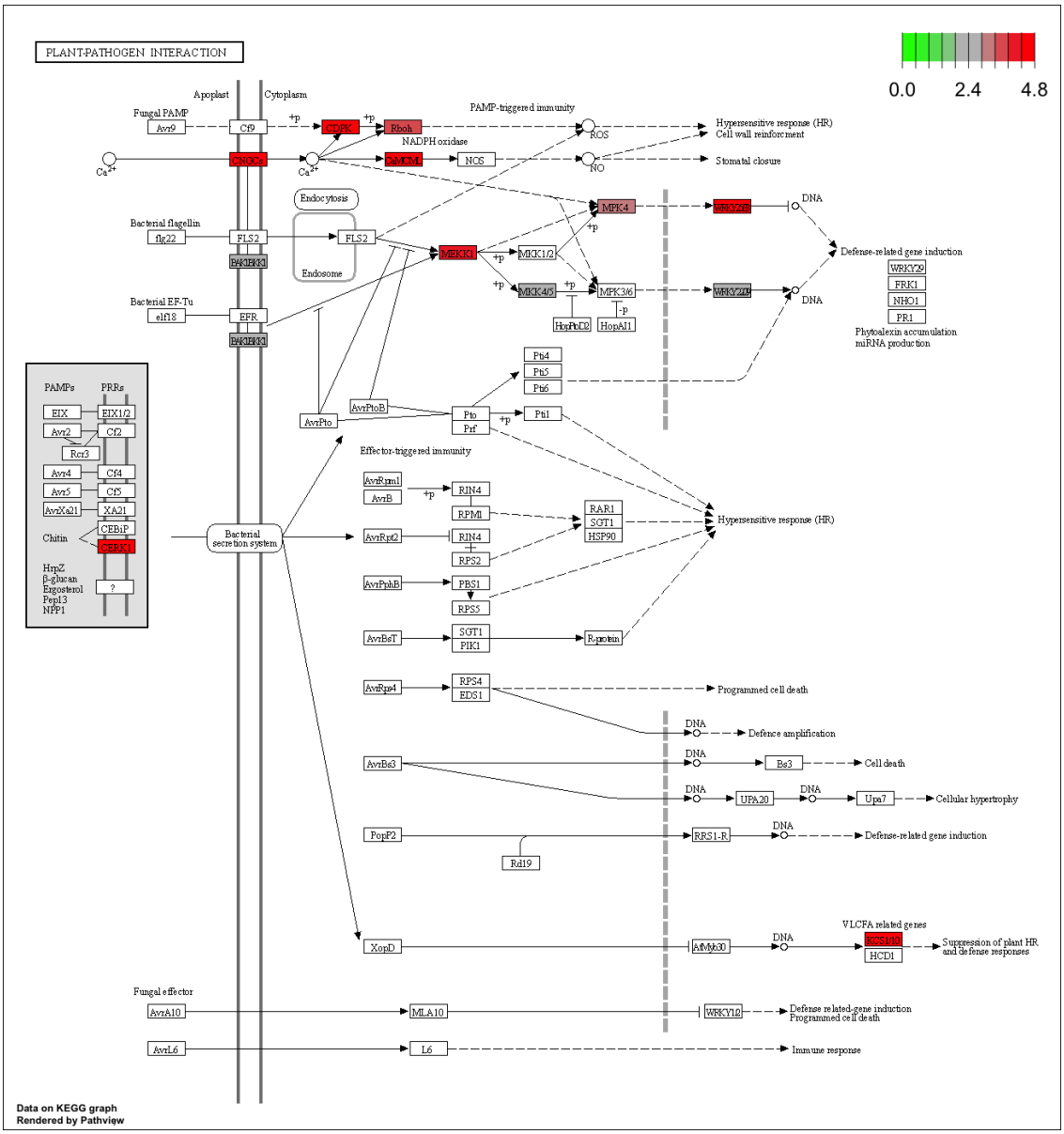

Figure 28. Illustration of the fold change of genes involved in "plant-pathogen interaction" pathway based on KEGG orthology enrichment information. In red are illustrated the upregulated genes according to the color-scale described in upper right corner of the figure.

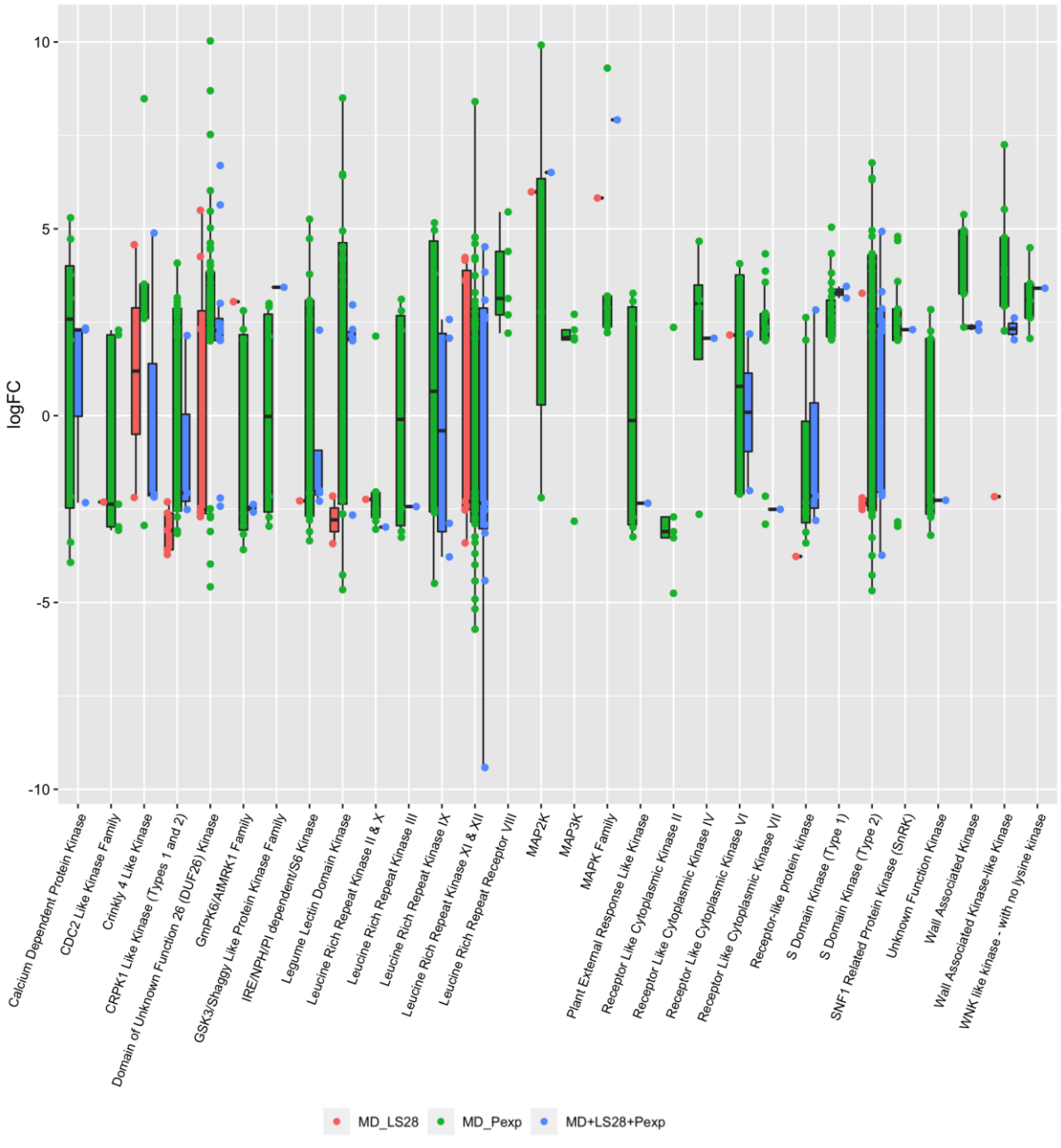

Figure 29. *Malus domestica* differentially expressed genes encoding for protein kinase grouped by classes according to the fold change value on y axis.

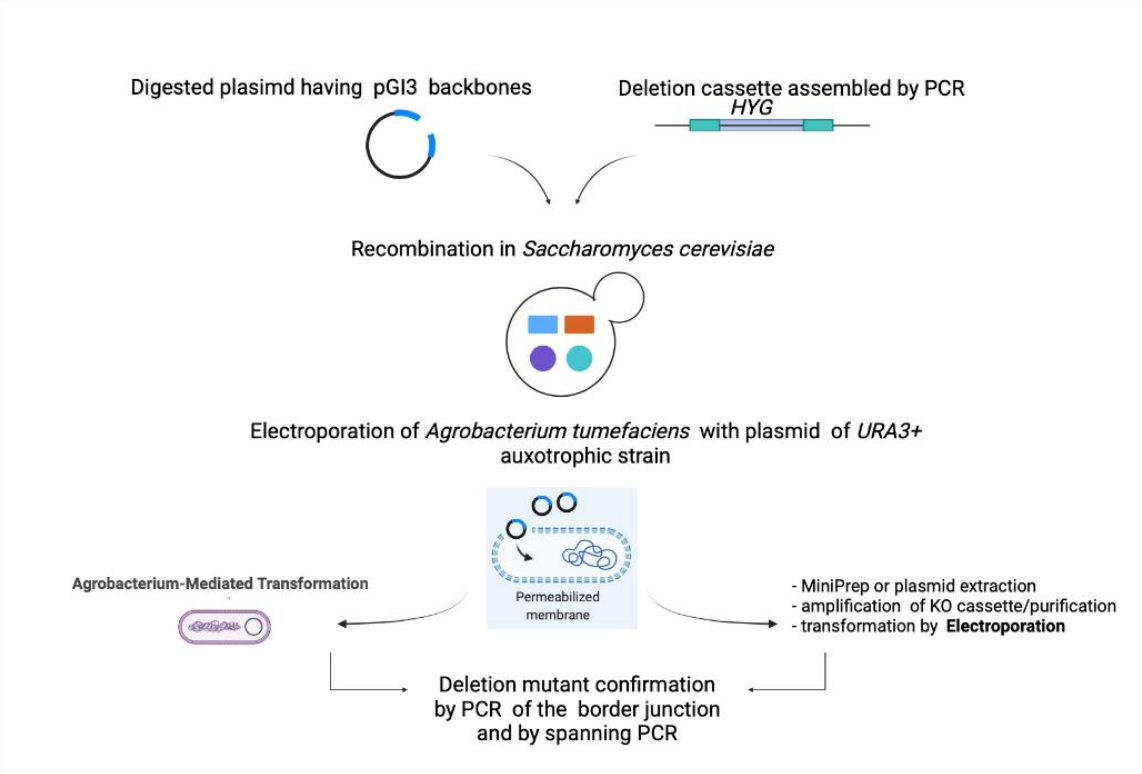

Figure 30. Scheme followed for the generation of deletion mutants in *Papiliotrema terrestris* strain LS28.

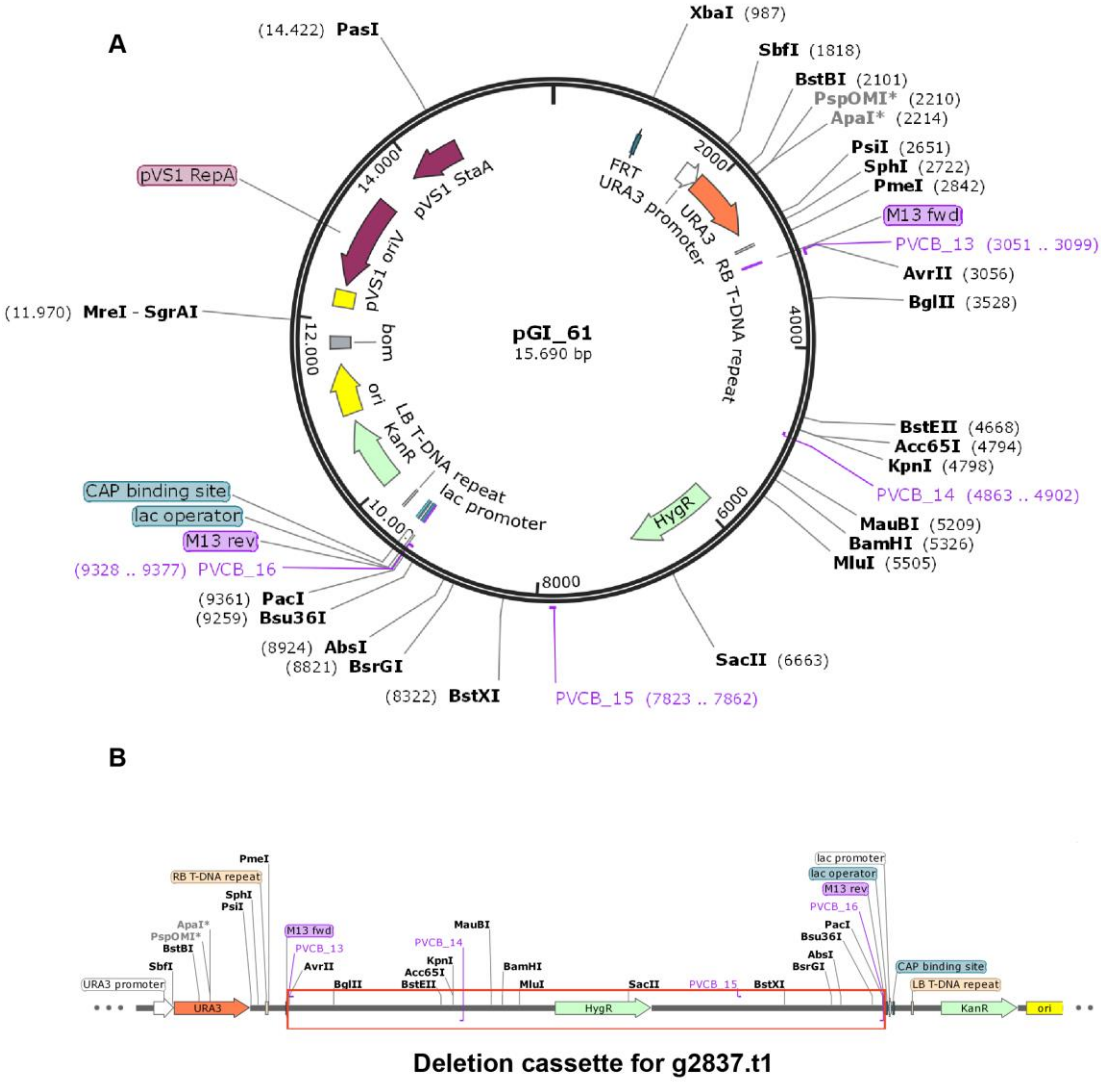

Figure 31. A, Plasmid map pGI61, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

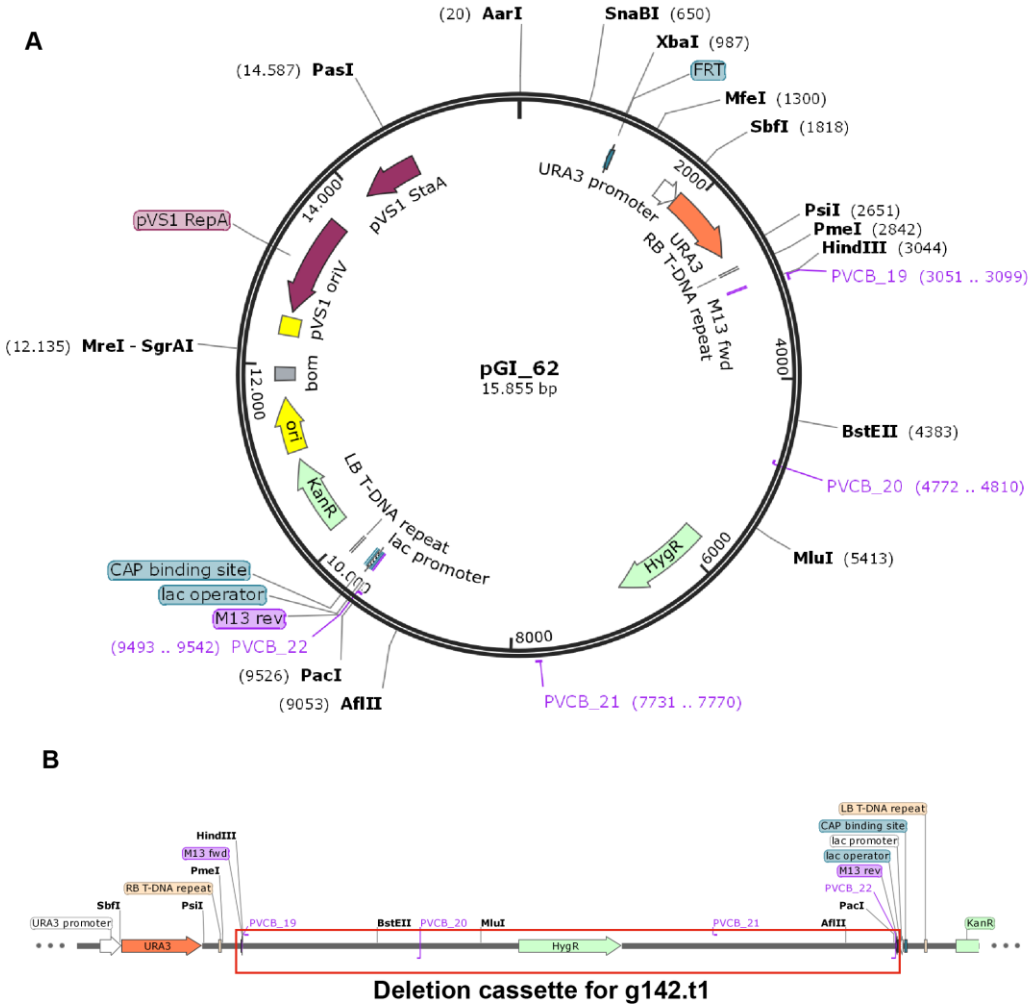

Figure 32. Plasmid map pGI62, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

