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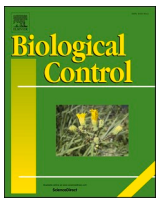

Metschnikowia pulcherrima as an efficient biocontrol agent of *Botrytis cinerea* infection in apples: Unraveling protection mechanisms through yeast proteomics

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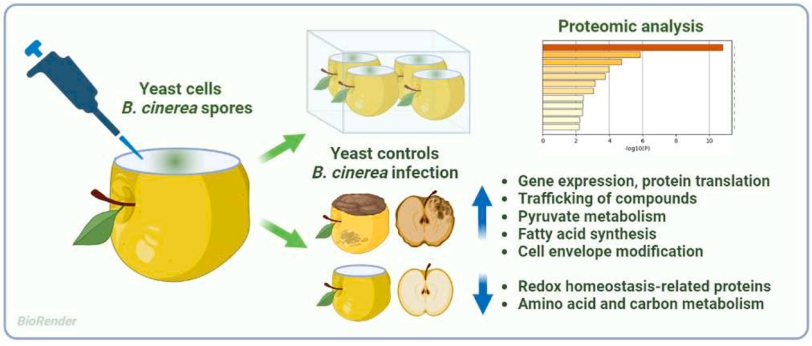
HIGHLIGHTS

- *Metschnikowia pulcherrima* Mp-30 prevents *Botrytis cinerea* infection in apples.
- Multiple yeast mechanisms are involved in the gray mold biocontrol ability.
- There is a reprogramming of yeast metabolism involved in *B. cinerea* inactivation.
- Membrane trafficking of yeast compounds is enhanced in the fungal presence.
- Yeast envelope composition has a major role in the antagonistic interaction.

GRAPHICAL ABSTRACT



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ABSTRACT

The results obtained in this study show that the Mp-30 strain of *Metschnikowia pulcherrima* is able to completely prevent *Botrytis cinerea* infection in apples, which is a major postharvest disease of fruits throughout the world. We have observed that although Mp-30 is able to rapidly colonize wounds, sequestrate iron and secrete antifungal compounds, other unknown mechanisms that occur in the early phase of the yeast-fungal interaction must be implicated in the biocontrol response. The main objective of this study was to identify the pathways involved in the mechanism of action of Mp-30 against *B. cinerea* in apples. Therefore, differentially accumulated yeast proteins in the presence/absence of *B. cinerea* on wounded apples were studied to elucidate Mp-30 biocontrol mechanisms and regulation at the protein level. A comparative proteomic analysis showed that 114 yeast proteins were increased and 61 were decreased. The Mp-30 antagonistic response mainly showed the increase of (1) gene expression and protein translation related proteins, (2) trafficking and vesicle-mediated transport related proteins, (3) pyruvate metabolism and mitochondrial proteins related to energy and amino acid production, (4)

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fatty acid synthesis, and (5) cell envelope related proteins. On the other hand, redox homeostasis, and amino acid and carbon metabolism were downregulated. Since there is no yeast growth enhancement associated with the presence of *B. cinerea*, such regulation mechanisms may be related to the reprogramming of metabolism, synthesis of new compounds and reorganization of yeast cell structure. Indeed, the results show that several pathways cooperate in restructuring the plasma membrane and cell wall composition, highlighting their major role in the antagonistic interactions for apple protection against gray mold proliferation. These results are of great interest since they provide a clear insight into the yeast mechanisms involved in *B. cinerea* inactivation during the first hours of contact in the wounded fruit. They shed light on the unknown yeast molecular biocontrol mechanisms.

1. Introduction

Apples are the fourth most widely consumed fruit in the world (FAO Statistical database FAO Stat https://www.fao.org/faostat/en/#home). In recent years, the fresh apple market has witnessed considerable growth due to increasing demand for fresh produce in emerging and developing nations across the world. In fact, the global production of fresh apples is projected to reach an annual average rate of growth in revenue (CAGR) of 4.0% during the 2022–26 period (The Business Research Company, 2022). To provide fruit throughout the year, fresh apples are stored after harvest, typically for up to 6 months at 1 °C, but can be kept for one year in controlled-atmosphere storage where temperature, oxygen, and carbon dioxide are maintained at specific levels (Robinson et al., 2014). Extended storage times increase the susceptibility of apples to various fungal diseases, resulting in escalating economic losses (Xiao and Boal, 2009). More than 90 fungal species may be agents of postharvest infections on apple fruit, and some of them cause devastating losses that can reach up to 25% (Jijakli and Lepoivre, 2004; Sansone et al., 2018).

Gray mold caused by the necrotrophic pathogen *Botrytis cinerea* affects more than 500 plant hosts and it is a major postharvest disease of apples and other fruits throughout the world (Testempasis et al., 2021), that results in economic losses ranging from $10 to $100 billion worldwide (Hua et al., 2018). This fungus is difficult to control because of its broad host range, high survival capacity in the environment and various attack modes (Hua et al., 2018). Control of gray mold in apples is generally achieved via cultural practices and fungicide application (Hua et al., 2018; Xiao and Boal, 2009). However, the fungus may remain a threat due to its ability to reproduce rapidly and survive adverse conditions for extended periods of time, and its potential to mutate at high frequencies to overcome fungicide treatments. Indeed, it has been observed that *B. cinerea* isolates from apples carry mutations in genes related to chemical resistance, making chemical treatments more complicated (Elad et al., 2015; Jurick et al., 2017; Malandrakis et al., 2022).