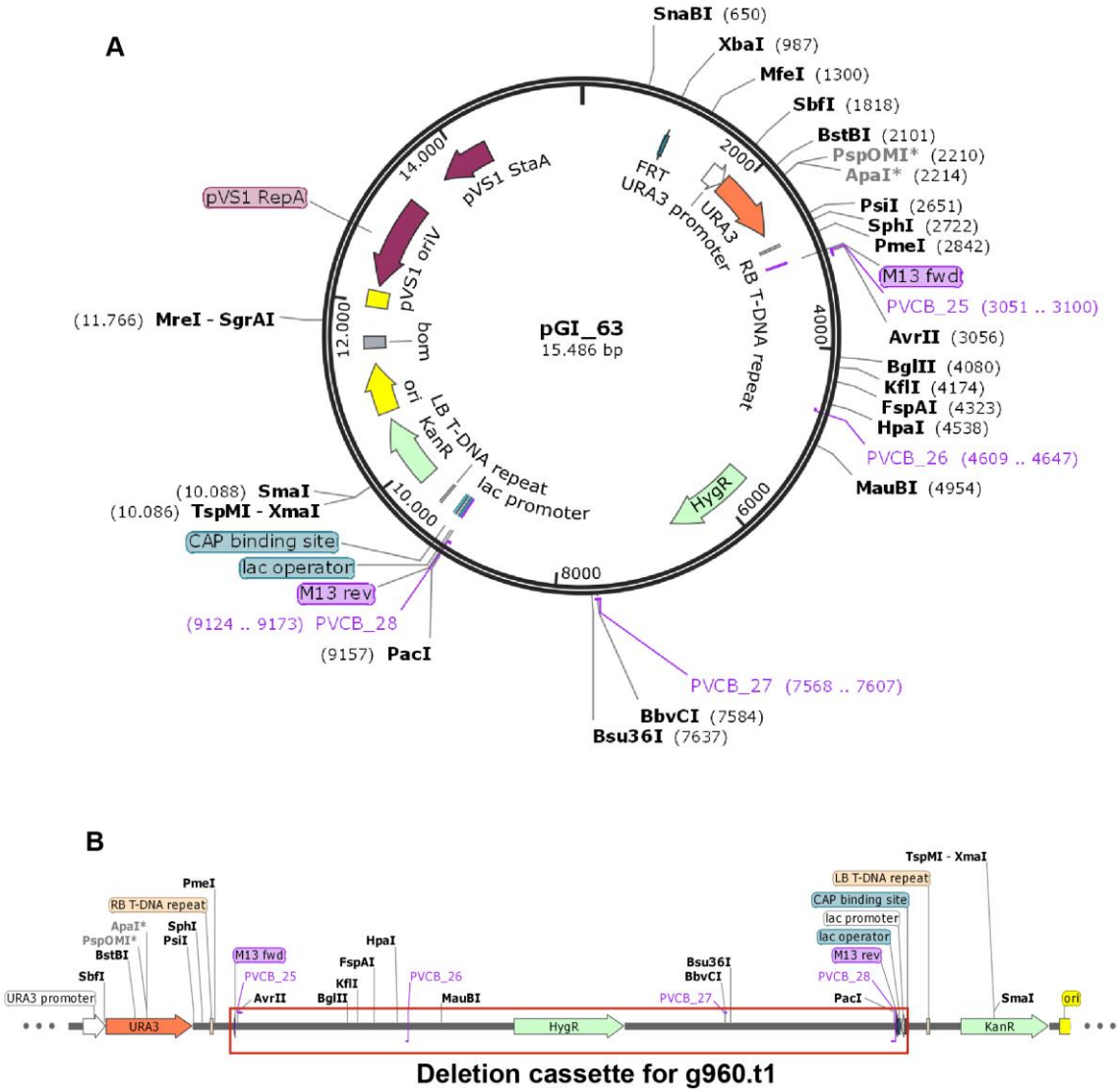

Figure 33. Plasmid map pGI63, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

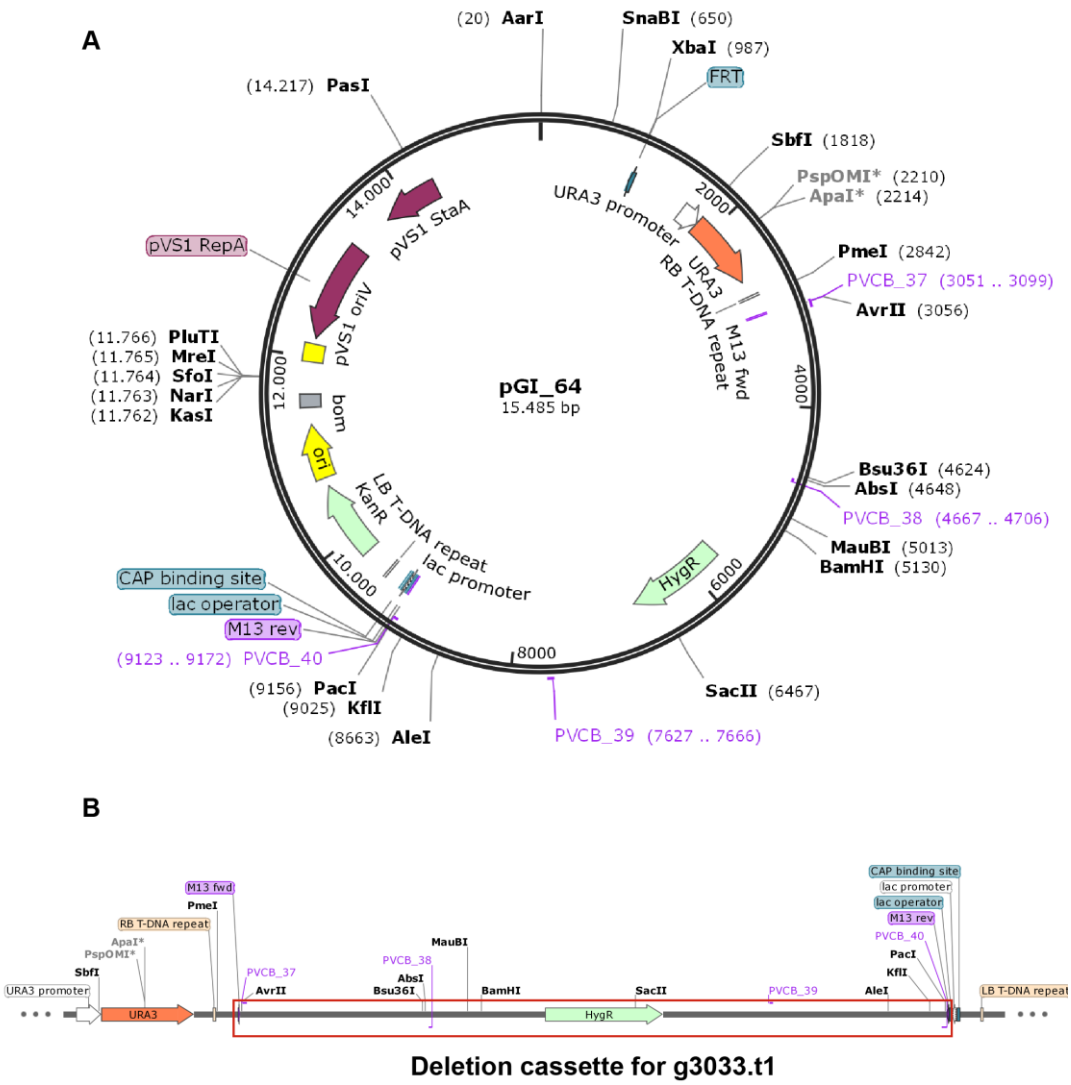

Figure 34. Plasmid map pGI64, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

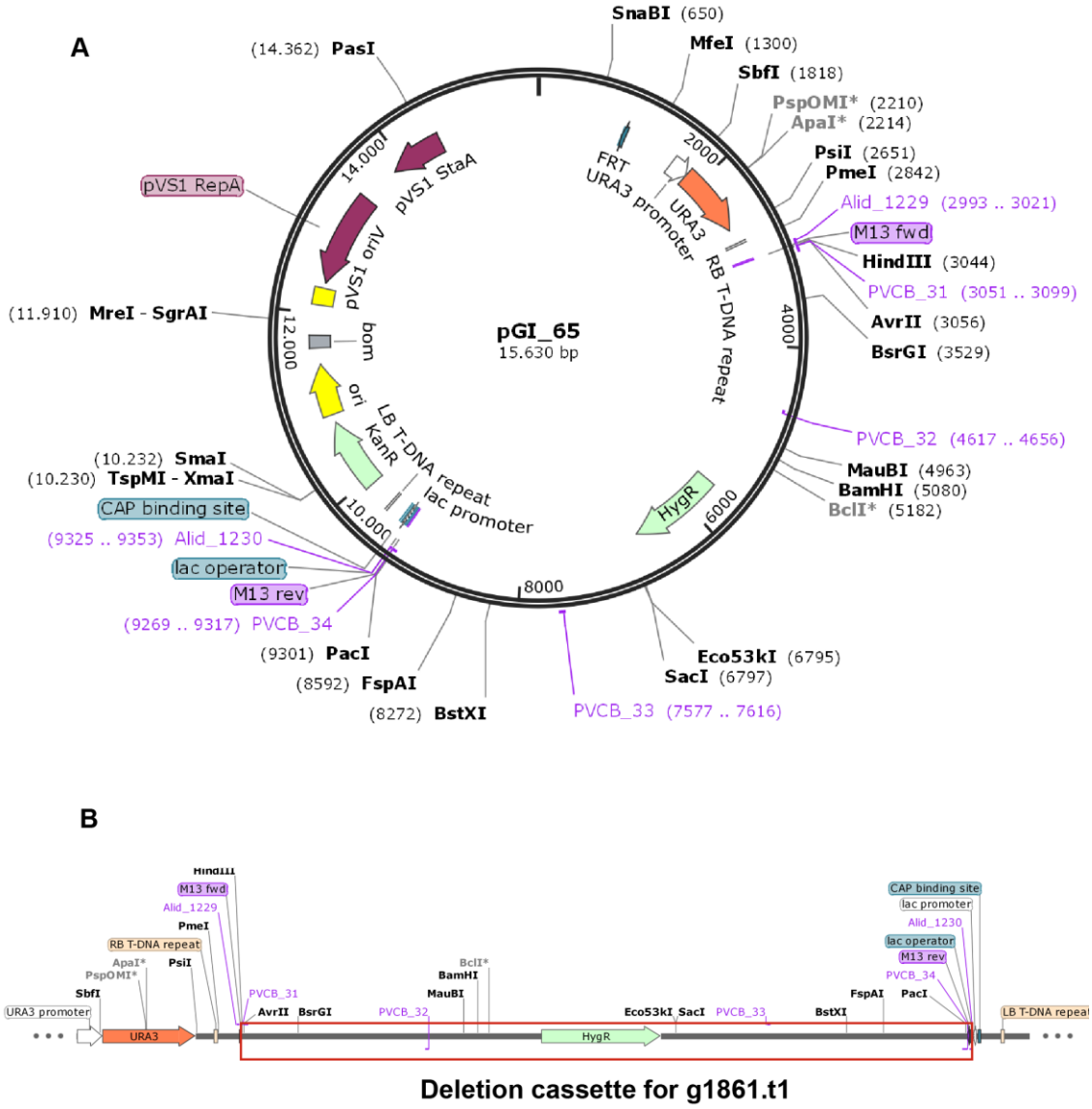

Figure 35. Plasmid map pGI65, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

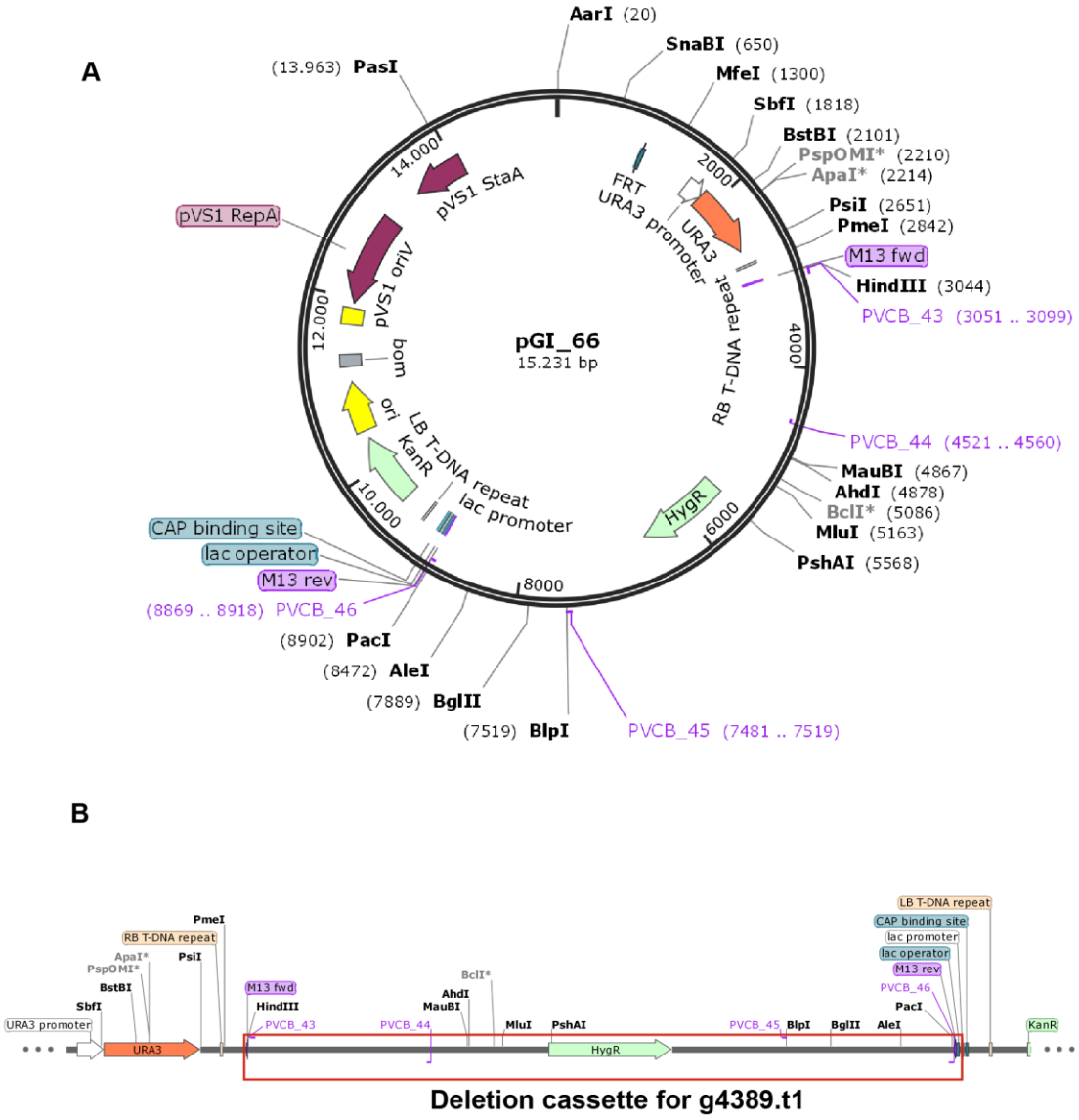

Figure 36. Plasmid map pGI66, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

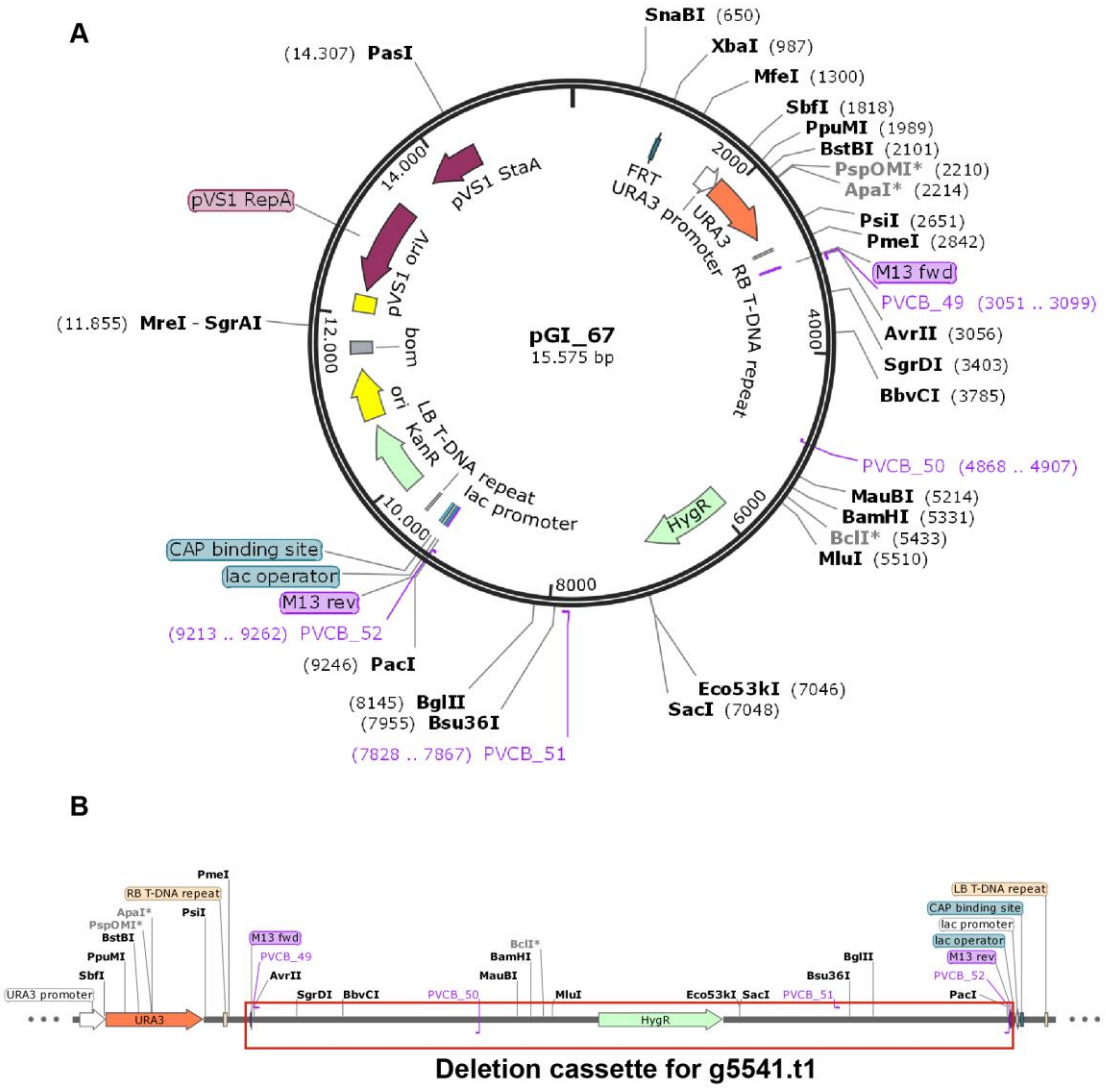

Figure 37. Plasmid map pGI67, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

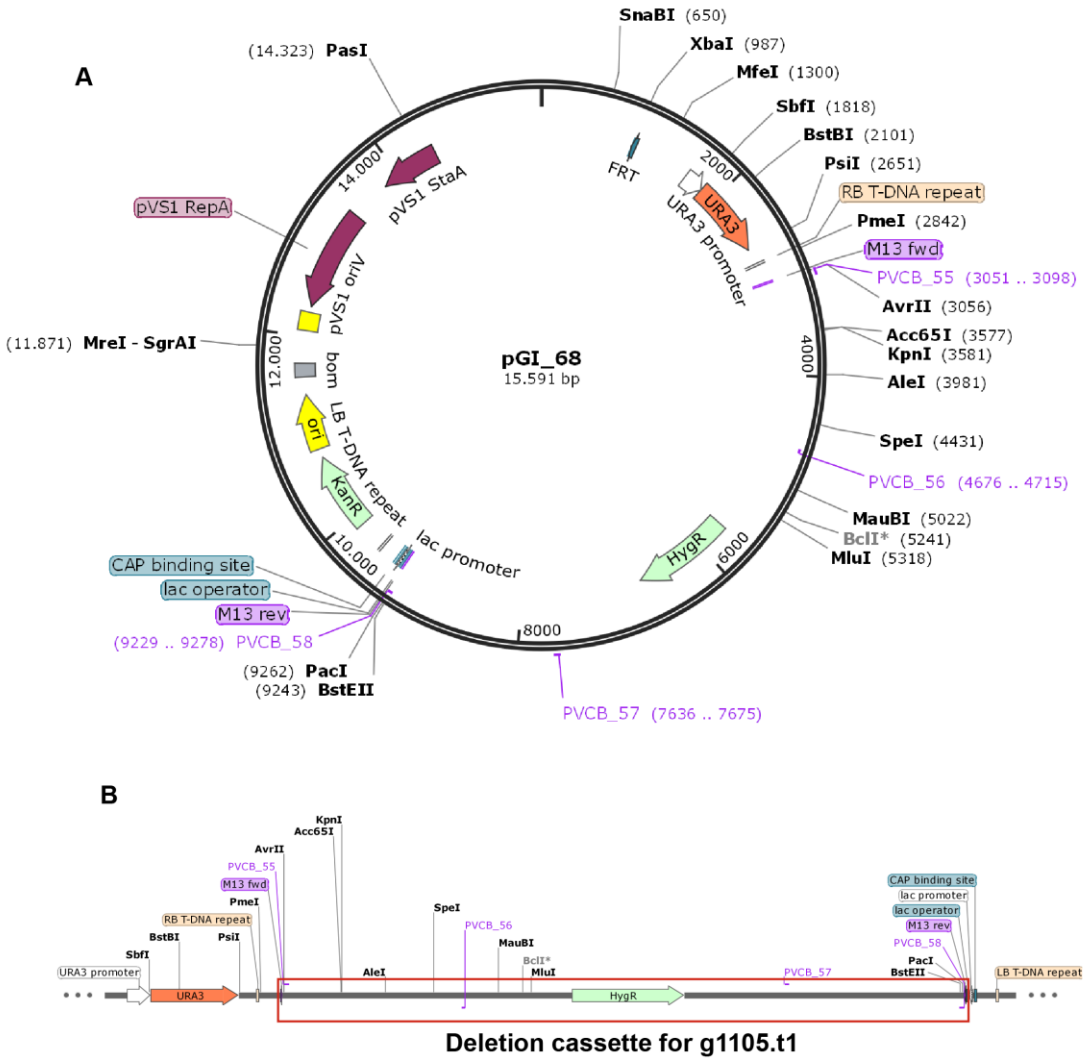

Figure 38. Plasmid map pGI68, based on pGI3 backbones. B, deletion cassette amplified by PCR and electroporated in LS28.

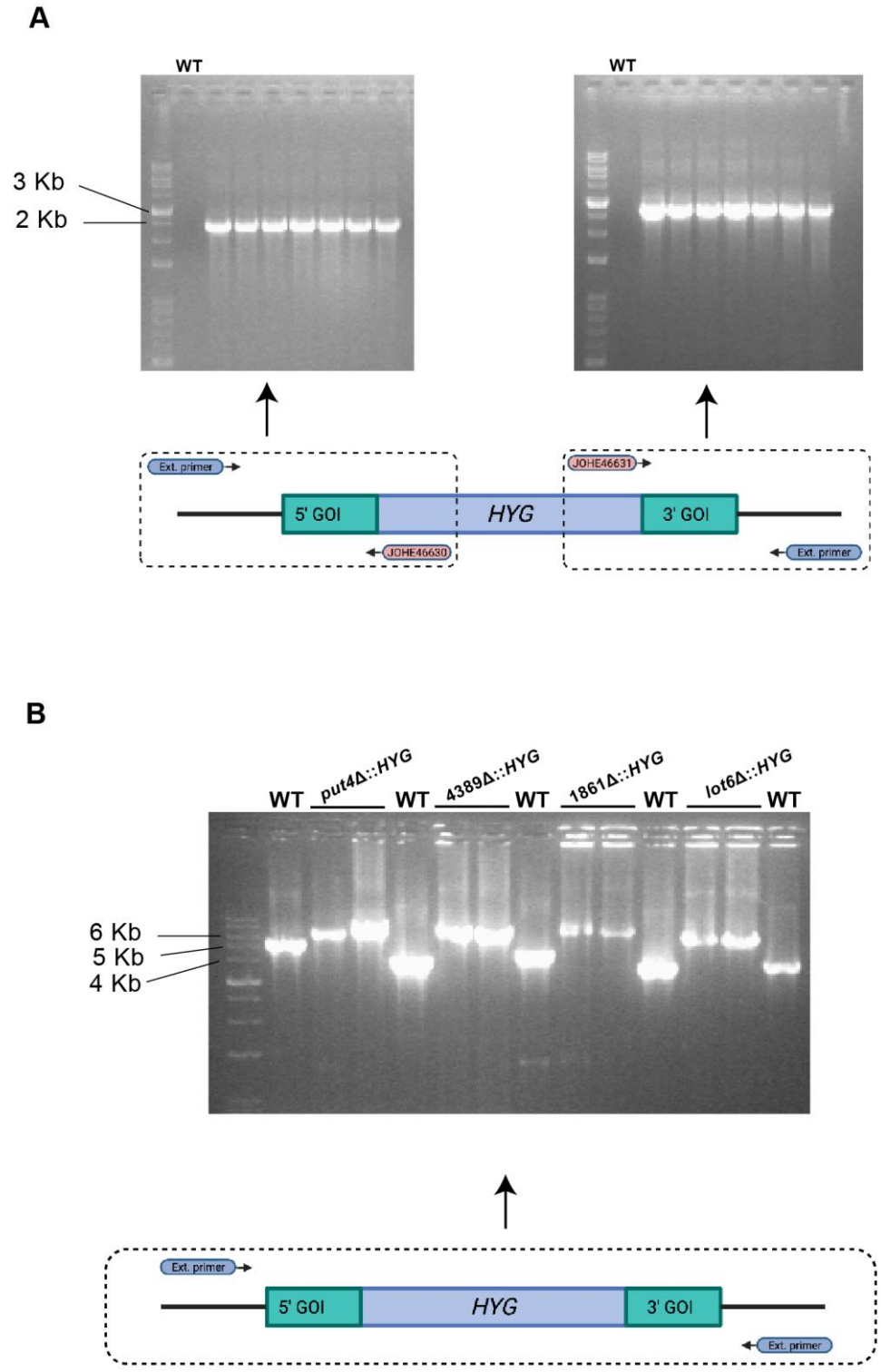

Figure 39. A, border junction confirmation on DNA extracted from LS28 transformants. Accordingly with figure 2 the expected size was 1.8/2 kilobases. B, spanning PCR performed using external primers only previously designed for each LS28 deletion mutant (see table 20).

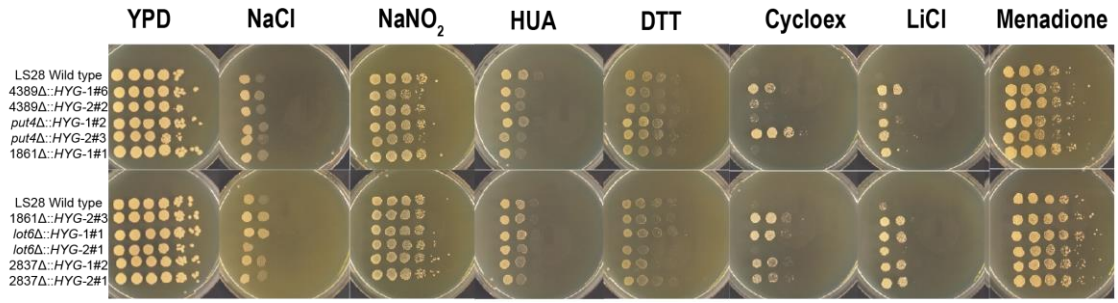

Figure 40. Spotting assay on deletion mutants of *P. terrestris* strain LS28, on Yeast extract Peptone Dextrose agar (YPD), sodium chloride (NaCl), sodium nitrite ($NaNO_2$), hydroxyurea (HUA), dithiothreitol (DTT), cycloheximide (Cyclohex), lithium chloride (LiCl) and menadione (for each concentration see material and methods, paragraph 3.4.2).

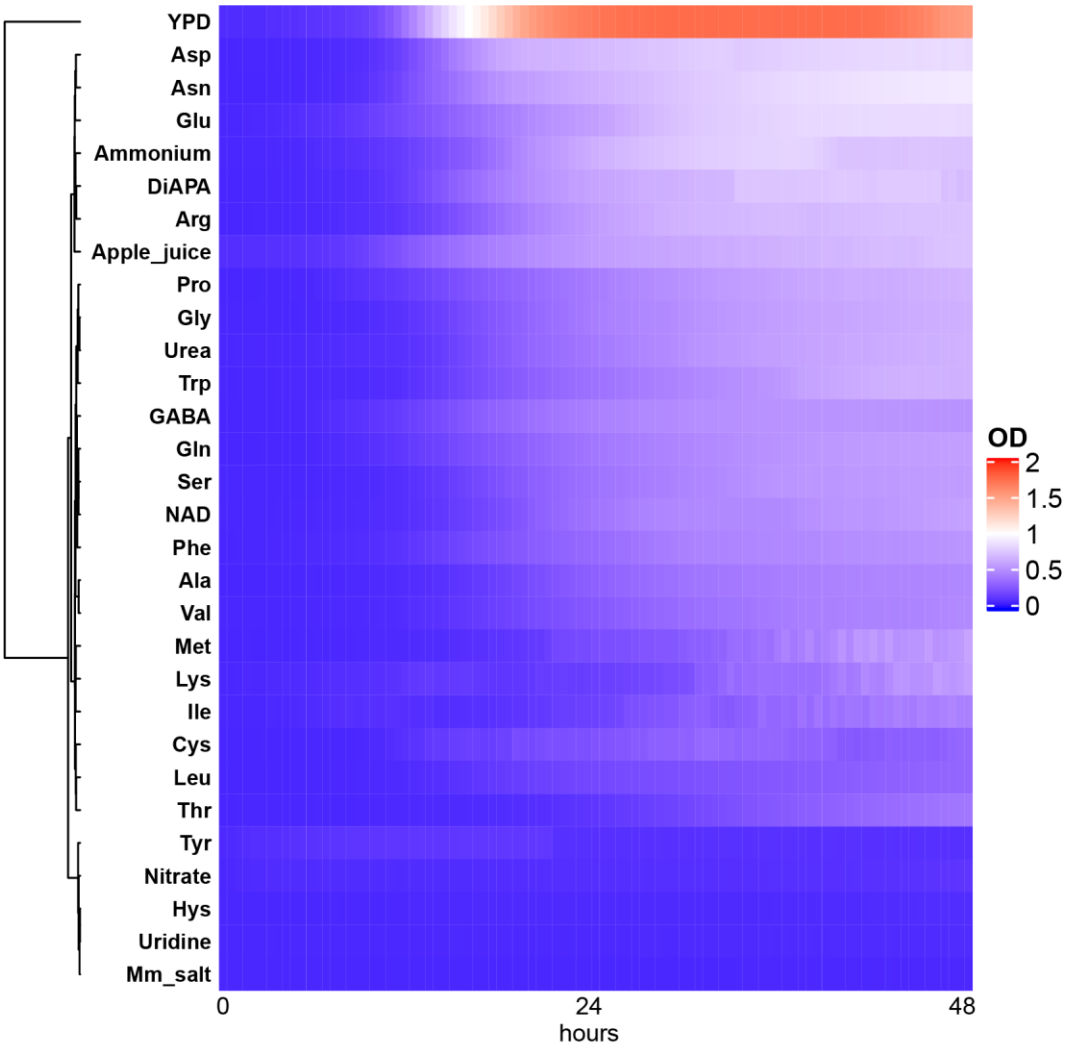

Figure 41. Heatmap representing the growth kinetics of LS28. Growth kinetics was assessed by measuring the optical density (OD_{600}), which were referred to a color scale on the right. The concentration used is reported in the table 22 along with the corresponding amino acid abbreviation.

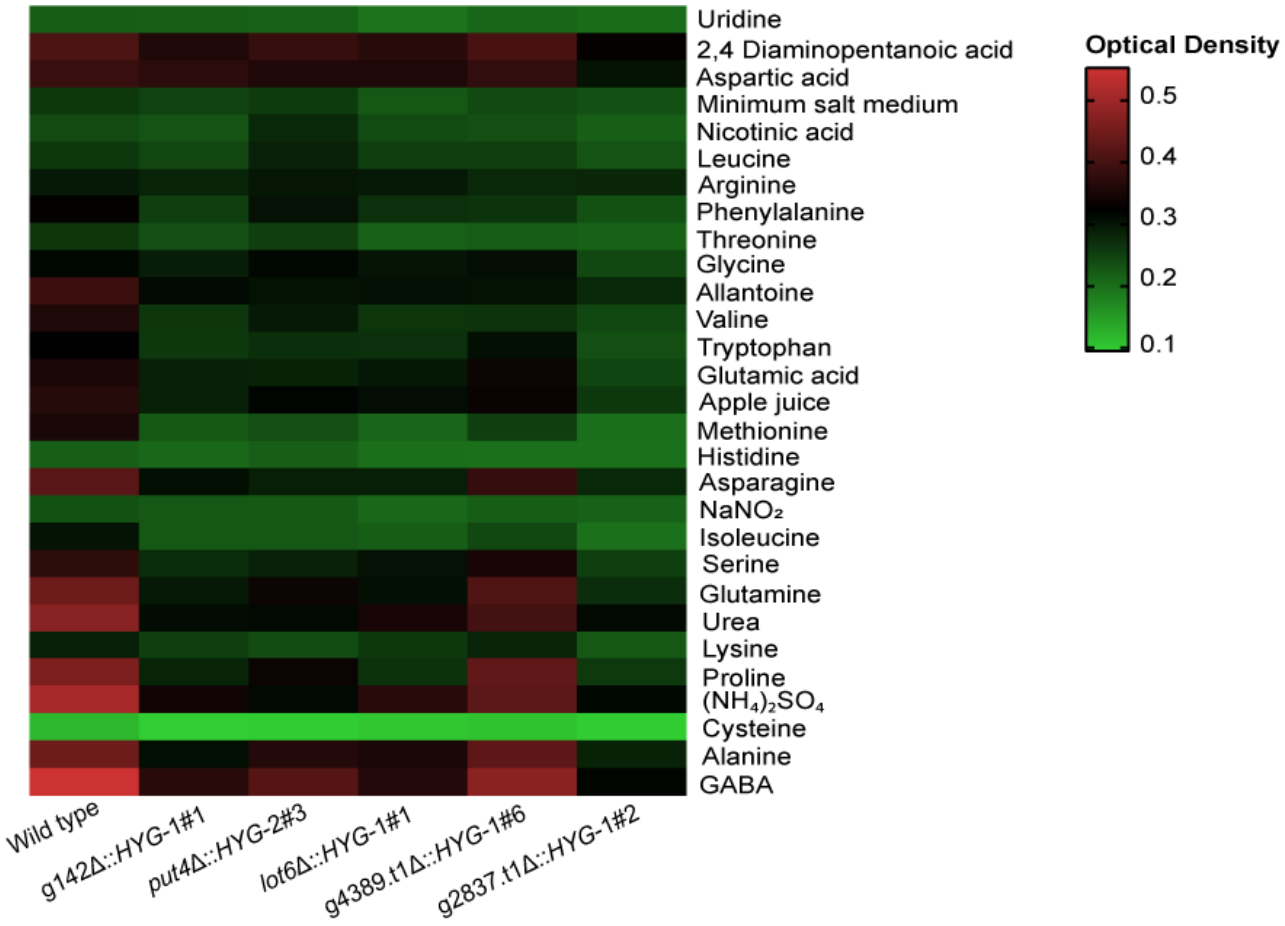

Figure 42. Growth data of deletion mutants after 24 hours in minimal medium modify with amino acids (only L-enantiomers were used) and other inorganic nitrogen sources, keep the concentration at 1.5 mM.

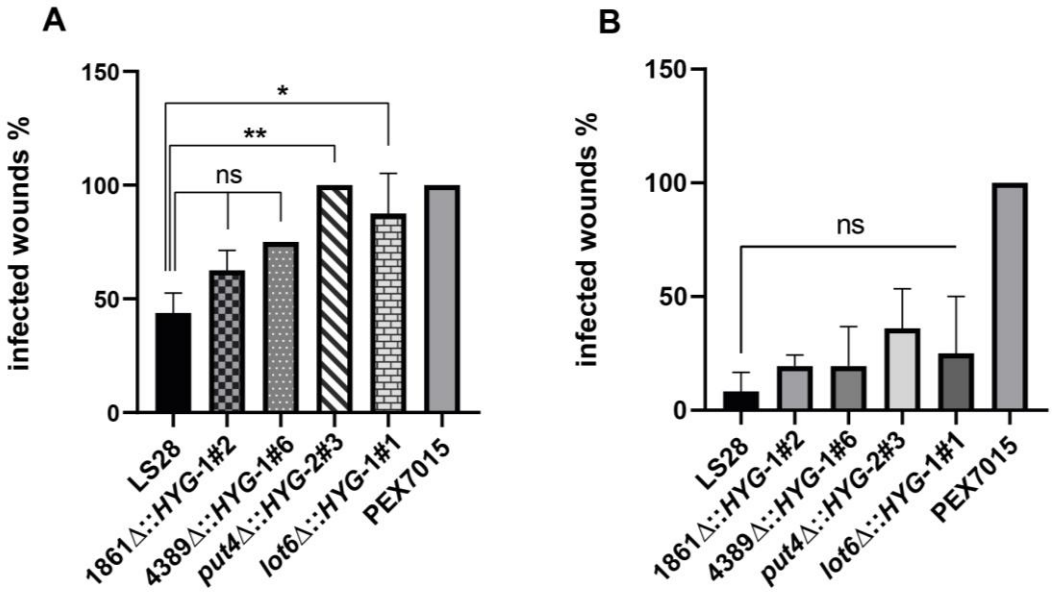

Figure 43. Biocontrol experiment at two different cell concentrations. A, the BCA was tested at the concentration of 5 x 10^6 CFU mL^{-1} and B, 1 x 10^8 CFU mL^{-1}. (ns = not significant, (*) $P < 0.05$, (**) $P < 0.01$; $1 - \alpha = 95\%$).

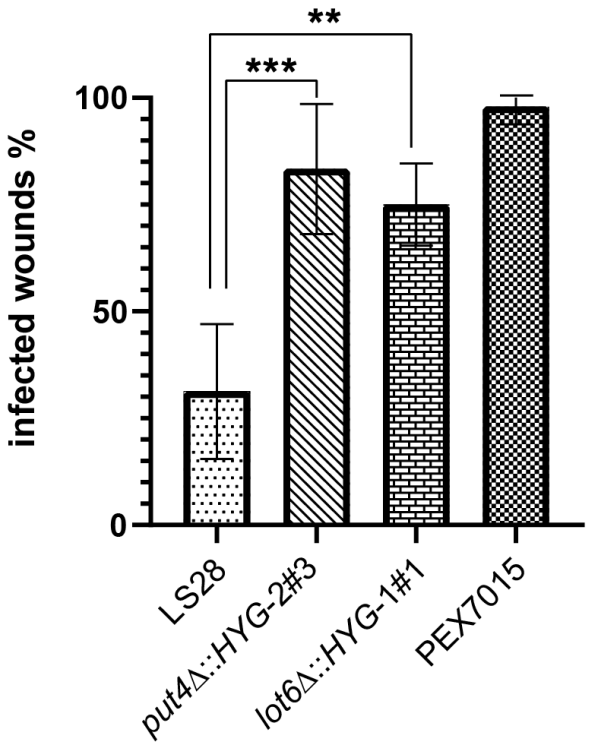

Figure 44. The biocontrol experiment was repeated three times and data were collected at 7 days post inoculation. The BCA was applied at sub-optimal concentration (5 x 10^6 CFU ml^{-1}). (**) P < 0.01, (***) P < 0.001, (****) P < 0.0001; 1 - α = 99,9 %.