Among the new strategies for the management of *B. cinerea* is the use of biocontrol agents (BCAs) (Abbey et al., 2019; Roca-Couso et al., 2021). In recent years, there has been an increase in the use of BCAs as an alternative to synthetic fungicides to contain fruit postharvest decay (Droby et al., 2016). In the case of apple fruit, several studies have proposed BCAs to reduce apple decay during storage (Błaszczyk et al., 2022; Kheireddine et al., 2018; Leng et al., 2022; Li et al., 2011; Piano et al., 1997; Sansone et al., 2018, 2005; Yu et al., 2008). In particular, yeasts have attracted considerable interest because of their ability to survive on fruit surfaces for a long period and rapidly colonize wounded tissues (Perez et al., 2019; Rodriguez Assaf et al., 2020). Yeasts also produce antifungal volatiles (e.g. 2-nonanone or phenylethyl alcohol), metabolites (e.g. aureobasidins or liamocins), biofilms, and killer toxins, inhibit spore germination and mycelial growth of pathogens and may induce pathogen resistance in fruits (Freimoser et al., 2019; Hernandez-Montiel et al., 2021; Leng et al., 2022; Muccilli and Restuccia, 2015; Ruiz-Moyano et al., 2020; Spadaro and Droby, 2016; Zhang et al., 2020). Additionally, biocontrol yeasts are acceptable to the general public because they are perceived as being more environmentally safe than other BCAs (Droby et al., 2009). Indeed, several species have been reported to exert biocontrol against gray mold infection in apples (reviewed in Leng et al., 2022): *Candida oleophila, Meyerozyma guilliermondii, Metschnikowia pulcherrima, Pichia caribbica, Pichia angusta* and *Rhodotorula glutinis*. Although commercial postharvest biocontrol products have been developed for apples, their success in the marketplace has been limited (Leng et al., 2022; Palmieri et al., 2022). In general, the application of yeasts has shown inconsistent efficacy compared to synthetic fungicides, mostly due to our poor knowledge of the molecular mechanisms of yeast-induced responses (Ferreira-Saab et al., 2018; Massart et al., 2015). The mechanisms of action by which yeasts exert their antifungal control still remain largely unknown due to the variety of interactions developed (Droby et al., 2009). In fact, it has been recognized that the antagonistic activity involves an interaction between the BCA, pathogen, host, resident microflora and the environment, which suggests that the inhibition of infection by a BCA is synergistic and a complex process (Droby et al., 2022). For marketing purposes, understanding the modes of action of BCAs is one of the relevant parameters to improve performance and reliability of formulations (Droby et al., 2009; Spadaro and Droby, 2016). Therefore, a comprehensive understanding of the mechanisms of action of BCAs is crucial to realize their potential use, safety and commercial development (Köhl et al., 2019; Parafati et al., 2016; Roca-Couso et al., 2021).

Among several yeast strains selected in our previous studies for their excellent biocontrol abilities against *B. cinerea* on postharvest tomato and grapes, *M. pulcherrima* Mp-30 shows the best antagonistic performance (Fernandez-San Millan et al., 2021). Indeed, its antifungal capacity was also demonstrated in tomato against another important soil pathogen, *Fusarium oxysporum*. The biocontrol mechanisms of the Mp-30 strain have been partially characterized, and as expected, multiple mechanisms seem to be involved: synthesis of cell wall-degrading enzymes, nutrient solubilization, development of biofilms, and secretion of iron chelating agents and antifungal metabolites (Fernandez-San Millan et al., 2022a, 2022b, 2021, 2020). The characterization of its exometabolome showed that potent antifungal metabolites are differentially secreted by the Mp-30 strain in the presence of *B. cinerea*. Indeed, three of these metabolites have been selected for their agronomic interest because they were able to control gray mold infection in tomato fruits (Fernandez-San Millan et al., 2022a). However, to gain a complete perspective of the metabolic pathways that are involved in its biocontrol abilities, a proteomic study is necessary. Proteomics have been used before to shed light on mechanisms responsible for biological control of several postharvest diseases from the pathogen or the fruit point of view (Belay and James Caleb, 2022; Chan et al., 2007; Liu et al., 2018; Sarethy and Saharan, 2021). However, very few studies have investigated the proteomic changes triggered in biocontrol yeasts during their antagonistic interactions (Gu et al., 2020; Kwasiborski et al., 2014) and this field remains unexplored.

Therefore, the main objective of this investigation was to study the potential of the Mp-30 yeast strain as a gray mold biocontrol agent in apples and to understand the molecular mechanisms and main cellular pathways involved in its antifungal abilities. For this purpose, a qualitative and quantitative proteomic approach was followed among other characterization assays. Differentially accumulated proteins in yeast

were studied in response to the presence/absence of *B. cinerea* in an *in vivo* three-way apple interaction model. The results have allowed us to describe the changes in the proteome of the Mp-30 strain that lead to *B. cinerea* inactivation during the first hours of contact in the wounded fruit.

2. Materials and methods

2.1. Yeast and fungal strains

The *Metschnikowia pulcherrima* Mp-30 yeast strain was originally isolated from grapes (*Vitis vinifera* cv. Grenache) (Fernandez-San Millan et al., 2020, 2021). Cryovials (VWR, Spain) were used for long-term storage of the yeast cells at −80 °C. Yeast cells were plated onto Yeast Mannitol Agar solid medium (YMA; Condalab, Spain) for solid culture at 28 °C for 2 d. When liquid culture was required, cells were grown in YPD broth (Yeast Peptone Dextrose; Condalab, Spain), and incubated at 28 °C on a rotary shaker at 150 rpm for 2 d. To determine the cell concentration a TC20 cell counter (Bio-Rad, USA) was used.

The 20754 strain of the fungus *Botrytis cinerea* was obtained from CECT (Paterna, Spain) and used for apple infection and confrontation in the proteomic analysis. The fungus was grown on Potato Dextrose Agar (PDA; Condalab, Spain) medium plates at 23 °C. The fungal spores were isolated as previously described (Fernandez-San Millan et al., 2021). Other *B. cinerea* fungal strains were isolated from grapes (Bc1-4) and molecularly identified at the University of Valencia (Spain) by 5.8S rRNA intergenic sequence and RPB2 amplification by PCR and sequencing (its1: 5′-TCCGTAGGTGAACCTGCGG-3′ and its4: 5′-TCCTCCGCTTATTGATATGC-3′; RPB2-5F2: 5′-GGGGWGAYCAGAAGAAGGC-3′ and fRPB2-7cR: 5′-CCCATRGCTTGTYYRCCCAT-3′). The presence or absence of Boty and Flipper transposons (Giraud et al., 1999) associated with *B. cinerea* virulence was analyzed by PCR to characterize the four *B. cinerea* different strains (Boty B4: 5′- CAGCTGCAGTA TACTGGGGGA-3′ and Boty R4: 5′- GGTGCTCAAAGTGTTACGGGAG-3′; F300: 5′- GCACAAAACCTA CAGAAGA-3′ and F1560: 5′-ATTCGTTTCTTGGACTGTA3′). All strains had the transposon Boty and only Bc1 and Bc4 the transposon Flipper. These four *B. cinerea* strains were selected among others for being highly pathogenic for apples under postharvest conditions.

2.2. In vivo biocontrol assay of M. pulcherrima Mp-30 against B. cinerea in apples

M. pulcherrima Mp-30 was assayed for postharvest antifungal properties against *B. cinerea* 20754 strain infection on apples (*Malus* × *domestica* cv. Golden delicious). This cultivar is one of the most cultivated in the world and in previous assays was highly susceptible to several *B. cinerea* strains. Fruits were selected based on absence of injury and uniformity of size. Briefly, apples were rinsed with tap water before the surface disinfection with 70% (v/v) ethanol for 1 min and 1% (v/v) sodium hypochlorite for 15 min. Apples were rinsed with sterile water and dried in a laminar flow cabinet. Four wounds (1 mm wide × 2 mm deep) were made around the fruit stalk with a scalpel tip. Then, each wound was inoculated with the live yeast (5 μL of the yeast culture at different dilutions to adjust the number to 10^6, 10^4 or 10^2 cells per wound) or YPD medium (5 μL) in negative control apples. Dead yeast cells (10^6 cells per wound) were also inoculated and were obtained by previous heating at 80 °C during 30 min. Yeast cell-free filtrates were obtained by filtration with a 0.22 μm pore diameter membrane filter. Wounds were dried for 30 min and inoculated with a conidial suspension (5 μL of 10^4 conidia mL^{-1}) of *B. cinerea*. Infected apples were placed in plastic containers. To maintain high relative humidity, water-soaked paper was included at the bottom of each container. Six apples per treatment were used. As positive controls of the infection, a set of apples were inoculated with the pathogenic fungus alone. Apples were kept in a dark chamber at constant temperature (25 °C). The disease incidence (DI) was measured after 7 days as the percentage of infected wounds with the following formula (Parafati et al., 2015): DI % = (n° infected wounds/n° total wounds) × 100. The disease severity (DS) was calculated as the average value of the diameters of the lesions for each treatment. The experiment was repeated three times.

Mp-30 biocontrol ability against four additional pathogenic strains of *B. cinerea* (Bc1-4) was assayed in the same way as described before by using the minimum amount of yeast live cells (10^4 cells per wound) that resulted in protection against the *B. cinerea* 20754 strain.

2.3. Colonization of apple fruit wounds by yeast cells

Apple fruits were wounded as described before. Each wound was treated with 10^2 yeast cells to observe the yeast growth ratio inside the wound until reaching the growth limit of 10^7 cells per wound (as we previously observed for the Mp-30 strain). A set of fruits was subsequently infected with *B. cinerea* 20754 spores whereas another set remained uninfected. All fruits were incubated at 25 °C as described previously. Samples were taken 1 h after treatment and at 1, 2, 3 and 5 d post-inoculation. Equal amounts of wounded apple tissues (1 g including the wound and the surrounding apple healthy tissue) were extracted with a sterile cork borer and macerated in 1 mL of sterile 0.85% NaCl solution with a pestle. The resulting solution was serially diluted (up to10^5, depending on the time of sampling) and plated on YMA. After incubation for 48 h at 28 °C, the yeast colonies were counted to calculate population densities as CFU/wound (Huang et al., 2021; Liu et al., 2019). Three fruits were used per treatment and the whole experiment was repeated twice.

2.4. Effect of M. pulcherrima Mp-30 on B. cinerea spore germination

Suspensions of yeast (100 μL of 10^8 cells mL^{-1}) and pathogen (100 μL of 10^6 conidia mL^{-1}) were added to sterile glass tubes containing 5 mL of YPD. For this experiment, the *B. cinerea* 20754 conidia concentration was increased in relation to the one used in the apple infection protocol (10^4 conidia mL^{-1}) to facilitate observation and counting under the microscope. A control with only fungal conidia was also cultured. After a 24 h incubation on a rotary shaker (100 rpm) at 28 °C, a total of 100 conidia were observed with a light microscope (Zeiss Axio) in four different fields. Germination of *B. cinerea* conidia was considered when the germ tube length was equal to or longer than the conidium length (Lutz et al., 2013). The germination inhibition percentage was calculated. Three replicates were observed for each treatment and the experiment was repeated twice.

2.5. Antifungal capacity of the yeast volatiles and extracellular fraction

To analyze the biocontrol effect of yeast volatile compounds on infected apples, a protocol previously described was followed (Fernandez-San Millan et al., 2021). Apples were surface sterilized, wounded and inoculated with *B. cinerea* 20754 as described before. However, instead of inoculating the wounds with yeast cells, a YPD plate (without the lid) seeded with the yeast was placed on each plastic tray. The control was an unseeded YPD plate.

The antifungal capacity of yeast extracellular substances on infected apples was measured by growing the yeast in YPD on a rotary shaker (150 rpm) at 28 °C for 2 days. Then the culture was centrifuged at 2,000 *g* for 5 min and the supernatants were filtered (0.22 μm of pore size). Supernatants were applied to wounded apples as described before for yeast living cells. Both experiments were carried out in triplicate with six apples per replicate.

2.6. Induction of apple resistance against B. cinerea by M. pulcherrima Mp-30

To assess the effect of the elicitation of defense resistance in apples, two protocols were assayed. The first one was reported by Zhang et al. (2011). Briefly, intact fruits were dipped into a yeast suspension (10^8 cells mL^{-1}) for 5 min, air dried and kept at 25 °C for 72 h. Then the fruits were wiped for 2 min with 75% ethanol to eliminate the yeast cells from the surface and rinsed with sterile water. Apples were wounded and inoculated with *B. cinerea* 20754 spores as described before. Apples treated with YPD medium without yeast cells served as controls. After seven days at 25 °C, the DS was determined.

The second protocol was based on experiments described by Droby et al. (2002). Fruits were wounded as described before, and a 20 μL aliquot of 10^8 cells mL^{-1} of Mp-30 was pipetted into each wound. For controls, wounds were treated with the same volume of YPD. After 72 h incubation at 25 °C, new wounds (made at 1 cm from the yeast-treated or the control wounds) were inoculated with *B. cinerea* as described before. Both experiments were carried out in triplicate with four apples per replicate.

2.7. In vitro and in vivo competition for iron between M. pulcherrima Mp-30 and B. cinerea

The effect of yeast cells on *B. cinerea* 20754 spore germination was measured as previously described Cabañas et al. (2020) with slight modifications. Aliquots of a conidial suspension (100 μL of 10^5 conidia mL^{-1}) were spread out on standard PDA plates or supplemented with 5, 10, 20 or 40 μg mL^{-1} $FeCl_3$. After drying, a drop (5 μL of 10^5 CFU mL^{-1}) of 2-day-old yeast cultures were deposited on the same plate. The drop cultures were replicated three times and the plates were incubated at 28 °C for 4 d. The reduction in spore germination was observed as a halo zone around the yeast colony. The experiment was performed three times.