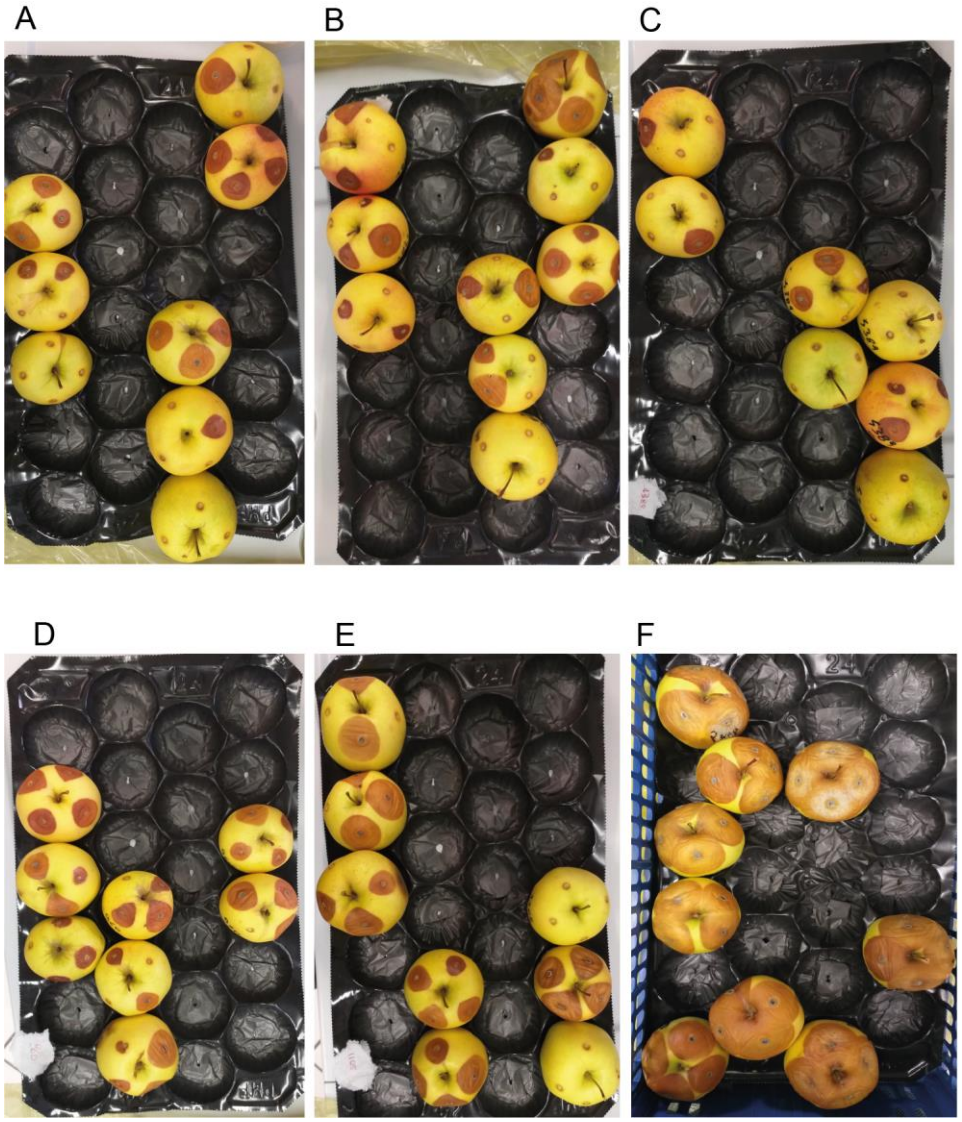

Figure 45. Biocontrol experiment performed at the concentration of 5 x 10^6 CFU ml^{-1} (10 days post inoculation). In these photos are showed two replicates for each treatment of the experiment referred to the graph in figure 41, A. The apples were treated according to the letters: A, LS28 wild type. B, g1861Δ::*HYG*-1#2. C, g4389Δ::*HYG*-1#6. D, *lot6*Δ::*HYG*-1#1. E, *put4*Δ::*HYG*-2#3. F, *Penicillium expansum*.

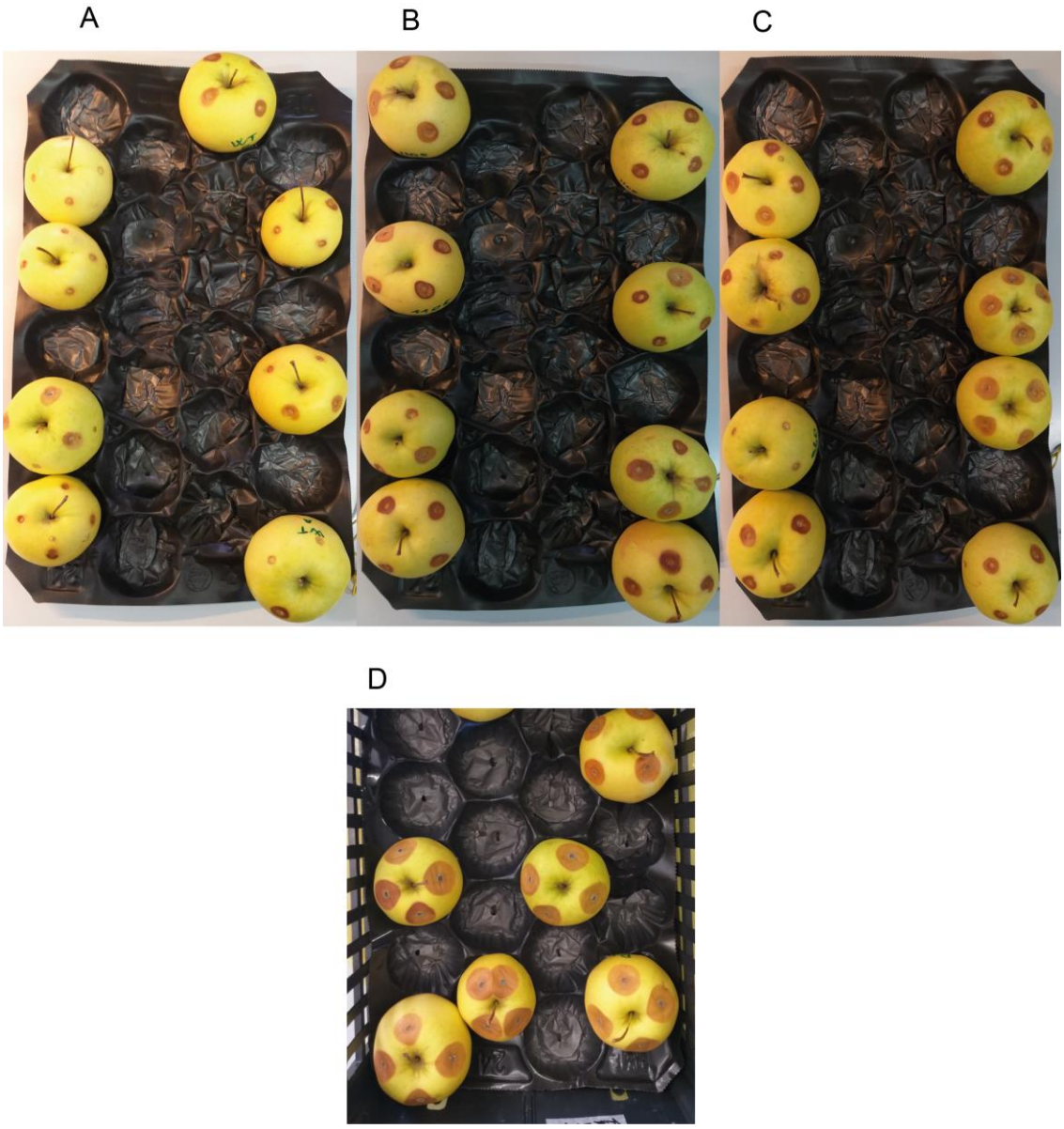

Figure 46. Confirmation of the biocontrol experiment at 5 x 10^6 CFU ml^{-1}. In these images are photographed only two replicates for each treatment of the experiment in figure 42. The images refer to the 5 days post inoculation. The apples were treated

according to the letters: A, LS28 wild type. B, *put4*Δ::*HYG*-2#3. C, *lot6*Δ::*HYG*-1#1. D, *Penicillium expansum*.

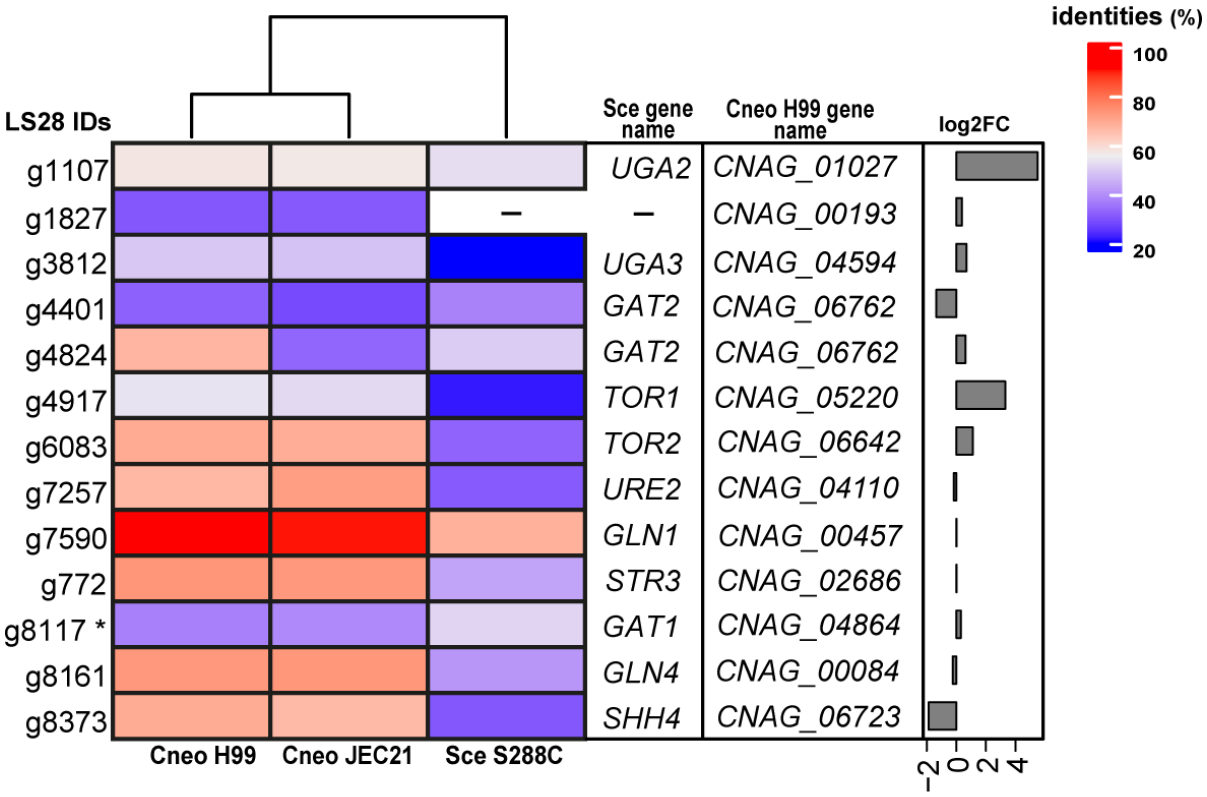

Figure 47. Result of direct and reversal blast of the main regulator of nitrogen metabolism performed against *S. cerevisiae* and *Cryptococcus neoformans* var. *neoformans* strain JEEC21 and *Cryptococcus neoformans* var. *grubii* strain H99. The value of the fold change is referred to the BCA alone in wounded apple.

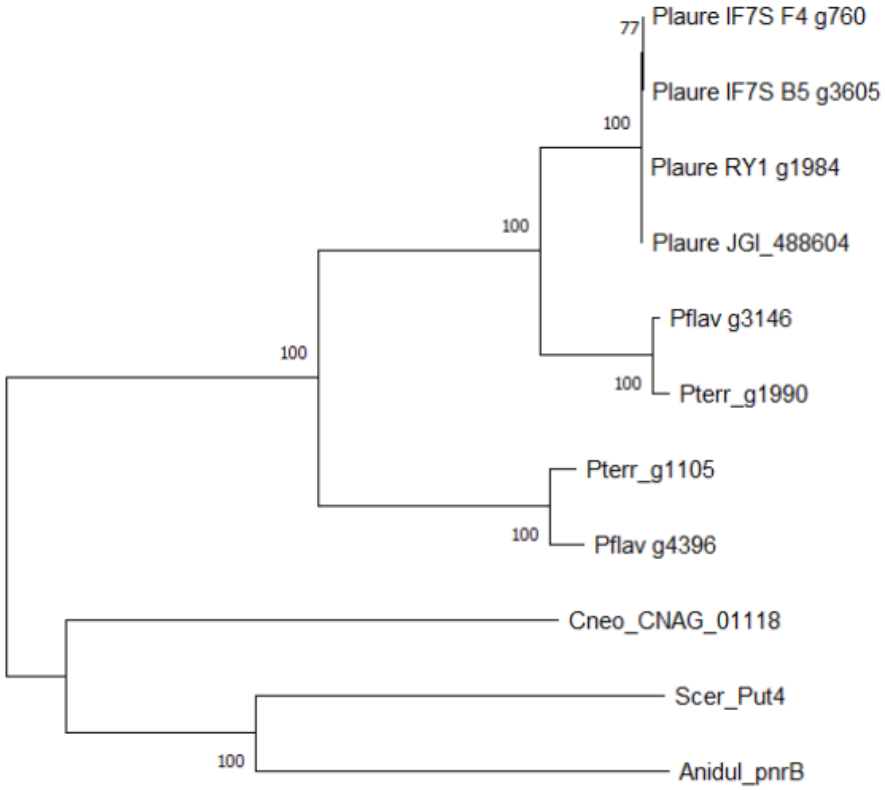

Figure 48. Phylogenetic reconstruction of proline transporters paralogs of Put4. These paralogs are present also in *P. flavescens*, but not in *P. laurentii* , *C. neoformans*, and *S. cerevisiae*.

8 Tables

Table 1. Mapping statistic.

Sample #replicate	Mapped against the genome of	Reads that mapped to one location (%)	Reads that mapped to multiple locations (%)	Unmapped reads (%)
P. terrestris #1	*P. terrestris*	95.49	0.07	4.42
P. terrestris #2	*P. terrestris*	96.54	0.07	3.38
P. expansum #1	*P. expansum*	52.44	0.12	47.44
P. expansum #2	*P. expansum*	93.76	0.16	6.08
P. expansum #3	*P. expansum*	92.72	0.16	7.12
M. domestica #1	*M. domestica*	70.24	20.06	9.64
M. domestica #2	*M. domestica*	64.94	25.95	9.04
M. domestica + *P. terrestris* #2	*M. domestica*	73.74	7.93	18.26
	P. terrestris	12.71	0.04	87.24
M. domestica + *P. terrestris* #3	*M. domestica*	82.92	4.75	12.24
	P. terrestris	8.04	0.02	91.93
M. domestica + *P. expansum* #1	*M. domestica*	73.77	11.74	14.44
	P expansum	8.53	0.01	91.46
M. domestica + *P. expansum* #2	*M. domestica*	71.45	8.05	20.45
	P. expansum	14.97	0.02	85.01
M. domestica + *P. expansum* #3	*M. domestica*	78.43	6.77	14.73
	P. expansum	7.81	0.01	92.17
M. domestica + *P. terrestris* + *P. expansum* #1	*M. domestica*	74.77	4.36	20.80
	P. expansum	1.29	0.00	98.70
	P. terrestris	14.44	0.02	85.53
M. domestica + *P. terrestris* + *P. expansum* #2	*M. domestica*	68.32	4.70	26.92
	P. expansum	2.14	0.00	97.85
	P. terrestris	17.60	0.03	82.36
M. domestica + *P. terrestris* + *P. expansum* #3	*M. domestica*	76.84	4.18	18.88
	P. expansum	1.24	0.00	98.76
	P. terrestris	11.46	0.02	88.51

Table 2. Main genes of LS28 expressed alone in apple (included only DEGs with logFC >4). Sorted for LogFC; extracted from file S1. Hypothetical proteins with no detected domains have not been included in this table and can be found in tables S1.

Gene	**SGD Gene name**	**logFC**	**GO Description and/or SGD-NCBI annotation**	**GO Biological Process**
g5076.t1	*PHO89*	7.79	Plasma membrane Na+/Pi cotransporter	Transmembrane transport/monovalent inorganic cation transport/oxidation-reduction process
g4427.t1	#N/A	7.33	Glycoside hydrolase/Signal peptide N-region/ Signal peptide H-region/ Signal peptide C-region	Carbohydrate metabolic process
g1997.t1	*SEO1*	6.90	MFS permease member of the allantoate transporter subfamily	Transmembrane transport/oxidation-reduction process
g379.t1	*TNA1*	6.57	Nicotinic acid plasma membrane permease	Transmembrane transport/oxidation-reduction process
g3163.t1	*PGC1*	6.53	Phosphatidyl Glycerol phospholipase C/ Signal peptide N-region/ Signal peptide H-region/ Signal peptide C-region	Oxidation-reduction process
g6316.t1	*TAL1*	5.97	Transaldolase b of the non-oxidative pentose phosphate pathway	Carbohydrate metabolic process
g8163.t1	*JLP1*	5.66	Fe(II)-dependent sulfonate/alpha-ketoglutarate dioxygenase	Oxidation-reduction process
g1017.t1	*SFC1*	5.24	Mitochondrial succinate-fumarate transporter	Transmembrane transport
g3018.t1	#N/A	5.21	MARVEL domain-containing protein	#N/A
g6408.t1	*FRE3*	5.15	Ferric reductase	Transmembrane transport/oxidation-reduction process
g7493.t1	*STL1*	4.98	Glycerol proton symporter of the plasma membrane	Transmembrane transport/carbohydrate transport
g4598.t1	*AIM2*	4.70	Cytoplasmic protein involved in mitochondrial function related to a dienelactone hydrolase	#N/A

Gene	SGD Gene name	logFC	GO Description and/or SGD-NCBI annotation	GO Biological Process
g4211.t1	*FET3*	4.69	Ferro-O2-oxidoreductase; multicopper oxidase	Oxidation-reduction process
g4787.t1	#N/A	4.58	Zinc transporter	Transmembrane transport/carbohydrate transport
g4520.t1	#N/A	4.44	beta-lactamase/transpeptidase-like protein	#N/A
g6360.t1	#N/A	4.39	Proteasome regulatory subunit	#N/A
g6245.t1	*DAL5*	4.32	MFS allantoate and ureidosuccinate permease	Transmembrane transport/carbohydrate metabolic process/trehalose metabolic process/disaccharide metabolic process/oxidation-reduction process

Table 3. Common DEGs of LS28 in apple wound with and without *P. expansum* (included only DEGs with logFC >4). DEGs sorted for LogFC; extracted from tables S1. Hypothetical proteins with no detected domains have not been included in this table and can be found in tables S1.