To test the effect of iron on antagonist efficacy *in vivo*, apples were wounded as described before and the protocol described by Zhang et al. (2011) was followed and served as guide for the $FeCl_3$ concentrations used. Briefly, aliquots of 10 μL of $FeCl_3$ (0.05 mM or 0.5 mM) were pipetted into each wound site. After 3 h incubation at 25 °C, yeast cells (5 μL of 10^4 cells mL^{-1}) and *B. cinerea* (5 μL of 10^4 conidia mL^{-1}) were added to each wound sequentially. Wounds inoculated only with the pathogen served as positive control. Fruits were kept for seven days at 25 °C and the DS was measured. Ten apples were used for each treatment and the experiment was repeated twice.

2.8. Three-way interaction assay and proteomic analysis

2.8.1. Three-way interaction assay

To study the yeast proteome, a triple pathogen/antagonist/apple interaction *in situ* assay was performed based on studies previously described (Kwasiborski et al., 2012, 2014). The experiment was carried out on *Malus* × *domestica* cv. Golden Delicious apples. Three successive subcultures of Mp-30 yeast cells on YMA for 24 h at 28 °C were made before scraping the colonies from plates and suspending them in isotonic water (0.85% NaCl). Apples were surface sterilized as described before and dried under the air flow cabinet. A round piece (diameter of 50 mm) of tissue was removed from the longitudinal section of the apples and covered with a Supor 450 membrane (0.45 μm, 47 mm, PALL, New York, USA). Membranes were treated with a mixture of yeast cells (400 μL of 10^7 CFU mL^{-1}) and *B. cinerea* 20754 conidia (400 μL of 10^6 CFU mL^{-1}) or with yeast cells (400 μL of 10^7 CFU mL^{-1}) and 400 μL of water. The yeast and fungal concentrations used were adjusted to follow a population dynamics similar to the protocol described by Kwasiborski et al. (2014). Inoculations of apples with yeast cells alone were used as controls to discriminate the differentially accumulated proteins in the Mp-30 - *B. cinerea* interaction. Inoculations of apples with fungal spores alone were used as infection controls. To ensure a high relative humidity, the apples were placed in plastic trays containing paper towels moistened with water. Apples were stored at 25 °C for 14 h (yeast growth in the exponential phase). Three apples were used per treatment and the experiment was repeated twice. Then, the membranes were placed carefully in 15 mL tubes with 5 mL of isotonic water and vortexed for 30 s. After centrifugation at 2,000 *g* for 10 min at 4 °C, yeast cells in the pellets were washed (1 mL of double distilled water) and frozen at −80 °C until use. The mycelium development after a ten-day incubation of apples inoculated with yeasts in the presence of *B. cinerea* or with *B. cinerea* alone (infection control) was assessed to verify the inhibitory

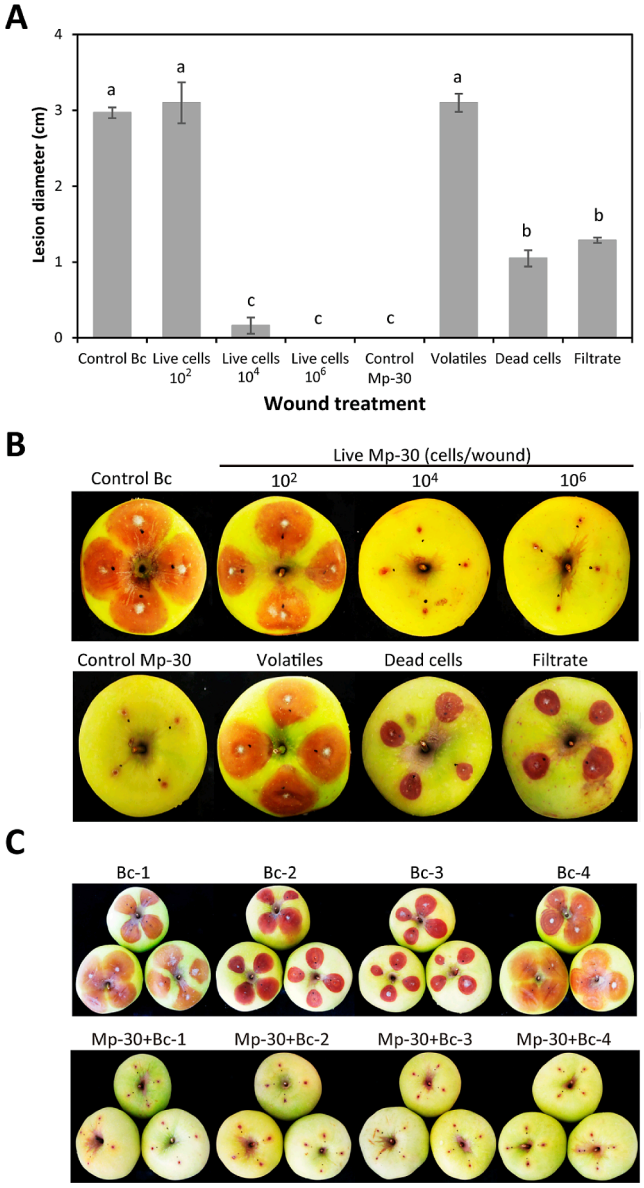

Fig. 1. *In vivo* protection assay of Mp-30 against *B. cinerea* infection in apples. (A) Disease severity determined according to lesion diameters in wounded apples inoculated with *B. cinerea* spores (strain 20754) and protected by live yeast cells (10^2-10^4-10^6 cells/wound), dead cells, cell-free culture filtrates or yeast volatile compounds. Data are presented as the means ± SE (n = 24). (B) Rot development in wounded apples under different treatments. Apples inoculated only with fungal spores (Control Bc) or yeast cells (Control Mp-30) are shown as controls. (C) Mp-30 biocontrol ability against four additional pathogenic strains of *B. cinerea* (Bc1-Bc4). The upper panel shows apples without Mp-30 and the lower panel apples protected with the yeast strain. Data were recorded seven days after fungal inoculation.

effect of yeast cells on *B. cinerea* infection (Fig. S1A).

To ensure yeast growth in the exponential phase (Kwasiborski et al., 2014) at the time when samples were taken (14 h after inoculation), growth curves were previously established in the presence or absence of *B. cinerea*. Yeast cells were recovered at different moments (0, 3, 6, 9, 12 and 15 h) after apple treatment and dilutions were plated on YMA medium for counting CFUs. Three apples were used for each treatment and the experiment was repeated twice (Fig. S1B).

2.8.2. Protein extraction and proteomic analysis

Two independent proteomic experiments were carried out with three experimental replicates per sample. Protein extraction, data acquisition and data analysis were performed as previously described (Marqués et al., 2022). An UltiMate 3000 UHLPC System (Thermo Scientific, San Jose, CA) fitted with an Aurora packed emitter column (IonOpticks, 25 cm × 75 μm ID, 1.6 μm C18) was used for peptide mixtures separation by reverse phase chromatography. Raw files were processed with MaxQuant (Cox and Mann, 2008) v 2.0.1 and the iAndromeda search engine (Cox et al., 2011). Data were searched against the UniProt proteome as described (Marqués et al., 2022). Mp-30 proteins were identified from apple samples inoculated with this strain alone. Proteins from both organisms (Mp-30 and *B. cinerea*) were identified in the protein extracts of apple samples inoculated with yeast and fungal cells. In order to study the main changes in differentially accumulated yeast proteins, identified proteins from *B. cinerea* (Table S1) were discarded in the following analysis. Perseus software (version 1.6.14.0) was used for the statistical analysis (Tyanova et al., 2016). An unpaired Student *t*-test ($p < 0.05$) was used for comparisons between two groups of samples. A 1% peptide false discovery rate threshold was stablished and an absolute fold change of < 0.77 (downregulation) or > 1.3 (upregulation) was considered to be significantly differentially accumulated.

Manual functional annotation of the differential dataset was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO) and DAVID Bioinformatics Resources 6.7. Using *S. cerevisiae* orthologs as input, pathway mapping and protein interactomes were analyzed using Metascape (Zhou et al., 2019) and STRING (Szklarczyk et al., 2021).

2.9. Data analysis

One-way ANOVA with Tukeýs pairwise comparison ant t-tests ($p < 0.05$) were used for estimating differences among treatments. Experiments were analyzed with SPSS.24 software (SPSS, Chicago).

3. Results

3.1. In vivo protection assay of Mp-30 yeast strain against B. cinerea infection in apples

The *M. pulcherrima* Mp-30 strain was assayed for biocontrol effects against *B. cinerea* in apple fruits using different concentrations of yeast cells. Non-viable yeast cells, cell-free culture filtrates and yeast volatile compounds were also assayed for protection. Fig. 1 (A-B) shows the DS after 7 days of the fungal infection. In the control apples, no lessions were produced by Mp-30 cells alone or brown lesions developed rapidly around the inoculation site in the presence of *B. cinerea* alone. In contrast, it was observed that the yeast cells were able to absolutely prevent the fungal infection when 10^4 or 10^6 cells were added per wound. Lower amounts of yeast cells or the volatile compounds produced by this yeast strain provided no protection against the infection. However, a certain degree of protection was observed with hot-inactivated cells or cell-free culture filtrates, with lesions significantly smaller than in control apples without any treatment. The DI was 100% in all treatments except for 10^4 or 10^6 cells/wound, where it was null. Protection of apples by Mp-30 remained unaltered after storage for 1 month at 25 °C (data not shown).

On the basis of the results obtained in the antagonistic assays, four additional highly pathogenic *B. cinerea* strains were used to analyze the antifungal spectrum of the yeast strain. The results demonstrate that Mp-30 provided complete protection against the four *B. cinerea* strains at 10^4 cells/wound, thus indicating broad antifungal activity against gray mold (Fig. 1C).

3.2. Apple wound colonization by Mp-30 yeast strain

Because there is a relationship between the protective effect and the amount of yeast cells needed for disease control on apples, a wound colonization assay was performed to determine whether the treatment of 10^2 yeast cells/wound was ineffective due to a low colonization rate. Therefore, the population dynamics of the Mp-30 yeast strain on artificially wounded apples was recorded as shown in Fig. 2. Starting with an inoculum of 10^2 cells/wound, the yeast cell concentration rapidly increased in an exponential phase reaching about 10^4 and 10^6 CFU/wound after the first 12 and 24 h, respectively. Subsequently, a stationary phase during the four following days was observed with a progressive reduction in the slope of the curve. Therefore, apple wounds were colonized rapidly in 48 h by the Mp-30 yeast strain. Five days after inoculation, the yeast cell concentration was on average 10^7 CFU/wound. Yeast cell measurements were slightly higher in the presence of *B. cinerea* spores, but no statistically significant differences were observed.

3.3. Effect of Mp-30 yeast strain on B. cinerea spore germination and iron availability

The presence of Mp-30 cells resulted in complete inhibition of spore germination and mycelial growth of *B. cinerea* in liquid coculture conditions, as shown in Fig. 3A-B. Since *M. pulcherrima* is able to sequester iron from the surrounding medium (Sipiczki, 2020), we performed an experiment to establish whether Mp-30 antagonism towards *B. cinerea* during germination might be related to iron depletion around spores. Mp-30 cells were inoculated in plates supplemented with various amounts of $FeCl_3$ and seeded with *B. cinerea* spores. A halo zone of 3–4 mm was observed around the yeast colonies in control plates with no addition of $FeCl_3$ (Fig. 3C-D). Outside the inhibition zones, the spores germinated uniformly and formed a dense mycelium (Fig. 3D). It was

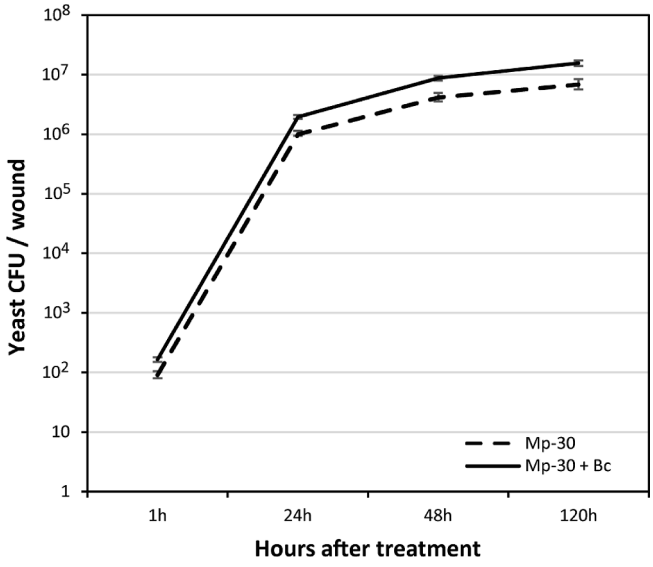

Fig. 2. Population dynamics of Mp-30 in wounds (100 cells/wound) of apple fruits in the presence or absence of *B. cinerea* spores (10^4 spores/wound). The colonies were counted and population densities expressed as CFU/wound. Data are presented as the means ± SE (n = 3).