Gene	**SGD Gene name**	**logFC *P. terrestris* + *P. expansum***	**logFC *P. terrestris***	**GO Description and/or SGD-NCBI annotation**	**GO Biological Process**
g3033.t1	#N/A	12.21	10.31	NAD(P)-binding Rossmann-fold domain	#N/A
g4389.t1	#N/A	11.48	14.16	Transmembrane domains	#N/A
g5541.t1	*OPT1*	11.27	8.75	Proton-coupled oligopeptide transporter of the plasma membrane; also transports glutathione and phytochelatin	Transmembrane transport/ion transport/organic anion transport/establishment of localization
g1105.t1	*PUT4*	10.70	10.59	Proline permease; required for high-affinity transport of proline	Transmembrane transport/polyamine transport/ammonium transport/oxidation-reduction process
g3330.t1	YGL039W	10.38	7.50	Nucleoside-diphosphate-sugar epimerase	Oxidation-reduction process
g7506.t1	*ARG4*	10.37	8.01	Argininosuccinate lyase that catalyzes the final step in the arginine biosynthesis pathway	Transmembrane transport/ion transport/establishment of localization/oxidation-reduction process
g6692.t1	YMR226C	9.69	7.57	NADP(+)-dependent serine dehydrogenase and carbonyl reductase	Oxidation-reduction process
g4159.t1	*THI73*	9.61	9.09	MFS putative plasma membrane permease	Transmembrane transport/ion transport/establishment of localization
g6691.t1	#N/A	9.53	7.37	Short-chain dehydrogenase/reductase like protein yusS	#N/A
g7507.t1	*MAL31*	9.42	6.66	Maltose permease	Transmembrane transport/ion Transport/establishment of localization
g5806.t1	*AYR1*	9.06	5.49	Bifunctional triacylglycerol lipase and 1-acyl DHAP phosphate reductase involved in phosphatidic acid biosynthesis	Oxidation-reduction process
g5540.t1	#N/A	8.77	6.61	Phosphoribosylaminoimidazolecarboxamide	Transmembrane transport/ion transport/establishment of

Gene	SGD Gene name	logFC *P. terrestris* + *P. expansum*	logFC *P. terrestris*	GO Description and/or SGD-NCBI annotation	GO Biological Process
				formyltransferase/IMP cyclohydrolase	localization/oxidation-reduction process
g1251.t1	*FUI1*	8.66	7.02	High affinity uridine permease	Transmembrane transport
g4160.t1	*HOL1*	8.55	9.67	Putative MFS transporter	Transmembrane transport/ion transport/organic hydroxy compound transport/establishment of localization/oxidation-reduction process
g7495.t1	YHL008C	8.30	9.19	Formate nitrite transporter	Transmembrane transport/ion transport/establishment of localization
g3623.t1	#N/A	7.74	7.93	Putative dehydrogenases with a glycine-rich NAD(P)-binding motif	#N/A
g4444.t1	*GOR1*	7.77	8.79	Glyoxylate reductase	Oxidation-reduction process
g7073.t1	#N/A	7.73	6.02	Glutathione-dependent formaldehyde-activating gfa protein	#N/A
g5118.t1	#N/A	7.71	7.60	MFS general substrate transporter	Transmembrane transport/establishment of localization/oxidation-reduction process
g3727.t1	*ENV9*	7.61	7.12	Protein proposed to be involved in vacuolar functions	Oxidation-reduction process
g6644.t1	*GAL7*	7.59	6.55	Galactose-1-phosphate uridyl transferase	Oxidation-reduction process
g2444.t1	#N/A	7.52	5.40	Flavin containing amine oxidoreductase	Transmembrane transport/ion transport/establishment of localization/oxidation-reduction process
g1770.t1	*PTR2*	7.33	6.59	Integral membrane peptide transporter	Transmembrane transport/ion transport/establishment of localization
g2090.t1	*AMF1*	7.10	5.30	Low affinity NH4+ transporter	Transmembrane transport/establishment of localization
g5935.t1	*AMD2*	7.10	7.92	Putative amidase	Oxidation-reduction process
g7494.t1	#N/A	7.00	7.64	Nitrite reductase NiiA	Transmembrane transport/ion transport/establishment of localization/oxidation-reduction process

Gene	SGD Gene name	logFC *P. terrestris* + *P. expansum*	logFC *P. terrestris*	GO Description and/or SGD-NCBI annotation	GO Biological Process
g840.t1	*AMF1*	6.90	3.98	Low affinity NH4+ MFS transporter	Transmembrane transport/establishment of localization
g5936.t1	*DUR3*	6.82	7.59	Plasma membrane transporter for both urea and polyamines	Transmembrane transport/ localization/oxidation-reduction process
g4538.t1	*GAP1*	6.77	8.15	General amino acid permease	Transmembrane transport/polyamine transport/establishment of localization
g4212.t1	YCR102C	6.73	6.98	Putative zinc-binding oxidoreductase ToxD involved in copper metabolism	Oxidation-reduction process
g5563.t1	*MEP2*	6.67	7.56	Ammonium permease	Ammonium transmembrane transport/establishment of localization
g1937.t1	#N/A	6.50	6.16	Marvel domain containing protein	#N/A
g1952.t1	*EXG1*	6.48	8.46	Major exo-1,3-beta-glucanase of the cell wall	Oxidation-reduction process
g3542.t1	#N/A	4.47	4.32	Glutathione-dependent formaldehyde-activating enzyme/centromere protein V	#N/A
g1926.t1	#N/A	6.38	4.91	Putative carboxypeptidase	#N/A
g1250.t1	#N/A	6.28	4.32	Putative methyltransferase	#N/A
g1043.t1	#N/A	6.24	3.00	Intradiol ring-cleavage dioxygenase	Transmembrane transport/establishment of localization/oxidation-reduction process
g1860.t1	*FLR1*	6.21	4.63	Plasma MFS membrane transporter	Transmembrane transport/polyamine transport/ammonium transport/establishment of localization
g7238.t1	*HNM1*	6.16	6.72	Plasma membrane transporter for choline, ethanolamine, and carnitine	Transmembrane transport/ammonium transport/organic cation transport/establishment of localization/oxidation-reduction process
g3941.t1	YEL023C	6.15	6.81	Putative short chain dehydrogenase reductase	#N/A
g5317.t1	*GAL1*	6.14	5.23	Galactokinase	Oxidation-reduction process

Gene	SGD Gene name	logFC *P. terrestris* + *P. expansum*	logFC *P. terrestris*	GO Description and/or SGD-NCBI annotation	GO Biological Process
g5890.t1	*AGP2*	6.10	6.33	Plasma membrane regulator of polyamine and carnitine transport	Transmembrane transport/ammonium transport/organic cation transport/establishment of localization/oxidation-reduction process
g1673.t1	YKL071W	6.08	4.87	NAD(P)-binding Rossmann-fold domain containing	Oxidation-reduction process.
g1180.t1	*DIP5*	6.07	5.25	Dicarboxylic amino acid permease	Transmembrane transport/ion transport/establishment of localization
g2435.t1	*GOR1*	6.05	5.95	Glyoxylate reductase	Oxidation-reduction process
g5468.t1	*OPT2*	5.90	4.85	OPT oligopeptide transporter	Transmembrane transport/establishment of localization
g3316.t1	*HXT2*	5.81	10.43	High-affinity glucose MFS transporter	Transmembrane transport/ion transport/establishment of localization/oxidation-reduction process
g6237.t1	*NIT1*	5.80	6.19	Aliphatic nitrilase	#N/A
g1700.t1	*MAL11*	5.75	7.49	High-affinity maltose transporter	Transmembrane transport/ion transport/establishment of localization
g137.t1	*ICL1*	5.73	6.15	Isocitrate lyase that catalyzes the formation of succinate and glyoxylate from isocitrate, a key reaction of the glyoxylate cycle	Oxidation-reduction process
g1870.t1	*KIN3*	5.73	6.66	Serine/threonine protein kinase	Oxidation-reduction process
g1990.t1	*PUT4*	5.60	6.47	Proline permease	Transmembrane transport/polyamine transport/ammonium transport/oxidation-reduction process
g7322.t1	#N/A	5.58	4.65	Lactonase	#N/A
g5243.t1	*SNF3*	5.58	6.72	High-affinity plasma membrane glucose transporter	Transmembrane transport/ion transport/establishment of localization
g7895.t1	*DAL4*	5.57	6.69	Allantoin permease	Transmembrane transport/nucleobase transport/establishment of localization
g1078.t1	*DAL5*	5.49	4.53	Allantoate permease	Transmembrane transport/ establishment of localization

Gene	SGD Gene name	logFC *P. terrestris* + *P. expansum*	logFC *P. terrestris*	GO Description and/or SGD-NCBI annotation	GO Biological Process
g6407.t1	*FOX2*	5.49	5.10	3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase; multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway	Oxidation-reduction process
g2105.t1	*THI73*	5.47	7.23	Putative plasma membrane permease; proposed to be involved in carboxylic acid uptake and repressed by thiamine.	Transmembrane transport/establishment of localization
g4715.t1	YLL056C	5.43	3.31	Putative NADH-flavin oxidoreductase	#N/A
g5249.t1	*MAL11*	5.39	6.83	High-affinity maltose transporter	Transmembrane transport/ion transport/establishment of localization
g5400.t1	*FSH2*	5.37	6.75	Putative serine hydrolase	#N/A
g1699.t1	#N/A	5.35	8.13	Putative endoglucanase	#N/A
g730.t1	#N/A	5.39	4.34	Putative short chain dehydrogenase	
g5796.t1	#N/A	5.23	5.10	Glutamyl-tRNA amidotransferase	Oxidation-reduction process
g6201.t1	#N/A	5.20	6.96	2-oxoglutarate-dependent ethylene/succinate-forming enzyme	Transmembrane transport/establishment of localization/oxidation-reduction process
g2664.t1	*ITR2*	5.15	8.12	Myo-inositol transporter	Transmembrane transport/establishment of localization/oxidation-reduction process
g5672.t1	#N/A	5.11	6.39	Chromate transmembrane transporter	Transmembrane transport/establishment of localization/oxidation-reduction process
g2284.t1	#N/A	5.10	5.11	Inner membrane transport protein yeiJ	Transmembrane transport/establishment of localization/oxidation-reduction process
g5568.t1	#N/A	5.04	8.56	Glycoside hydrolase family 79 protein	#N/A
g6694.t1	#N/A	4.99	5.30	Alpha-galactosidase C	Oxidation-reduction process
g5688.t1	*DAL5*	4.99	5.59	Allantoate permease	Transmembrane transport.
g7491.t1	#N/A	4.93	4.66	FAD dependent oxidoreductase	Transmembrane transport/establishment of

Gene	SGD Gene name	logFC *P. terrestris* + *P. expansum*	logFC *P. terrestris*	GO Description and/or SGD-NCBI annotation	GO Biological Process
					localization/oxidation-reduction process
g2051.t1	YMR034C	4.93	11.60	Putative sodium/bile acid cotransporter 7-B/bile acid cotransporter 7-B	Oxidation-reduction process
g5745.t1	*TPO3*	4.92	4.80	Polyamine MFS transporter	Transmembrane transport/polyamine transport/ammonium transport/establishment of localization
g3186.t1	#N/A	4.83	4.58	MFS general substrate transporter	Transmembrane transport/establishment of localization/oxidation-reduction process
g3611.t1	#N/A	4.82	4.39	Myo-inositol oxygenase	Oxidation-reduction process
g6404.t1	#N/A	4.71	4.19	Glutamine synthetase guanido kinase	Glutamine family amino acid metabolic process
g1175.t1	#N/A	4.66	4.00	Acidic repeat-containing protein	Oxidation-reduction process
g6294.t1	#N/A	4.65	8.74	Carbonic anhydrase	Transport/establishment of localization
g258.t1	*SER3*	4.60	5.47	3-phosphoglycerate dehydrogenase that catalyzes the first step in serine and glycine biosynthesis	Oxidation-reduction process
g2744.t1	#N/A	4.59	6.04	N-methyl-L-tryptophan oxidase	Oxidation-reduction process
g1107.t1	*UGA2*	4.56	5.50	Succinate semialdehyde dehydrogenase involved in the utilization of gamma-aminobutyrate (GABA) as a nitrogen source	Oxidation-reduction process/glutamine family amino acid metabolic process
g3457.t1	#N/A	4.55	5.93	Glycoside hydrolase	Glutamine family amino acid metabolic process
g5485.t1	*TNA1*	4.53	4.97	High affinity nicotinic acid plasma membrane MFS permease	Transmembrane transport/establishment of localization
g2442.t1	#N/A	4.53	5.96	Uric acid-xanthine permease	Transmembrane transport/nucleobase transport/establishment of localization
g8313.t1	*JEN1*	4.52	8.01	Monocarboxylate/proton MFS symporter of the plasma membrane	Transmembrane transport/ion transport/establishment of localization/organic hydroxy compound transport

Gene	SGD Gene name	logFC *P. terrestris* + *P. expansum*	logFC *P. terrestris*	GO Description and/or SGD-NCBI annotation	GO Biological Process
g6121.t1	*LYS9*	4.50	5.57	Saccharopine dehydrogenase (NADP+, L-glutamate-forming) involved in lysine biosynthesis	Oxidation-reduction process
g4109.t1	*SEO1*	4.48	5.97	Putative MFS permease	Transmembrane transport/establishment of localization
g5778.t1	*DAL4*	4.47	4.45	Allantoin permease	Transmembrane transport/nucleobase transport/establishment of localization
g826.t1	*OPT1*	4.47	4.31	Proton-coupled oligopeptide transporter of the plasma membrane; also transports glutathione and phytochelatin	Transmembrane transport/ion transport/organic anion transport/establishment of localization
g6361.t1	YIL166C	4.44	6.02	Putative protein with similarity to allantoate MFS permease	Transmembrane transport/ion transport/establishment of localization
g7289.t1	#N/A	4.30	4.71	NADPH-dependent FMN reductase ArsH	Oxidation-reduction process
g2154.t1	*GLO4*	4.30	4.35	Mitochondrial hydroxyacylglutathione hydrolase	Transmembrane transport/establishment of localization/oxidation-reduction process
g6693.t1	*MAL31*	4.27	4.61	Maltose permease	Transmembrane transport/ion transport/establishment of localization
g6785.t1	#N/A	4.25	4.81	Serine/threonine protein kinase	#N/A
g1800.t1	#N/A	4.25	6.41	NADP-dependent mannitol dehydrogenase	Oxidation-reduction process
g4708.t1	*IRC24*	4.20	6.73	Putative benzyl reductase similar to short-chain dehydrogenase/reductases	Oxidation-reduction process
g2622.t1	*DUR3*	4.20	6.66	Plasma membrane transporter for both urea and polyamines	Transmembrane transport/polyamine transport/ammonium transport/establishment of localization/oxidation-reduction process
g2623.t1	*DUR3*	4.19	7.05	SSS family solute:Na+ symporter	Transmembrane transport/establishment of localization
g7286.t1	*YRM1*	4.17	4.09	Zinc finger transcription factor involved in multidrug resistance	Regulation of transcription by RNA polymerase II

Gene	SGD Gene name	logFC *P. terrestris* + *P. expansum*	logFC *P. terrestris*	GO Description and/or SGD-NCBI annotation	GO Biological Process
g2450.t1	*ASP3-4*	4.13	8.92	Cell-wall L-asparaginase II involved in asparagine catabolism	Glutamine family amino acid metabolic process
g4161.t1	#N/A	4.13	4.67	N-substituted formamide deformylase	Transmembrane transport/glutamine family amino acid metabolic process/establishment of localization
g7813.t1	#N/A	4.11	6.05	Chromate transmembrane transporter	Transmembrane transport/establishment of localization/oxidation-reduction process
g825.t1	*OPT1*	4.05	4.63	Proton-coupled oligopeptide transporter of the plasma membrane; also transports glutathione and phytochelatin	Transmembrane transport/ion transport/organic anion transport/establishment of localization
g7969.t1	YIL166C	4.04	6.25	Putative MFS protein with similarity to allantoate permease	Transmembrane transport/establishment of localization/oxidation-reduction process

Table 4. Main genes of LS28 expressed in dual interaction with *P. expansum* in apple (included only DEGs with logFC >4). DEGs sorted for LogFC; extracted from tables S1. Hypothetical proteins with no detected domains have not been included in this table and can be found in tables S1.

Gene	SGD Gene name	logFC	GO Description and/or SGD-NCBI annotation	GO Biological Process
g3655.t1	#N/A	6.84	N-terminal signal peptide	Negative regulation of hydrolase activity
g2837.t1	#N/A	5.00	Unknown domains/HYR domain	#N/A
g142.t1	#N/A	4.92	Unknown glutathione S-transferase	Glutathione metabolic process
g1861.t1	#N/A	4.33	Predicted F-box domain	#N/A
g5316.t1	*GAL10*	4.14	UDP-glucose-4-epimerase	Galactose catabolic process
g8073.t1	*KIP2*	4.02	Kinesin-related motor protein involved in mitotic spindle positioning	Microtubule polymerization/ chemotaxis/positive regulation of protein phosphorylation/Vesicle-mediated transport

Table 5. List of *P. terrestrsis* LS28 transcription factors present in our DEGs, filtered for logFC > 2 identified by blastp using as input all the identified sequences as transcription factors from *Cryptococcus neoformans* var. grubii strain H99, downloaded from NCBI.

Condition	**Gene**	**SGD Gene name**	**logFC *P. terrestris* + *P. expansum***	**logFC *P. terrestris***	**NCBI annotation**
Common genes	g5687.t1	#N/A	3.59	3.64	C6 finger domain-containing protein
	g1106.t1	*ASG1*	3.55	3.02	Fungal transcriptional regulatory protein, N-terminal
	g713.t1	*PZF1*	2.94	3.04	Transcription factor iiia
	g5386.t1	*STE12*	2.92	3.12	Sexual development transcription factor SteA
	g7133.t1	*PPR1*	2.34	3.12	Transcriptional regulatory
	g2789.t1	#N/A	2.20	2.79	Fungal-specific transcription factor domain-domain-containing protein
	g6376.t1	*ASG1*	2.10	2.11	Fungal-specific transcription factor domain-domain-containing protein
	g1487.t1	#N/A	2.06	2.19	Fungal-specific transcription factor domain-domain-containing protein
P. terrestris	g7108.t1	#N/A	-	2.99	Transcription factor RfeD
	g337.t1	#N/A	-	2.39	Immunoreactive mannoprotein MP88
	g1120.t1	#N/A	-	2.08	Bacterial transferase hexapeptide
P. terrestris + *P. expansum*	g6686.t1	#N/A	2.04	-	#N/A

Table 6. KEGG enrichment analysis on *Papiliotrema terrestris* LS28. Upregulated DEGs.

Condition	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue
Papiliotrema terrestris	Valine, leucine and isoleucine degradation	7/20	30/848	1.88E-06	7.92E-05	6.15E-05
	Propanoate metabolism	4/20	16/848	3.43E-04	7.20E-03	5.60E-03
	ABC transporters	2/20	4/848	3.08E-03	4.32E-02	3.36E-02
	Biosynthesis of antibiotics	8/20	131/848	6.44E-03	6.76E-02	5.25E-02
	Sulfur metabolism	2/20	7/848	1.03E-02	8.69E-02	6.75E-02
Common genes	Phenylalanine metabolism	8/69	16/848	9.83E-06	6.00E-04	4.47E-04
	Arginine and proline metabolism	9/69	21/848	1.25E-05	6.00E-04	4.47E-04
	Styrene degradation	4/69	4/848	4.04E-05	1.29E-03	9.64E-04
	Glycine, serine and threonine metabolism	10/69	31/848	7.43E-05	1.78E-03	1.33E-03
	Aminobenzoate degradation	4/69	5/848	1.90E-04	3.64E-03	2.71E-03
	Tryptophan metabolism	7/69	18/848	2.70E-04	4.32E-03	3.22E-03
	Glyoxylate and dicarboxylate metabolism	7/69	19/848	4.00E-04	5.49E-03	4.09E-03
	Microbial metabolism in diverse environments	20/69	120/848	6.25E-04	7.50E-03	5.59E-03
	Alanine, aspartate and glutamate metabolism	7/69	21/848	8.09E-04	8.63E-03	6.43E-03
	Arginine biosynthesis	4/69	7/848	1.17E-03	1.12E-02	8.37E-03
	Tyrosine metabolism	6/69	17/848	1.41E-03	1.23E-02	9.19E-03
	Biosynthesis of antibiotics	20/69	131/848	2.06E-03	1.65E-02	1.23E-02
	Glycerolipid metabolism	5/69	14/848	3.47E-03	2.56E-02	1.91E-02

Condition	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue
Common genes	Biosynthesis of secondary metabolites	23/69	167/848	3.73E-03	2.56E-02	1.91E-02
	Nitrogen metabolism	3/69	5/848	4.59E-03	2.94E-02	2.19E-02
	Biosynthesis of amino acids	10/69	51/848	5.61E-03	3.28E-02	2.44E-02
	Tropane, piperidine and pyridine alkaloid biosynthesis	4/69	10/848	5.80E-03	3.28E-02	2.44E-02
	Phenylalanine, tyrosine and tryptophan biosynthesis	4/69	11/848	8.57E-03	4.15E-02	3.09E-02
	Isoquinoline alkaloid biosynthesis	4/69	11/848	8.57E-03	4.15E-02	3.09E-02
	Ascorbate and aldarate metabolism	3/69	6/848	8.64E-03	4.15E-02	3.09E-02
	Glycolysis / Gluconeogenesis	6/69	24/848	9.70E-03	4.43E-02	3.31E-02
	Lysine degradation	5/69	19/848	1.46E-02	6.37E-02	4.75E-02
	Galactose metabolism	4/69	13/848	1.64E-02	6.83E-02	5.10E-02
	Degradation of aromatic compounds	3/69	8/848	2.15E-02	8.59E-02	6.40E-02
	Metabolic pathways	40/69	389/848	2.41E-02	9.25E-02	6.90E-02
Papiliotrema terrestris + *P expansum*	Chemical carcinogenesis	1/3	4/848	1.41E-02	6.35E-02	1.91E-02
	Ascorbate and aldarate metabolism	1/3	6/848	2.11E-02	6.35E-02	1.91E-02
	Metabolism of xenobiotics by cytochrome P450	1/3	6/848	2.11E-02	6.35E-02	1.91E-02
	Drug metabolism - cytochrome P450	1/3	7/848	2.46E-02	6.35E-02	1.91E-02
	Hepatocellular carcinoma	1/3	8/848	2.81E-02	6.35E-02	1.91E-02
	Inositol phosphate metabolism	1/3	11/848	3.85E-02	6.35E-02	1.91E-02
	Fluid shear stress and atherosclerosis	1/3	11/848	3.85E-02	6.35E-02	1.91E-02

Condition	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue
Papiliotrema terrestris + P expansum	Drug metabolism - other enzymes	1/3	12/848	4.19E-02	6.35E-02	1.91E-02
	Platinum drug resistance	1/3	12/848	4.19E-02	6.35E-02	1.91E-02
	Galactose metabolism	1/3	13/848	4.53E-02	6.35E-02	1.91E-02
	Glutathione metabolism	1/3	15/848	5.22E-02	6.64E-02	2.00E-02
	Pathways in cancer	1/3	18/848	6.24E-02	7.28E-02	2.19E-02
	Amino sugar and nucleotide sugar metabolism	1/3	26/848	8.93E-02	9.62E-02	2.89E-02

Table 7. Genes of *Papiliotrema terrestris* wrongly annotated by KEGG as “antibiotic biosynthesis”.