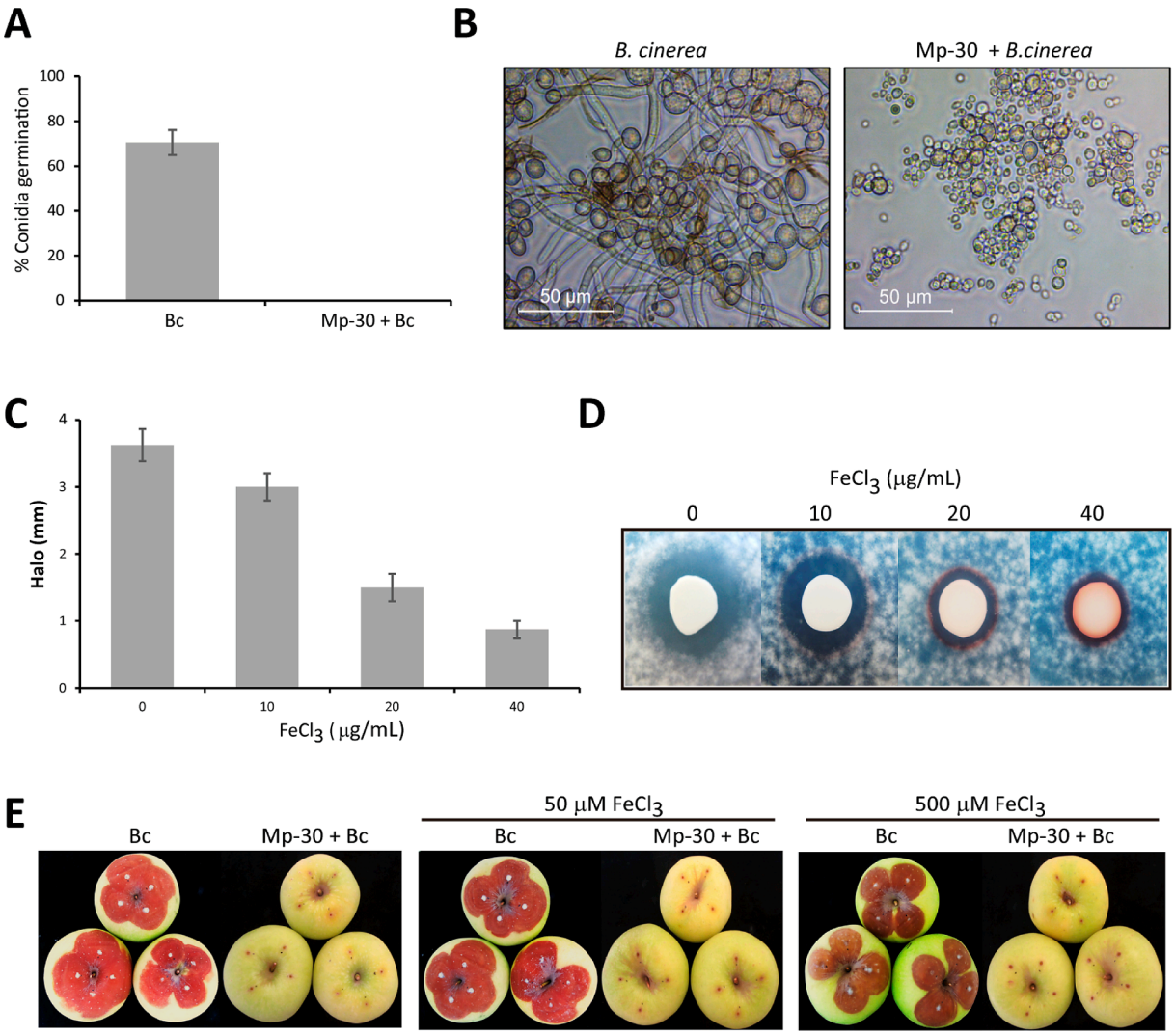

Fig. 3. Germination of *B. cinerea* spores under different conditions. (A) Percentage of spores germinated in the absence (control) or presence of Mp-30 yeast cells after 24 h coculture at 25 °C. Data are presented as the means ± SE (n = 4). (B) Germination of spores observed under the microscope. Bar = 50 µm. (C) Effect of the presence of iron on the halo zone produced by Mp-30 on *in vitro* spore germination. Data are presented as the means ± SE (n = 6). (D) Spore germination inhibition zones (halo) produced by the yeast cells at different concentrations of $FeCl_3$ in the culture media. (E) Effect of $FeCl_3$ addition (50–500 µM) to the same wound inoculated with *B. cinerea* spores and with Mp-30 yeast cells.

observed that the greater the amount of iron added, the narrower the halos and inhibition zones were. At an $FeCl_3$ concentration of 40 $\mu g mL^{-1}$, the halo almost disappeared, and the growth of *Botrytis* was not inhibited around the yeast colony. The reduction in the sizes of the inhibition zones following iron supplementation suggests that the Mp-30 strain was able to inhibit *B. cinerea* growth by depleting the iron in the medium. The yeast colonies also increased pigmentation following supplementation with $FeCl_3$ in the plate due to pulcherrimin production (Mažeika et al., 2021; Sipiczki, 2006) and the halo at 10 µg mL^{-1} was already slightly red. At an $FeCl_3$ concentration of 40 µg mL^{-1}, the yeast colonies had changed from pale to dark red, suggesting that the pigment was also retained in the cells. However, supplementation of apple wounds with $FeCl_3$ in the *in vivo* assay of *B. cinerea* infection showed that $FeCl_3$ (50–500 µM) added to the wounds in the presence of the yeast cells had no significant effect on disease development due to *B. cinerea* (Fig. 3E). Therefore, competition for iron was not important for Mp-30 gray mold biocontrol under these *in vivo* infection conditions.

3.4. Induction of apple resistance against B. cinerea by Mp-30 yeast strain

Two different methods for resistance induction with the yeast cells were assayed: soaking apples in a yeast cell suspension or pre-inoculation of the yeast cells into a wound 1 cm away from the *B. cinerea* infection point. The diameter of the lesions produced by *B. cinerea* were not significantly lower from those of the control treatment (Fig. S2). This result indicates that in this antagonistic interaction, Mp-30 did not cause an induction of disease resistance to *B. cinerea* in apples.

3.5. Three-way interaction assay and proteomic analysis

To study the changes produced in the Mp-30 proteome that could be involved in its biocontrol abilities, a triple antagonist/pathogen/apple interaction *in situ* assay was developed based on studies previously described (Kwasiborski et al., 2014). Inoculations of apples with a mixture of yeast cells and *B. cinerea* spores were made. Another set of apples with yeast cells alone were used as controls to discriminate the

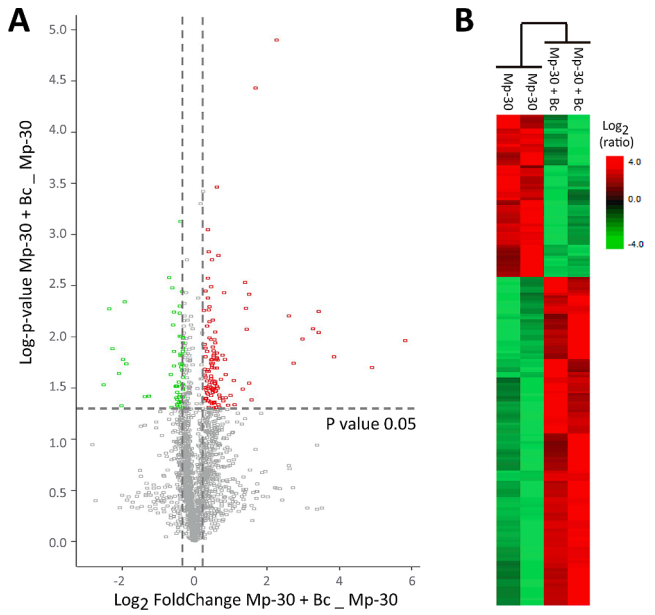

Fig. 4. Differentially accumulated proteins in groups of Mp-30 and Mp-30 cocultured with *B. cinerea* spores (Mp-30 + Bc). (A) Volcano plot representing the fold change of identified proteins with associated P values from the pairwise quantitative comparison. In green, significantly decreased proteins and, in red, increased proteins ($P < 0.05$). The unchanged proteome is represented by grey dots. (B) Heatmap representation showing both clustering and the degree of change for the differentially accumulated proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentially accumulated proteins in Mp-30 cells. A total of 2,705 proteins were identified by mass spectrometry. As this study was focused on yeast proteins, identified proteins of *B. cinerea* (Table S1) were discarded from further analysis. From the 2,052 quantified proteins across all samples, 175 yeast proteins were differentially accumulated. Specifically, the hierarchical cluster analysis (Fig. 4) showed that 114 and 61 yeast proteins were increased and decreased, respectively (Table 1).

To extract biological insights, functional interactomes and pathway analysis were performed. Due to the inherent limitations found with protein codes associated with *Metschnikowia aff. pulcherrima*, protein identificators associated with the differential proteins were converted to *S. cerevisiae* orthologs. As shown in Table 1 and Figs. 5 and 6, the upregulated proteome mainly impacted functions related to five categories: (i) transcriptional events and DNA repair; (ii) ribosome biogenesis and ribonucleoprotein complex formation; (iii) membrane trafficking and vesicle-mediated transport; (iv) pyruvate metabolism; and (v) long-chain fatty-acid and glucan metabolism. In contrast, the down-modulated proteome was preferentially involved in oxidoreductase processes together with amino acid and carbon metabolism.

4. Discussion

M. pulcherrima Mp-30 has been described as a strong antagonist of *B. cinerea* infection in harvested tomatoes and grapes (Fernandez-San Millan et al., 2021). Several strains of this species show antagonism against other fungal postharvest pathogens such as *Penicillium* or *Alternaria* spp., among others (Gore-Lloyd et al., 2019; Hilber-Bodmer et al., 2017; Janisiewicz et al., 2001; Oztekin and Karbancioglu-Guler, 2021; Piano et al., 1997; Ruiz-Moyano et al., 2016; Saravanakumar et al., 2008; Sipiczki, 2020; Spadaro et al., 2002; Türkel et al., 2014). This background prompted us to study Mp-30′s antifungal performance and biocontrol mechanisms in apples, another fruit of high economic relevance. We have observed that Mp-30 cells were capable of completely preventing gray mold infection from five different *B. cinerea* strains when 10^4 or more yeast cells were inoculated per wound. Indeed, Mp-30 is able to colonize the wounds rapidly with an exponential growth from 10^2 to 10^6 cells per wound during the first 24 h after inoculation. However, although this rapid colonization probably results in competition with *B. cinerea* for nutrients and space, this mechanism cannot alone account for the antifungal effect since an inoculation with 10^2 cells did not offer protection against the fungal pathogen. Therefore, other mechanisms that occur during the first hours of the yeast-fungus interaction must be implicated in this apple infection model.

Previous *in vitro* studies showed that the Mp-30 strain displays a plethora of antifungal mechanisms (Fernandez-San Millan et al., 2021, 2020): synthesis of cell-wall degrading enzymes (chitinase and β-endoglucanase), solubilization of nutrients (phosphorous and zinc) and biofilm production. On the other hand, there are several studies that point to the production of the iron chelating agent pulcherrimin as the main antifungal mechanism of several *M. pulcherrima* strains (Gore-Lloyd et al., 2019; Saravanakumar et al., 2008; Sipiczki, 2020, 2006). However, our results point to the involvement of other processes in the Mp-30 strain. Although we have corroborated via *in vitro* assays that Mp-30 inhibits germination of *B. cinerea* spores and competes for iron in the surrounding medium, we have also observed that iron deficiency is not a relevant control mechanism under *in vivo* conditions. This is because when iron was added to infected apple wounds in the presence of Mp-30, the apples were also protected against *B. cinerea* infection. The production of volatile compounds and induction of apple resistance responses have also been discarded as the main protective mechanisms. However, hot-inactivated yeast cells or cell-free culture filtrates showed a certain degree of fungal protection, thereby indicating that there are intracellular compounds and substances secreted into the extracellular medium that have antifungal potential. Indeed, in a previous study it was shown that Mp-30 secretes potent antifungal metabolites into the extracellular medium (Fernandez-San Millan et al., 2022a, 2022b). Two of these (3-amino, 5-methylhexanoic acid and biphenyl-2,3 diol) result in total control of the gray mold infection in apples and could be of agronomic interest.