***Papiliotrema terrestris* in wound**		**KEGG annotation for antibiotic biosynthesis**	
Sigla	**GeneName**	**logFC**	**Description**
g6316.t1	*TAL1*	5.97	Transaldolase b
g4720.t1	*MET17*	3.09	O-acetylhomoserine aminocarboxypropyltransferase
g1977.t1	*ALD5*	2.84	NAD-dependent aldehyde dehydrogenase
g7591.t1	*KGD2*	2.72	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
g4593.t1	*PDA1*	2.65	2-oxoisovalerate dehydrogenase subunit alpha
g3441.t1	*PDB1*	2.60	2-oxoisovalerate dehydrogenase subunit beta, mitochondrial
g6037.t1	*MET3*	2.16	Sulfate adenylyltransferase
g7755.t1	#N/D	2.04	Medium-chain specific acyl-CoA dehydrogenase

Table 8. Main genes of *P. expansum* expressed alone in apple (include only those with logFC >4). Sorted for Log2FC; DEGs were selected based on the most enriched biological process and only the highest expressed were included in this table (with fold change > 4). The complete list is available in supplemental file S3.

Gene	**SGD Gene name**	**logFC**	**GO Description and/or SGD-NCBI annotation**	**GO Biological Process**	**Homologous retrieved in strain MD8**
PEX1_050910	#N/D	6.14	HMG high mobility group box	#N/A	PEX2_103030
PEX1_003930	*HNM1*	5.26	Amino acid transporter/Polyamine transporter I	Transmembrane transport	PEX2_100260
PEX1_005170	#N/D	5.09	FAD-linked oxidoreductase/patulin biosynthesis cluster protein O	#N/A	patO PEX2_082840
PEX1_003140	#N/D	4.99	IgE-binding protein (Fragment)/signal peptide	#N/A	PEX2_022560
PEX1_038390	*SNQ2*	4.68	Pleiotropic drug resistance protein, ABC superfamily	Transmembrane transport	PEX2_051090
PEX1_045200	*FAT1*	4.55	AMP-dependent synthetase/ligase	#N/A	PEX2_001320
PEX1_005150	*SNQ2*	4.31	CDR ABC transporter / Patulin biosynthesis cluster protein M	Transmembrane transport	patM PEX2_082820
PEX1_002430	*CEM1*	4.02	Acyl transferase/acyl hydrolase/lysophospholipase /Non reducing polyketide synthase, patulin biosynthesis cluster protein K	Fatty acid biosynthetic process	patK PEX2_082880
PEX1_037860	*ERG5*	4.02	Cytochrome P450, E-class, group I	#N/A	PEX2_066830

Table 9. DEGs common in *Penicillium expansum*-apple/ *Penicillium expansum*-BCA-apple interaction. DEGs were selected based on the most enriched biological process and only the highest expressed were included in this table (with fold change > 4). The complete list is available in supplemental file S3.

Gene	**SGD Gene name**	**logFC P. exp. + LS28**	**logFC P. exp**	**GO Description and/or SGD-NCBI annotation**	**GO Biological Process**	**Homologous retrieved in strain MD-8**
PEX1_083840	*ATG26*	13.75	13.71	UDP-glucuronosyl/UDP-glucosyltransferase	Oxidation-reduction process/Carbohydrate metabolic process/ Regulation of gene expression	PEX2_106790
PEX1_058740	*HOL1*	10.67	10.72	Major facilitator superfamily domain, general substrate transporter	Drug transport/Response to drug/ Transmembrane transport/ xenobiotic transport	PEX2_075270
PEX1_085620	#N/A	10.28	11.36	Pectin lyase fold/virulence factor/signal peptide	Glucan metabolic process/ lipid metabolic process/ Oxidation-reduction process	PEX2_092020
PEX1_010540	*CBR1*	9.62	11.22	Cytochrome b5	Oxidation-reduction process	PEX2_051790
PEX1_052260	YHL008C	9.58	8.90	Formate/nitrite transporter	Transmembrane transport/ nitrite transport/ nitrate transport	PEX2_065590
PEX1_023520	#N/A	9.47	8.22	Aromatic-ring-hydroxylating dioxygenase, alpha subunit	Oxidation-reduction process/Cellular metabolic process	PEX2_013010
PEX1_019820	#N/A	8.63	5.86	Pyrimidine monooxygenase RutA	Uracil catabolic process/Nitrogen utilization/	PEX2_084050
PEX1_053980	*HXT17*	8.59	4.70	General substrate transporter	Transmembrane transport	PEX2_015950
PEX1_088550	*PGU1*	8.54	9.95	Glycoside hydrolase, family 28	Carbohydrate metabolic process/ Macromolecule catabolic process	PEX2_031340 (Levin et al., 2019)
PEX1_010530	*ERG11*	8.31	10.29	Cytochrome P450	Transmembrane transport/ Oxidation-reduction process	PEX2_051800
PEX1_058130	*DAL5*	8.10	7.57	Major facilitator superfamily domain, general substrate transporter	Transmembrane transport	PEX2_110020

Gene	SGD Gene name	logFC P. exp. + LS28	logFC P. exp	GO Description and/or SGD-NCBI annotation	GO Biological Process	Homologous retrieved in strain MD-8
PEX1_025200	YDL144C	7.10	5.86	Ketopantoate reductase ApbA/PanE/ signal peptide	Pantothenate biosynthetic process/methylation/ Amide biosynthetic process	PEX2_000650
PEX1_083670	#N/A	6.81	7.17	Pectin lyase fold/virulence factor/signal peptide	Polysaccharide metabolic process/Pectin metabolic process/Cell wall organization	PEX2_107230 Levin et al., 2019)
PEX1_049460	*DUR1,2*	6.55	4.16	Amidase	Glutaminyl-tRNAGln biosynthesis via transamidation/mitochondrial translation	PEX2_039050
PEX1_059870	*FDH1*	5.82	6.35	D-isomer specific 2-hydroxyacid dehydrogenase, NAD-binding	Formate catabolic process/Oxalate metabolic process/leucyl-tRNA aminoacylation/oligosaccharide metabolic process	PEX2_073710
PEX1_045780	*PGU1*	4.54	6.71	Glycoside hydrolase, family 28/ signal peptide	Cell wall organization/Carbohydrate metabolic process/Drug catabolic process/Macromolecule catabolic process	PEX2_069880
PEX1_058750	YDR338C	4.43	5.21	Multi antimicrobial extrusion protein	Xenobiotic transport/Transmembrane transport	PEX2_075260
PEX1_096480	#N/A	4.24	3.73	Major facilitator superfamily domain	Transmembrane transport/Drug transport/	PEX2_003430
PEX1_080170	#N/A	4.10	2.96	Aminoglycoside phosphotransferase/ Kinase domain	Translation	PEX2_102360

Table 10. DEGs of *Penicillium expansum* highly expressed in apple only in presence of the BCA. DEGs were selected based on the most enriched biological process and only the highest expressed were included in this table (with fold change > 4). The complete list is available in supplemental file S3.

Gene	SGD Gene name	logFC	GO Description and/or SGD-NCBI annotation	GO Biological Process	Homologous retrieved in strain MD-8
PEX1_022400	#N/A	6.64	Glycoside hydrolase, family 2, N-terminal / signal peptide C- region	Polysaccharide catabolic process	PEX2_080380
PEX1_012350	#N/A	6.60	Sialidase family / signal peptide N- region	#N/A	PEX2_054830
PEX1_027760	#N/A	6.55	Fungal chitosanase / signal peptide N- region	#N/A	PEX2_093030
PEX1_023120	*HNM1*	6.51	AA_permease	Transmembrane transport	PEX2_041120
PEX1_010930	*TPO2*	6.28	Major facilitator superfamily domain, general substrate transporter	Transmembrane transport	PEX2_080560
PEX1_056040	#N/A	6.19	Fungal chitosanase / signal peptide N- region	#N/A	PEX2_081710
PEX1_012360	#N/A	6.15	Dihydrodipicolinate synthetase	#N/A	PEX2_054840
PEX1_065360	#N/A	5.69	RmlC-like jelly roll fold protein / signal peptide N- region	#N/A	PEX2_028180
PEX1_088900	*BDH1*	5.52	Alcohol dehydrogenase superfamily, zinc-type	#N/A	PEX2_044230
PEX1_019210	*CTS2*	5.30	Chitinases II / signal peptide N- region	Carbohydrate metabolic process	PEX2_053990
PEX1_005750	*HSP31*	4.81	ThiJ/PfpI	#N/A	PEX2_014470
PEX1_044380	#N/A	4.61	Acetoacetate decarboxylase	#N/A	PEX2_009020
PEX1_071780	#N/A	4.57	Aminotransferase, class IV	#N/A	PEX2_054800
PEX1_103690	#N/A	4.44	Transcription factor, fungi / Citrinin biosynthesis transcriptional activator	Regulation of transcription, DNA-templated	PEX2_030910
PEX1_072210	YFL054C	4.28	Major intrinsic protein	Transmembrane transport	PEX2_018710
PEX1_049140	#N/A	4.27	NADH-dependent flavin oxidoreductase nadA	#N/A	PEX2_101560
PEX1_093060	#N/A	4.11	N-acetyl-glucosamine-6-phosphate deacetylase	N-acetylglucosamine metabolic process	PEX2_084400
PEX1_004560	*TRP5*	4.09	Tryptophan synthase beta chain	Tryptophan metabolic process	PEX2_083770

Gene	SGD Gene name	logFC	GO Description and/or SGD-NCBI annotation	GO Biological Process	Homologous retrieved in strain MD-8
PEX1_063240	*MCH5*	4.08	Major facilitator superfamily domain, general substrate transporter	Transmembrane transport	PEX2_052010
PEX1_010380	*ALD5*	4.07	Aldehyde dehydrogenase, N-terminal	#N/A	PEX2_100500
PEX1_038110	#N/A	4.02	GPI-anchored CFEM domain protein B / signal peptide N-region / signal peptide C-region	#N/A	PEX2_029710

Table 11. KEGG *Penicillium expansum* uregulated DEGs

Condition	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue
Penicillium expansum	Fatty acid degradation	2/9	24/1373	9.79E-03	1.05E-01	8.71E-02
	Fatty acid metabolism	2/9	24/1373	9.79E-03	1.05E-01	8.71E-02
	Biosynthesis of enediyne antibiotics	1/9	2/1373	1.31E-02	1.05E-01	8.71E-02
	Aflatoxin biosynthesis	1/9	4/1373	2.60E-02	1.56E-01	1.30E-01
Common genes	Ribosome	51/252	72/1373	7.98E-24	1.30E-21	1.18E-21
	Phenylalanine metabolism	19/252	38/1373	6.86E-06	5.59E-04	5.09E-04
	Tropane, piperidine and pyridine alkaloid biosynthesis	8/252	11/1373	1.15E-04	6.27E-03	5.71E-03
	Arginine and proline metabolism	19/252	46/1373	1.93E-04	7.85E-03	7.15E-03
	Ribosome biogenesis in eukaryotes	17/252	41/1373	4.03E-04	1.31E-02	1.20E-02
	Alanine, aspartate and glutamate metabolism	12/252	27/1373	1.44E-03	3.91E-02	3.56E-02
	Isoquinoline alkaloid biosynthesis	8/252	15/1373	2.27E-03	5.28E-02	4.81E-02
	Tryptophan metabolism	15/252	41/1373	3.95E-03	7.23E-02	6.58E-02
	Lysine biosynthesis	7/252	13/1373	4.06E-03	7.23E-02	6.58E-02
	Microbial metabolism in diverse environments	50/252	196/1373	4.44E-03	7.23E-02	6.58E-02
	beta-Alanine metabolism	10/252	24/1373	6.37E-03	9.06E-02	8.25E-02
Penicillium expansum + P. terrestris	Amino sugar and nucleotide sugar metabolism	7/33	41/1373	3.01E-05	3.04E-03	2.82E-03
	Phenylalanine metabolism	5/33	38/1373	1.67E-03	8.44E-02	7.83E-02
	Isoquinoline alkaloid biosynthesis	3/33	15/1373	4.73E-03	1.59E-01	1.48E-01
	Phenylpropanoid biosynthesis	3/33	19/1373	9.43E-03	1.91E-01	1.78E-01
	Tyrosine metabolism	4/33	36/1373	9.47E-03	1.91E-01	1.78E-01

Table 12**.** Accession of *Penicillium expansum* highlighted by KEGG analysis during the interaction with *P. terrestris* in wound, annotated as "Amino sugar and nucleotide sugar metabolism".

NCBI ID	**Locus**	**logFC**	**Description**	**Annotation**
KGO70353.1	PEX1_022400	6.64	Exo-beta-D-glucosaminidase	Glycoside hydrolase, family 2, N-terminal
KGO47124.1	PEX1_027760	6.55	Endo-chitosanase C	Fungal chitosanase
KGO61070.1	PEX1_019210	5.30	Glycoside hydrolase	Glycoside hydrolase, superfamily
KGO73453.1	PEX1_093060	4.11	N-acetyl-glucosamine-6-phosphate deacetylase	#N/D
KGO70627.1	PEX1_010940	3.87	Glucokinase regulator family	N-acetylmuramic acid 6-phosphate etherase MurQ
KGO73456.1	PEX1_093090	3.81	Glucosamine-6-phosphate isomerase	#N/D
KGO58098.1	PEX1_007300	3.68	Phenol hydroxylase reductase	Cytochrome b5

Table 13. Most expressed genes of *Malus domestica* affected by BCA inoculation. The list of genes was filtered by fold change >4.

Gene	***Arabidopsis* Gene name**	***M. domestica* + *P. terrestris* logFC**	**GO Description and/or TAIR annotation**	**GO Biological Process**
MD13G1133200	*POT5*	5.06	Potassium transporter	Cellular potassium ion transport
MD07G1254600	*SKU5*	4.94	Monocopper oxidase-like protein	Oxidation-reduction process
MD06G1147000	*BGLU13*	4.78	Beta-glucosidase 13	Carbohydrate metabolic process
MD13G1148400	*GA2OX6*	4.76	Gibberellin 2-oxidase 6	Metabolic process/Oxidation-reduction process

Table 14. Most expressed Common DEGs. DEGs were selected based on the most enriched biological process and only those highest expressed were included in this table (with fold change > 4). The complete list is available in supplemental file S5.

Gene	*Arab.* Gene name	logFC[a]	logFC[b]	logFC[c]	GO Description and/or TAIR annotation	GO Biological Process
MD03G1142500	#N/A	13.81	8.92	6.41	NAD+ ADP-ribosyltransferases	Metabolic process
MD12G1211300	*BGLU17*	13.14	7.69	3.06	Beta glucosidase 17	Carbohydrate metabolic process
MD14G1140400	*DLO2*	13.03	9.81	4.65	Protein DMR6-LIKE OXYGENASE 2	Metabolic process/ Oxidation-reduction process
MD14G1141000	*DLO2*	12.41	7.31	7.81	Protein DMR6-LIKE OXYGENASE 2	Metabolic process/ Oxidation-reduction process
MD05G1285000	*CYP710 A1*	11.83	7.18	7.07	Cytochrome P450	Metabolic process/ Oxidation-reduction process
MD12G1211500	*BGLU17*	11.43	6.17	2.90	Beta glucosidase 17	Carbohydrate metabolic process
MD16G1215200	#N/A	11.38	7.74	7.29	1-aminocyclopropane-1-carboxylate oxidase homolog1	Oxidation-reduction process
MD10G1155000	*CAD7*	11.09	9.01	7.15	Elicitor-activated gene 3-1/ ELI3-1	Oxidation-reduction process
MD05G1287200	*AGL62*	10.97	6.83	5.99	Agamous-like MADS-box protein AGL62	Positive regulation of transcription by RNA polymerase II
MD16G1278900	*XTH30*	10.84	5.27	6.34	Probable xyloglucan endotransglucosylase /hydrolase protein 30	Polysaccharide metabolic process
MD03G1204700	*BGLU15*	10.30	4.94	-	Beta-glucosidase 15	Carbohydrate metabolic process
MD00G1145300	*BGLU17*	10.10	4.43	-	Beta glucosidase 17	Carbohydrate metabolic process
MD06G1043900	*BAM3*	8.71	9.40	6.46	Beta-amylase 3, chloroplastic	Polysaccharide metabolic process

Gene	*Arab.* Gene name	logFC[a]	logFC[b]	logFC[c]	GO Description and/or TAIR annotation	GO Biological Process
MD09G1072200	*CSLB3*	8.43	4.77	3.59	Cellulose synthase-like protein B3	Cellulose biosynthetic process
MD11G1146000	*CRK8*	8.42	7.35	6.79	Cysteine-rich RLK (RECEPTOR-like protein kinase) 8	DNA integration
MD10G1196700	*CRK8*	7.97	5.56	-	Cysteine-rich RLK (RECEPTOR-like protein kinase) 8	DNA integration
MD05G1287100	*AGL29*	7.78	4.77	5.43	AGAMOUS-like 29	Positive regulation of transcription by RNA polymerase II
MD12G1227900	*BON3*	7.74	5.74	4.76	Calcium-dependent phospholipid-binding Copine family protein	Cell death/ Negative regulation of cell death
MD17G1141300	*SHP1*	7.43	4.76	4.00	K-box region and MADS-box transcription factor family protein	Positive regulation of transcription by RNA polymerase II
MD12G1228300	*BON3*	6.42	3.20	-	Calcium-dependent phospholipid-binding Copine family protein	Cell death/ Negative regulation of cell death
MD05G1320800	#N/A	6.24	4.17	4.98	Polyphenol oxidase	Pigment metabolic process
MD16G1212000	*CSLG2*	5.91	3.01	2.11	Glycosyltransferase (Fragment)	Cellulose metabolic process/ Polysaccharide metabolic process
MD05G1320500	#N/A	5.90	4.31	5.39	Polyphenol oxidase	Pigment metabolic process
MD04G1142600	*LAC7*	5.60	2.86	2.40	Laccase-7	Aromatic compound catabolic process/ Lignin catabolic process/ Phenylpropanoid catabolic process
MD02G1095700	*CSLE1*	5.59	2.97	-	Cellulose synthase-like protein E1	Cellulose metabolic process/ Polysaccharide metabolic process
MD12G1157100	*LAC7*	5.44	3.72	-	Laccase-7	Aromatic compound catabolic process/ Lignin catabolic

Gene	*Arab.* Gene name	logFC[a]	logFC[b]	logFC[c]	GO Description and/or TAIR annotation	GO Biological Process
						process/ Phenylpropanoid catabolic process
MD12G1228200	*BON3*	4.71	2.68	-	Calcium-dependent phospholipid-binding Copine family protein	Cell death/ Negative regulation of cell death
MD04G1142900	*LAC7*	4.68	-	3.02	Laccase-7	Aromatic compound catabolic process/ Lignin catabolic process/ Phenylpropanoid catabolic process
MD00G1014900	*LAC14*	4.67	-	3.42	Laccase-14	Aromatic compound catabolic process/ Lignin catabolic process/ Phenylpropanoid catabolic process
MD02G1076800	*CDA1*	4.42	2.43	2.08	Cytidine deaminase 1	Aromatic compound catabolic process
MD07G1307400	*TT10*	4.29	-	2.13	Laccase/Diphenol oxidase family protein/Laccase-15	Lignin catabolic process/ Phenylpropanoid catabolic process
MD02G1295200	#N/A	4.09	2.74	2.27	Mitochondrial transcription termination factor family protein	DNA integration

logFC [a,b,c]=fold change of M. domestica inoculated with Penicillium expansum([a]),Tritrophic condition([b]), inoculated with Papiliotrema terrestris ([c])

Table 15: Most expressed DEGs of *Malus domestica* when inoculated with *P. expansum* plus genes shared with Tritrophic treatment. DEGs were selected based on the most enriched biological process and only those highest expressed were included in this table (with fold change > 4). The complete list is available in supplemental file S5.

Gene	***Arabidopsis* Gene name**	***M. domestica* + *P. expansum***	**GO Description and/or TAIR annotation**	**GO Biological Process**
MD05G1275700	*SBT1.7*	10.10	Subtilisin-like protease SBT1.7	Organonitrogen compound metabolic process
MD10G1248500	*WAK2*	7.25	Wall-associated receptor kinase 2	Cellular protein metabolic process/Phosphorus metabolic process
MD16G1010800	*GAD4*	7.04	Glutamate decarboxylase 4	Organonitrogen compound metabolic process
MD16G1065700	*CM3*	6.91	Chorismate mutase 3, chloroplastic	Aromatic amino acid family metabolic process
MD06G1232800	*PUB21*	6.79	U-box domain-containing protein 21	Cellular protein metabolic process/ Macromolecule modification
MD11G1254600	*LECRK91*	6.46	L-type lectin-domain containing receptor kinase IX.1	Phosphate-containing compound metabolic/ Cellular protein metabolic process/ Macromolecule modification
MD05G1144900	#N/A	6.42	Concanavalin A-like lectin protein kinase family protein	Phosphate-containing compound metabolic/ Cellular protein metabolic process/ Macromolecule modification
MD09G1098900	#N/A	6.36	G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130	Cell recognition/ Cellular protein metabolic process/ Macromolecule modification
MD01G1027900	*CRK26*	6.02	Cysteine-rich receptor-like protein kinase 26	Phosphate-containing compound metabolic process/Phosphorus metabolic process /Phosphorylation
MD03G1134800	*ADT6*	5.71	Arogenate dehydratase	Aromatic amino acid family metabolic process/L-phenylalanine biosynthetic process
MD13G1066600	*CM3*	5.61	Chorismate mutase 3, chloroplastic	Aromatic amino acid family metabolic process/ chorismate metabolic process

Gene	*Arabidopsis* Gene name	*M. domestica* + *P. expansum*	GO Description and/or TAIR annotation	GO Biological Process
MD01G1033900	#N/A	5.50	Phospho-2-dehydro-3-deoxyheptonate aldolase	Aromatic amino acid family metabolic process
MD08G1186700	#N/A	5.28	Phospho-2-dehydro-3-deoxyheptonate aldolase	Aromatic amino acid family metabolic process
MD15G1076500	#N/A	4.80	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080	Multi-multicellular organism process/Pollen-pistil interaction/Recognition of pollen/Reproduction
MD02G1047500	#N/A	4.62	Lectin protein kinase family protein	Cell recognition/ multi-multicellular organism process / Pollen – pistil interaction / Recognition of pollen
MD07G1157600	*KCS1*	4.25	3-ketoacyl-CoA synthase	Fatty acid metabolic process.
MD15G1391000	#N/A	4.15	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080	Cell recognition/ multi-multicellular organism process / Pollen – pistil interaction / Recognition of pollen
MD05G1039400	#N/A	4.12	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300	Cell recognition/ multi-multicellular organism process / Pollen – pistil interaction / Recognition of pollen

Table 16: List of DEGs of *Malus domestica* significantly upregulated exclusively in Tritrophic condition (apple treated with LS28 + *P. expansum*).

Locus	**Gene_name**	**LogFC**	**Description**
MD00G1105300	#N/D	4.31	Plant invertase/pectin methylesterase inhibitor superfamily protein
MD15G1038200	*CLPB4*	3.01	casein lytic proteinase B4
MD02G1144800	*SYP112*	2.73	syntaxin of plants 112
MD08G1222100	#N/D	2.47	#N/D
MD06G1212900	*PCR6*	2.44	PLAC8 family protein
MD09G1181800	#N/D	2.37	#N/D
MD08G1026600	#N/D	2.34	P-loop containing nucleoside triphosphate hydrolases superfamily protein
MD07G1266700	#N/D	2.28	#N/D
MD12G1082100	*CAF1*-5	2.20	Polynucleotidyl transferase
MD05G1351400	*CSC1*	2.18	ERD (early-responsive to dehydration stress) family protein
MD13G1073400	#N/D	2.13	Mog1/PsbP/DUF1795-like photosystem II reaction center PsbP family protein
MD00G1067900	*ATACA2*	2.12	alpha carbonic anhydrase 7
MD13G1210100	#N/D	2.08	Protein of unknown function (DUF668)
MD09G1267700	#N/D	2.06	HXXXD-type acyl-transferase family protein
MD03G1258300	*HSFA6B*	2.06	heat shock transcription factor A6B
MD12G1181900	*SDR2A*	2.03	NAD(P)-binding Rossmann-fold superfamily protein
MD11G1296800	*NPL41*	2.01	NPL4-like protein 1

Table 17. Most enriched KEGG pathway of *Malus domestica*.

Condition	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue
Malus domestica+P. terrestris	Cyanoamino acid metabolism	3/10	95/6998	2.71E-04	2.38E-03	1.67E-03
	Phenylpropanoid biosynthesis	4/10	258/6998	3.18E-04	2.38E-03	1.67E-03
	Diterpenoid biosynthesis	2/10	47/6998	1.92E-03	9.60E-03	6.74E-03
	Starch and sucrose metabolism	3/10	208/6998	2.66E-03	9.98E-03	7.00E-03
	Other glycan degradation	1/10	21/6998	2.96E-02	8.89E-02	6.24E-02
Common genes	Platinum drug resistance	17/171	74/6998	1.22E-12	2.83E-10	2.56E-10
	Drug metabolism - cytochrome P450	16/171	70/6998	6.31E-12	7.32E-10	6.61E-10
	Chemical carcinogenesis	15/171	73/6998	1.52E-10	1.17E-08	1.06E-08
	Metabolism of xenobiotics by cytochrome P450	15/171	75/6998	2.28E-10	1.32E-08	1.19E-08
	Phenylpropanoid biosynthesis	26/171	258/6998	5.08E-10	2.04E-08	1.84E-08
	Drug metabolism - other enzymes	17/171	106/6998	5.27E-10	2.04E-08	1.84E-08
	Hepatocellular carcinoma	16/171	108/6998	5.78E-09	1.92E-07	1.73E-07
	Longevity regulating pathway - worm	18/171	148/6998	1.55E-08	4.51E-07	4.07E-07
	Fluid shear stress and atherosclerosis	16/171	133/6998	1.20E-07	3.09E-06	2.79E-06
	Pathways in cancer	18/171	186/6998	5.34E-07	1.24E-05	1.12E-05
	Glutathione metabolism	15/171	132/6998	6.42E-07	1.35E-05	1.22E-05
	Monoterpenoid biosynthesis	10/171	57/6998	9.34E-07	1.81E-05	1.63E-05
	alpha-Linolenic acid metabolism	10/171	70/6998	6.52E-06	1.16E-04	1.05E-04
	Tyrosine metabolism	9/171	63/6998	1.92E-05	3.18E-04	2.87E-04
	Cyanoamino acid metabolism	10/171	95/6998	9.81E-05	1.52E-03	1.37E-03
	Biosynthesis of various secondary metabolites - part 1	4/171	13/6998	2.07E-04	3.00E-03	2.71E-03

Condition	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue
Common genes	Systemic lupus erythematosus	7/171	55/6998	3.47E-04	4.74E-03	4.28E-03
	Alcoholism	9/171	95/6998	4.87E-04	6.28E-03	5.67E-03
	Isoquinoline alkaloid biosynthesis	5/171	33/6998	1.12E-03	1.37E-02	1.23E-02
	Neutrophil extracellular trap formation	8/171	103/6998	3.57E-03	4.14E-02	3.74E-02
Malus domestica +*P. terrestris*+ *P. expansum*	Plant-pathogen interaction	34/425	206/6998	6.47E-08	1.97E-05	1.79E-05
	MAPK signaling pathway - plant	27/425	169/6998	2.93E-06	4.45E-04	4.04E-04
	Biosynthesis of amino acids	41/425	341/6998	1.63E-05	1.65E-03	1.50E-03
	alpha-Linolenic acid metabolism	14/425	70/6998	6.28E-05	4.77E-03	4.33E-03
	Taurine and hypotaurine metabolism	6/425	17/6998	3.37E-04	1.85E-02	1.68E-02
	Glycine, serine and threonine metabolism	14/425	82/6998	3.66E-04	1.85E-02	1.68E-02
	Phenylalanine, tyrosine and tryptophan biosynthesis	12/425	65/6998	4.57E-04	1.98E-02	1.80E-02
	Glycerophospholipid metabolism	18/425	138/6998	1.63E-03	6.01E-02	5.46E-02
	Synaptic vesicle cycle	9/425	47/6998	1.78E-03	6.01E-02	5.46E-02
	Sphingolipid metabolism	8/425	39/6998	2.01E-03	6.05E-02	5.49E-02
	Glycerolipid metabolism	15/425	109/6998	2.30E-03	6.05E-02	5.49E-02
	Nicotinate and nicotinamide metabolism	7/425	32/6998	2.59E-03	6.05E-02	5.49E-02
	Glucosinolate biosynthesis	5/425	17/6998	2.71E-03	6.05E-02	5.49E-02
	beta-Alanine metabolism	9/425	50/6998	2.79E-03	6.05E-02	5.49E-02
	Linoleic acid metabolism	6/425	26/6998	3.94E-03	7.98E-02	7.24E-02

Table 18. Summary of the genes selected for targeted mutagenesis. The symbol ”-“ in the last two columns means that the mutant has yet to be obtained.

Locus	**logFC**	**Description**	**Gene name**	**Condition**	**plasmid tag**	***Agrobacterium* mediated transformation**	**Electroporation**
g2837.t1	5.01	-	-	LS28+P.exp	pGI61	-	KO mutant
g142.t1	4.92	Glutathione S-transferase	-	LS28+P.exp	pGI62	-	KO mutant
g960.t1	3.54	Transcriptional repressor involved in the control of multidrug resistance	*LOT6*	LS28+P.exp	pGI63	-	KO mutant
g3033.t1	12.22	-	-	Common	pGI64	-	-
g1861.t1	4.33	F-box domain protein	-	LS28+P.exp	pGI65	-	KO mutant
g4389.t1	11.48	-	-	Common	pGI66	-	KO mutant
g5541.t1	11.27	Oligopeptide transporter	*OPT1*	Common	pGI67	-	-
g1105.t1	10.70	General amino acid permease	*PUT4*	Common	pGI68	KO mutant	KO mutant

Table 19. Information retrieved from INTERPRO (https://www.ebi.ac.uk/interpro/) related to each gene of *P. terrestris* LS28 selected for the target mutagenesis.

Gene	Encoded AA residues	Predictors			start	end
g2837.t1	279	MobiDBLite	mobidb-lite	consensus disorder prediction	251	279
g2837.t1	279	MobiDBLite	mobidb-lite	consensus disorder prediction	47	144
g2837.t1	279	MobiDBLite	mobidb-lite	consensus disorder prediction	101	125
g2837.t1	279	MobiDBLite	mobidb-lite	consensus disorder prediction	216	279
g2837.t1	279	MobiDBLite	mobidb-lite	consensus disorder prediction	251	279
g142.t1	233	SUPERFAMILY	SSF47616	GST C-terminal domain-like	86	221
g142.t1	233	Pfam	PF02798	GST N-terminal domain	39	79
g142.t1	233	SUPERFAMILY	SSF52833	Thioredoxin-like	1	90
g960.t1	228	SUPERFAMILY	SSF52218	Flavoproteins	8	200
g960.t1	228	Pfam	PF03358	NADPH-dependent FMN reductase	8	177
g3033.t1	328	Phobius	SIGNAL_PEPTIDE_N_REGION	N-terminal region of a signal peptide.	1	4
g3033.t1	328	SUPERFAMILY	SSF51735	NAD(P)-binding Rossmann-fold domains	4	300
g3033.t1	328	Gene3D	G3DSA:3.40.50.720	-	1	129
g3033.t1	328	Phobius	NON_CYTOPLASMIC_DOMAIN	Region of a membrane-bound protein predicted to be outside the membrane, in the extracellular region.	26	328

Gene	Encoded AA residues	Predictors			start	end
g1861.t1	-	-	-	-	-	-
g4389.t1	127	TMHMM	TMhelix	Region of a membrane-bound protein predicted to be embedded in the membrane.	47	65
g4389.t1	127	Phobius	NON_CYTOPLASMIC_DOMAIN	Region of a membrane-bound protein predicted to be outside the membrane, in the extracellular region.	127	127
g4389.t1	127	TMHMM	TMhelix	Region of a membrane-bound protein predicted to be embedded in the membrane.	109	126
g4389.t1	127	Phobius	CYTOPLASMIC_DOMAIN	Region of a membrane-bound protein predicted to be outside the membrane, in the cytoplasm.	101	106
g5541.t1	826	Phobius	NON_CYTOPLASMIC_DOMAIN	Region of a membrane-bound protein predicted to be outside the membrane, in the extracellular region.	153	157
g5541.t1	826	TMHMM	TMhelix	Region of a membrane-bound protein predicted to be embedded	159	177

Gene	Encoded AA residues	Predictors			start	end
				in the membrane.		
g5541.t1	826	TMHMM	TMhelix	Region of a membrane-bound protein predicted to be embedded in the membrane.	561	583
g5541.t1	826	Phobius	TRANSMEMBRANE	Region of a membrane-bound protein predicted to be embedded in the membrane.	505	525
g5541.t1	826	TMHMM	TMhelix	Region of a membrane-bound protein predicted to be embedded in the membrane.	727	749
g5541.t1	826	Pfam	PF03169	OPT oligopeptide transporter protein	129	782
g1105.t1	546	Phobius	CYTOPLASMIC_DOMAIN	Region of a membrane-bound protein predicted to be outside the membrane, in the cytoplasm.	501	546
g1105.t1	546	Phobius	TRANSMEMBRANE	Region of a membrane-bound protein predicted to be embedded in the membrane.	106	126
g1105.t1	546	Phobius	TRANSMEMBRANE	Region of a membrane-bound protein predicted to be embedded in the membrane.	74	94

Gene	Encoded AA residues	Predictors			start	end
g1105.t1	546	Phobius	NON_CYTOPLASMIC_DOMAIN	Region of a membrane-bound protein predicted to be outside the membrane, in the extracellular region.	398	402
g1105.t1	546	Phobius	TRANSMEMBRANE	Region of a membrane-bound protein predicted to be embedded in the membrane.	156	177
g1105.t1	546	Phobius	TRANSMEMBRANE	Region of a membrane-bound protein predicted to be embedded in the membrane.	330	355
g1105.t1	546	Phobius	CYTOPLASMIC_DOMAIN	Region of a membrane-bound protein predicted to be outside the membrane, in the cytoplasm.	356	375
g1105.t1	546	Phobius	TRANSMEMBRANE	Region of a membrane-bound protein predicted to be embedded in the membrane.	448	469
g1105.t1	546	Pfam	PF00324	Amino acid permease	46	505

Table 20. List of primers used for “Knock-Out” cassette development. In red adaptors for *HYG* marker. In blue adaptors for plasmid vector. In the last column the corresponding gene of interest (GOI) to be deleted.

Nucleotide sequence	Type of primer	GOI
GCGCGCCTAGGCCTCTGCAGGTCGACTCTGATGTTCAGCACCATGACCG	Fw 5’ fragment::	g2837.t1
TATATCGACAGTGGCTTCTCGCTTGGCGTCCTTATCTTCG	Rev 5’ fragment::	
AACAATCTGCGCAGTGCTGAGAGATCCCCGAGCCTTTGAT	Fw 3’ fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGGTGCTGACACCGTAGCCGAC	Rev 3’ fragment::	
GGCTGATAGAATGCTCACGC	Fw – Ext. screening	
TTCACCTCACTCCAACCGG	Rev – Ext. screening	
GCGCGCCTAGGCCTCTGCAGGTCGACTCTGCGCACATGGGTCTGAACTG	Fw 5’ fragment::	g142.t1
TATATCGACAGTGGCTTCTCCTTTGGAGCCAGCGTTGTC	Rev 5’ fragment::	
AACAATCTGCGCAGTGCTGACCACTCTGGCGATTTGATCT	Fw 3’ fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGATGAGGGCATTGCAACGATC	Rev 3’ fragment::	
CGGAGGCGTTTATTGACCAG	Fw – Ext. screening	
GACGAGGTGGCTGATGAGG	Rev – Ext. screening	
GCGCGCCTAGGCCTCTGCAGGTCGACTCTGTCACGGAAGATACCTCGGT	Fw 5’ fragment::	g960.t1
TATATCGACAGTGGCTTCTCGTCTTGCCTAGCGTTGTGG	Rev 5’ fragment::	
AACAATCTGCGCAGTGCTGAGGAACGGATGAAAGGTGACG	Fw 3’ fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGACATCTCCTCAACGCAAAGC	Rev 3’ fragment::	
GAGGTAGCGGGATTATTGCG	Fw – Ext. screening	
CCTCGTTATGTCGTCCAGGA	Rev – Ext. screening	
GCGCGCCTAGGCCTCTGCAGGTCGACTCTCACAAGCAGAGATCGACGTG	Fw 5’ fragment::	g1861.t1
TATATCGACAGTGGCTTCTCTCTTCGGTGTCATCCTCCTG	Rev 5’ fragment::	
AACAATCTGCGCAGTGCTGAGAGACATCCTGCCTCGAATC	Fw 3’ fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGGAATGTGCGGGTCATGCTG	Rev 3’ fragment::	
TCTACTCGTCGGGCATATCG	Fw – Ext. screening	
CTTGAACCGTGTCAGACTGC	Rev – Ext. screening	
GCGCGCCTAGGCCTCTGCAGGTCGACTCTCACGCAGGGTTAAAGGGTTA	Fw 5’ fragment::	g3033.t1

Nucleotide sequence	Type of primer	GOI
TATATCGACAGTGGCTTCTCGCGCTTGATCAGGTCTTCAT	Rev 5' fragment::	
AACAATCTGCGCAGTGCTGAAGCCTCATCAACGCAGAGAT	Fw 3' fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGCTTGTCGCAACAACATACGG	Rev 3' fragment::	
ATTGATGCTCCACAGGGAAG	Fw – Ext. screening	
ACGACACCACGACTCGCTAT	Rev – Ext. screening	
GCGCGCCTAGGCCTCTGCAGGTCGACTCTGAGCGGTCTTGATAACAGCA	Fw 5' fragment::	g4389.t1
TATATCGACAGTGGCTTCTCTGTTGTCTTGACGGTGATGG	Rev 5' fragment::	
AACAATCTGCGCAGTGCTGACACATTGACCATCCGAAGC	Fw 3' fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGTCAATGGCGAGATAGACGTG	Rev 3' fragment::	
GGAAGTGCGGTGTAGCTGAT	Fw – Ext. screening	
CGAAGACGACGAAGAGAAGG	Rev – Ext. screening	
GCGCGCCTAGGCCTCTGCAGGTCGACTCTATGGCGTATCGGTATCCTCC	Fw 5' fragment::	g5541.t1
TATATCGACAGTGGCTTCTCGTCTCCCTCCACATTCCACT	Rev 5' fragment::	
AACAATCTGCGCAGTGCTGATGGTTCATCTTCGGCTTCCT	Fw 3' fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGAGATTCGCTCACTCAGGCTT	Rev 3' fragment::	
CGGCTCTCATTCTCCTCGTT	Fw – Ext. screening	
TGATGGCGGAACTGAGTGAC	Rev – Ext. screening	
GCGCGCCTAGGCCTCTGCAGGTCGACTCTAGGTGGGTACTTCTCGTGC	Fw 5' fragment::	g1105.t1
TATATCGACAGTGGCTTCTCCGACTTCATCTTCGCCTACT	Rev 5' fragment::	
AACAATCTGCGCAGTGCTGAGCTACCGACCTGAACAGTCA	Fw 3' fragment::	
TGATTACGAATTCTTAATTAAGATATCGAGGTCACCTCGGCAAAGACATT	Rev 3' fragment::	
AGCAATGGTTCTGAGCCTCT	Fw – Ext. screening	
CCTCGAACTACAGGTCAGGG	Rev – Ext. screening	
Check correct recombination in *S. cerevisiae* within marker and pGI3 R border'		
CTGATCCAAGCTCAAGCTC	JOHE43279	
TCCTCCTCACTCCCATCATC	JOHE46630	
Check correct recombination in *S. cerevisiae* within marker and pGI3 L border		
CGGTAAGCCGTGTGACCTAT	JOHE46631	
GTTGGCCGATTCATTAATGC	JOHE43280	
First couple of primers used for the "split marker"		
GTAACGCCAGGGTTTTCCCAGTCACGACG	JOHE43263	

Nucleotide sequence	Type of primer	GOI
CTTCTACACAGCCATCGGTCCAG	HPH-Rv	
Second couple of primers used for the "split marker"		
GGAAGTGCTTGACATTGGGG	HPH-Fw	
GCGGATAACAATTTCACACAGGAAACAGC	JOHE43264	
Check the integration of the deletion cassette at target site in LS28 at R border		
Fw – Ext. screening (see above in the table)		
TCCTCCTCACTCCCATCATC	JOHE46630	
Check the integration of the deletion cassette at target site in LS28 at L border		
CGGTAAGCCGTGTGACCTAT	JOHE46631	
Rev – Ext. screening (see above in the table)		

Table 21. Summary score of the sensitivity tests. The score was referred to the wild type growth, in all the conditions. (+1)= tenfold growth more than WT; (+2)= one hundred fold growth more than WT; (-1)= tenfold less growth than WT; (-2)= one hundred fold less growth than WT; (1/2)= intermediate scenario according to + or -.

Strains	**Rich media**	**Cell wall stress**			**Nitrosative stress**	**Oxidative stress**		**Protein synthesis inhibitor**	**Genotoxic stress**	**ER stress inducer**
Ref to Wild type	YPD	NaCl	Congo red	LiCl	$NaNO_2$	Menadione	H_2O_2	Cycloheximide	Hydroxyurea (HUA)	DTT
4389Δ::*HYG*-1#6	=	=	+1	+2	-1	=	-1/2	+2	-1	-1/2
4389Δ::*HYG*-2#2	=	=	+1	+1	-1	=	-1	+1	-1	-1/2
*put4*Δ::*HYG*-1#2	=	=	=	+1.5	=	=	=	=	=	=
*put4*Δ::*HYG*-2#3	=	=	=	+1	-1	-1/2	=	+3	-1	=
1861Δ::*HYG*-1#1	=	=	=	+1.5	=	=	=	=	=	=
1861Δ::*HYG*-2#3	=	=	+1	+2	=	=	=	+2	=	=
*lot6*Δ::*HYG*-1#1	=	=	+1	+2	=	=	=	+2	=	=
*lot6*Δ::*HYG*-2#1	=	-1	=	+1	=	-1/2	=	=	=	-2
2837Δ::*HYG*-1#2	=	=	+1	+2	=	=	=	+2	-1/2	-2
2837Δ::*HYG*-2#1	=	=	+1	+2	=	=	=	+2	-1/2	=

Table 22. Amino acids and nitrogen containing compounds tested in the growth kinetics.

Compound	AA ID	final concentration
Alanine	Ala	10 mM -1.5 mM
Allantoin		1.5 mM
Ammonium sulphate	-	10 mM -1.5 mM
Apple mimicking	Apple_juice	-
Arginine	Arg	10 mM -1.5 mM
Asparagine	Asn	10 mM -1.5 mM
Aspartic acid	Asp	10 mM -1.5 mM
Cysteine	Cys	10 mM -1.5 mM
GABA	GABA	10 mM -1.5 mM
Glycine	Gly	10 mM -1.5 mM
Glutamic acid	Glu	10 mM -1.5 mM
Glutamine	Gln	10 mM -1.5 mM
Hystidine	Hys	10 mM -1.5 mM
Isoleucine	Ile	10 mM -1.5 mM
Leucine	Leu	10 mM -1.5 mM
Lysine	Lys	10 mM -1.5 mM
Methionine	Met	10 mM -1.5 mM
Minimum salt medium	MM_salt	-
Nicotinic acid	NAD	10 mM -1.5 mM
Ornithine	Diapa	10 mM -1.5 mM
Phenylalanine	Phe	10 mM -1.5 mM

Proline	Pro	10 mM -1.5 mM
Yeast peptone dextrose	YPD	-
Serine	Ser	10 mM -1.5 mM
Sodium nitrate	nitrate	10 mM -1.5 mM
Tyrosine	Tyr	0.5 mM
Threonine	Thr	10 mM -1.5 mM
Tryptophan	Trp	10 mM -1.5 mM
Urea	Urea	10 mM -1.5 mM
Uridine	Uridine	10 mM -1.5 mM
Valine	Val	10 mM -1.5 mM

Table 23. Multiple Tukey comparisons of Biocontrol experiment treatments

Tukey's multiple comparisons test	**Mean Diff,**	**99,90% CI of diff,**	**Significant?**	**Adjusted P Value**
LS28 *vs*. *put4*Δ::*HYG*-2#3	-52,08	-97,24 to -6,922	Yes	0,0003
LS28 *vs*. *lot6*Δ::*HYG* -1#1	-43,75	-88,91 to 1,411	No	0,0013
LS28 *vs*. PEX7015	-66,67	-111,8 to -21,51	Yes	<0,0001
*put4*Δ::*HYG*-2#3 *vs*. *lot6*Δ::HYG-1#1	8,333	-36,83 to 53,49	No	0,7685
*put*4Δ::HYG-2#3 *vs*. PEX7015	-14,58	-59,74 to 30,58	No	0,3663
*lot6*Δ::HYG -1#1 *vs*. PEX7015	-22,92	-68,08 to 22,24	No	0,0836

Table 24. List of DEGs belong to kinase class involved in Pathogen-trigger immunity (PTI) expressed by *Malus domestica* when treated with pathogen *P. expansum.*

Locus	**logFC**	**Gene name**	**Arabidopsis annotation**
WAK and _WAKL_ receptors			
MD10G1248500	7.25	*WAK2*	Wall-associated receptor kinase 2
MD17G1131600	5.52	*WAKL2*	Wall-associated receptor kinase-like 2
MD10G1251200	4.89	*WAK2*	Wall-associated receptor kinase 2
MD09G1145300	3.80	*WAKL2*	Wall-associated receptor kinase-like 2
MD09G1145500	2.93	*WAKL2*	Wall-associated receptor kinase-like 2
MD04G1239400	2.27	*WAKL1*	Wall-associated receptor kinase-like 1
MD00G1038300	2.27	*WAKL14*	Wall-associated receptor kinase-like 14
MD10G1251400	4.96	*WAK2*	Wall-associated receptor kinase 2
MD17G1132000	4.77	*WAKL9*	Wall-associated receptor kinase-like 9
MD09G1145400	4.32	*WAK2*	Wall-associated receptor kinase 2
MD10G1250900	3.94	*WAK5*	Wall-associated receptor kinase 5
MD10G1250500	3.78	*WAK2*	Wall-associated receptor kinase 2
MD05G1269900	3.26	*WAK2*	Wall-associated receptor kinase 2
MD15G1088400	2.37	*WAK2*	Wall-associated receptor kinase 2
Chitin/elicitor binding receptor _CERK_/_BAK_			
MD09G1111800	3.24	*CERK1*	Chitin elicitor receptor kinase 1
MD01G1178000	3.06	*CERK*	Ceramide kinase
MD17G1102100	2.71	*CERK1*	Chitin elicitor receptor kinase 1
MD08G1221700	2.13	*BAK1*	BRI1-associated receptor kinase
ROS inducer (_RBOHD_) and calcium dependent kinase			
MD13G1134500	3.74	*RBOHD*	Respiratory burst oxidase homolog protein D
MD12G1044400	5.30	*CPK2*	Calcium-dependent protein kinase 2
MD14G1043300	4.73	*CPK2*	Calcium-dependent protein kinase 2
MD03G1200500	3.77	*CPK9*	Calcium-dependent protein kinase 9
MD11G1217500	3.14	*CPK33*	calcium-dependent protein kinase 33
MD01G1227200	2.02	*CRK4*	CDPK-related kinase 4
lectin S-domain receptor kinase			
MD11G1254600	6.46	*LECRK91*	L-type lectin-domain containing receptor kinase IX.1
MD02G1107200	5.64	*LECRK32*	Putative inactive L-type lectin-domain containing receptor kinase III.2
MD03G1232900	4.94	*LECRK91*	L-type lectin-domain containing receptor kinase IX.1
MD04G1133500	4.48	*LECRK41*	L-type lectin-domain containing receptor kinase IV.1
MD03G1233600	4.22	*LECRK91*	L-type lectin-domain containing receptor kinase IX.1

Locus	logFC	Gene name	Arabidopsis annotation
MD03G1233100	3.76	*LECRK91*	L-type lectin-domain containing receptor kinase IX.1
MD03G1233700	4.20	*LECRK91*	L-type lectin-domain containing receptor kinase IX.1
MD06G1166300	3.47	*LECRKS1*	L-type lectin-domain containing receptor kinase S.1
MD02G1047600	2.51	*CES101*	Lectin protein kinase family protein
MD10G1308200	3.46	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD10G1308300	3.25	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD02G1260900	3.05	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD10G1291100	3.02	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD17G1273200	2.93	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD10G1307800	2.31	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD11G1231000	2.19	*SD11*	G-type lectin S-receptor-like serine/threonine-protein kinase SD1-1
MD10G1291200	2.88	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD10G1307600	2.75	*RKS1*	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
LRR receptor like			
MD00G1007000	3.82	*RKF1*	Probable LRR receptor-like serine/threonine-protein kinase RFK1
MD00G1006600	3.19	*LRR-RLK*	Probable leucine-rich repeat receptor-like serine/threonine-protein kinase At3g14840
MD10G1273400	2.99	*RKF1*	Probable LRR receptor-like serine/threonine-protein kinase RFK1
MD03G1246600	2.67	*LRR-RLK*	Probable leucine-rich repeat receptor-like serine/threonine-protein kinase At3g14840
MD00G1006700	2.31	*RKF1*	Probable LRR receptor-like serine/threonine-protein kinase RFK1
MD11G1267900	2.23	*LRR-RLK*	Probable leucine-rich repeat receptor-like serine/threonine-protein kinase At3g14840
MD11G1267700	2.10	*LRR-RLK*	Probable leucine-rich repeat receptor-like serine/threonine-protein kinase At3g14840
MD11G1267800	2.03	*LRR-RLK*	Probable leucine-rich repeat receptor-like serine/threonine-protein kinase At3g14840
MD11G1293500	2.37	*RKF3*	Probable LRR receptor-like serine/threonine-protein kinase RKF3
MD03G1272600	2.22	*RKF3*	Probable LRR receptor-like serine/threonine-protein kinase RKF3
RALF–FER pathway			

Locus	logFC	Gene name	Arabidopsis annotation
MD12G1256100	2.63	*FER*	Receptor-like protein kinase FERONIA
MD16G1025500	4.62	*RALFL33*	Protein RALF-like 33
MD12G1256200	2.02	*FER*	Receptor-like protein kinase FERONIA
MD12G1256100	2.63	*FER*	Receptor-like protein kinase FERONIA
MD12G1256200	2.02	*FER*	Receptor-like protein kinase FERONIA
Uncharacterized genes annotated as kinase			
MD05G1144900	6.42	#N/A	Concanavalin A-like lectin protein kinase family protein
MD09G1098900	6.36	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130
MD02G1047300	5.47	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11330
MD17G1249500	5.02	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g61370
MD02G1107300	4.84	#N/A	Concanavalin A-like lectin protein kinase family protein
MD15G1076500	4.80	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD02G1047500	4.62	#N/A	lectin protein kinase family protein
MD02G1107100	4.52	#N/A	Concanavalin A-like lectin protein kinase family protein
MD11G1024700	4.34	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD15G1226500	4.16	#N/A	Concanavalin A-like lectin protein kinase family protein
MD12G1151200	4.11	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD05G1263100	4.01	#N/A	S-locus lectin protein kinase family protein
MD05G1039500	3.86	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD17G1108200	3.56	#N/A	S-locus lectin protein kinase family protein
MD03G1020000	3.36	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD00G1203200	3.34	#N/A	S-locus lectin protein kinase family protein
MD03G1019900	3.29	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD12G1151600	3.22	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD05G1332300	3.02	#N/A	S-locus lectin protein kinase family protein
MD17G1108100	2.64	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130
MD05G1332400	2.64	#N/A	S-locus lectin protein kinase family protein
MD10G1202800	2.28	#N/A	S-locus lectin protein kinase family protein
MD11G1232500	2.23	#N/A	S-locus lectin protein kinase family protein
MD11G1025500	4.96	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080

Locus	logFC	Gene name	Arabidopsis annotation
MD15G1391000	4.15	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD05G1039400	4.12	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD11G1262700	3.44	#N/A	EP1-like glycoprotein 2
MD17G1249800	3.08	#N/A	S-locus lectin protein kinase family protein
MD11G1262600	2.97	#N/A	EP1-like glycoprotein 3
MD09G1094800	2.84	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130
MD03G1020200	2.81	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD03G1020300	2.41	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD10G1181800	2.33	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD17G1249300	2.19	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g61390
MD10G1307400	2.17	#N/A	Serine/threonine-protein kinase
MD03G1019800	2.13	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD15G1186100	2.06	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11330
MD09G1094900	2.00	#N/A	S-locus lectin protein kinase family protein
MD05G1144900	6.42	#N/A	Concanavalin A-like lectin protein kinase family protein
MD09G1098900	6.36	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130
MD02G1047300	5.47	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11330
MD17G1249500	5.02	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g61370
MD02G1107300	4.84	#N/A	Concanavalin A-like lectin protein kinase family protein
MD15G1076500	4.80	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD02G1047500	4.62	#N/A	lectin protein kinase family protein
MD02G1107100	4.52	#N/A	Concanavalin A-like lectin protein kinase family protein
MD11G1024700	4.34	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD15G1226500	4.16	#N/A	Concanavalin A-like lectin protein kinase family protein
MD12G1151200	4.11	#N/A	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD05G1263100	4.01	#N/A	S-locus lectin protein kinase family protein

Table 25. List of resistance protein encoding genes expressed by *Malus domestica* when treated with pathogen *P. expansum* being potentially involved in effector trigger immunity pathway (ETI).

Locus	**logFC**	**Gene name**	**Arabidopsis annotation**
MD11G1044500	6.95	RPPL1	Putative disease resistance RPP13-like protein 1
MD05G1207700	3.80	RPM1	Disease resistance protein RPM1
MD12G1053900	3.76	DIR21	Dirigent protein 21
MD00G1073000	3.70	RPPL1	Putative disease resistance RPP13-like protein 1
MD02G1288600	3.14	RPPL1	Putative disease resistance RPP13-like protein 1
MD02G1118100	2.97	DIR19	Dirigent protein 19
MD02G1249300	2.84	LRK10L-2.1	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.1
MD13G1037900	2.66	LRK10L-2.8	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.8
MD13G1038000	2.37	LRK10L-2.1	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.1
MD00G1073100	2.96	RPPL1	Putative disease resistance RPP13-like protein 1
MD07G1260700	2.81	SNC1	disease resistance protein (TIR-NBS-LRR class), putative
MD00G1072900	2.62	RPPL1	Putative disease resistance RPP13-like protein 1
MD10G1136500	2.48	DSC1	Disease resistance-like protein DSC1
MD00G1203600	2.35	RPPL1	Putative disease resistance RPP13-like protein 1
MD03G1009400	2.32	RPPL1	Putative disease resistance RPP13-like protein 1
MD02G1118400	2.30	DIR11	Dirigent protein 11
MD02G1249400	2.19	LRK10L-2.4	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.4
MD06G1218500	2.14	LRK10L-2.1	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.1

Locus	logFC	Gene name	Arabidopsis annotation
MD02G1253800	2.11	LRK10L-2.4	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.4

Table 26. List of resistance protein encoding genes expressed by *Malus domestica* when treated with LS28 being potentially involved in PTI and ETI.

Locus	logFC	Gene name	Arabidopsis annotation
MD07G1069300	3.00	AtRLP19	Receptor-like protein 19
MD07G1069600	2.78	AtRLP24	Receptor like protein 24
MD02G1107200	-4.07	LECRK32	Putative inactive L-type lectin-domain containing receptor kinase III.2
MD12G1256100	-3.77	FER	Receptor-like protein kinase FERONIA
MD03G1233700	-3.42	LECRK91	L-type lectin-domain containing receptor kinase IX.1
MD05G1328000	-2.69	RKS1	G-type lectin S-receptor-like serine/threonine-protein kinase RKS1
MD10G1306600	-2.57	B120	B120
MD10G1181800	-2.42	#N/D	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD11G1262400	-2.31	#N/D	EP1-like glycoprotein 4
MD15G1391000	-2.28	#N/D	G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080
MD05G1039400	-2.20	#N/D	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300
MD12G1256100	-3.77	FER	Receptor-like protein kinase FERONIA
MD02G1249400	-2.31	LRK10L-2.4	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.4
MD17G1132000	-2.17	WAKL9	Wall-associated receptor kinase-like 9
MD01G1057400	-2.33	#N/D	Disease resistance family protein / LRR family protein
MD02G1249400	-2.31	LRK10L-2.4	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.4
MD04G1161100	-2.24	#N/D	Disease resistance like protein
MD05G1224700	-2.12	#N/D	Disease resistance family protein / LRR family protein
MD02G1264800	-2.02	#N/D	Disease resistance protein (TIR-NBS-LRR class) family

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