To have a broader perspective and unravel the still unknown Mp-30 antifungal mechanisms, we have characterized the Mp-30 protein profile in the direct interaction with *B. cinerea* in apples. Proteomics has been used before to shed light on mechanisms underlying the biological control of several postharvest diseases, but usually, from the pathogen or the fruit point of view. For example, Chan et al. (2007) described the peach fruit proteins involved in resistance to gray mold in the presence of the yeast *Pichia membranefaciens* and salicylic acid. Liu et al. (2018) also showed several proteins implicated in the response of kiwifruit to *B. cinerea*. Gu et al. (2020) have also revealed the mechanisms involved in the enhanced biocontrol efficacy of the yeast *Rhodotorula mucilaginosa* induced by chitosan. Using an approach that resembled our study, Kwasiborski et al. (2014) compared the proteome of *Wickerhamomyces anomalus* grown on apple fruits in the presence or absence of *B. cinerea*. These results suggested an enhancement of the pentose phosphate pathway, thus indicating a modification in energetic metabolism that met the needs for higher levels of energy and nucleic acids associated with the increase in the metabolic activity of this yeast strain.

Our proteomic analysis showed an important readjustment of yeast metabolic pathways in response to the presence of *B. cinerea* spores in wounded apples. This response was independent of the Mp-30 growth status because no significant differences were found in cell density between Mp-30 growing alone or in the presence of *B. cinerea*.

4.1. Interaction with B. cinerea induces gene expression and membrane trafficking in the yeast

Interestingly, there were 36 increased proteins implicated in DNA replication, repair and maintenance as well as RNA synthesis, rRNA biogenesis and maturation, tRNA synthesis, and enhancement of translation, all pointing to an overall increase in gene expression. Among the

Table 1
Identification of *Metschnikowia pulcherrima* Mp-30 proteins with different levels in the presence of *B. cinerea* spores in wounded apples.

Protein name	Accession	Fold change (log_2)
Genome expression		
DNA replication and maintenance		
Non-structural maintenance of chromosomes element 1	A0A4P6XI24	1.41
MMS19 nucleotide excision repair protein	A0A4P6XKU3	0.52
WSTF, HB1, Itc1p, MBD9 motif 1	A0A4P6XL51	0.52
FACT complex subunit	A0A4P6XIM3	0.36
Replication factor C subunit 3/5	A0A4P6XDP9	0.28
Helix-loop-helix DNA-binding domain-containing protein	A0A4P6XW68	−2.09
Nucleotide metabolism		
Ras-related protein Rab-6A	A0A4P6XN64	0.62
Nucleolar GTP-binding protein 1	A0A4P6XVX3	0.56
CTP synthase	A0A4P6XP07	0.46
Transcription, RNA processing/degradation, ribosome biogenesis		
DNA-directed RNA polymerase II subunit RPB4	A0A4P6XU06	2.60
Small nuclear ribonucleoprotein Sm D2 (snRNP core protein D2)	A0A4V1AEE1	2.26
Threonylcarbamoyl-AMP synthase	A0A4P6XTE5	1.51
Putative transcription factor	QFZ29609 *	0.92
ATP-dependent RNA helicase DOB1	A0A4P6XN68	0.85
5′-3′ exoribonuclease	A0A4P6XLD9	0.85
Reticulon-like protein	A0A4V1AEN5	0.84
RNA cytidine acetyltransferase	A0A4P6XUZ1	0.64
DNA-directed RNA polymerase subunit beta	A0A4P6XPB2	0.62
5′-3′ exoribonuclease 1	A0A4P6XLP6	0.60
ATP-dependent helicase STH1/SNF2	A0A4P6XJ75	0.55
PAN2-PAN3 deadenylation complex subunit PAN3	A0A4P6XJY7	0.52
mRNA-splicing factor ATP-dependent RNA helicase DHX15/PRP43	A0A4P6XLN9	0.52
DNA-directed RNA polymerase III subunit RPC3	A0A4V1AEU1	0.48
DNA-directed RNA polymerase subunit	A0A4P6XR48	0.47
Pre-rRNA-processing protein TSR1	A0A4P6XIK4	0.45
Enhancer of mRNA-decapping protein 3	A0A4P6XQF3	0.45
KRR1 small subunit processome component	A0A4P6XT08	0.42
U3 small nucleolar RNA-associated protein 8	A0A4P6XRU6	0.41
DNA-directed RNA polymerase subunit	A0A4P6XX01	0.36
ATP-dependent RNA helicase DDX51/DBP6	A0A4V1ADG3	0.31
Valyl-tRNA synthetase	A0A4P6XQF9	0.29
Aspartate–tRNA ligase	A0A4P6XGX8	0.29
Zinc finger C-x8-C-x5-C-x3-H type and similar	A0A4V1ADW4	−0.39
RXT2-like, N-terminal	A0A4P6XM72	−0.39
40S ribosomal protein S26	A0A4V1AEB1	−0.41
BSD domain-containing protein	A0A4P6XQ89	−0.45
Transcriptional activator SPT8	A0A4P6XJK6	−0.46
RNA exonuclease 1	A0A4P6XUC6	−0.48
DNA-directed RNA polymerases I, II, and III subunit RPABC3	A0A4P6XP42	−0.48
BZIP domain-containing protein	A0A4P6XVZ8	−0.71
Cytochrome *b* pre-mRNA-processing protein 3	A0A4P6XJM2	−1.38
Ribosome biogenesis protein NSA2 homolog	A0A4P6XLQ6	−1.93
mRNA-capping enzyme subunit beta	A0A4V1ADZ5	−2.02
Translation		
Diphthamide synthase	A0A4P6XMZ1	1.09
GCN1, translational activator of GCN4	A0A4P6XV01	0.55
Eukaryotic translation initiation factor 3 subunit A (eIF3a)	A0A4P6XNR9	0.50
Pumilio family protein 6	A0A4P6XG45	0.47
Protein TIF31	A0A4P6XKL4	0.31
Eukaryotic translation initiation factor 3 subunit J (eIF3j)	A0A4P6XUV9	−0.33

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Table 1 (*continued*)

Protein name	Accession	Fold change (log_2)
Translation elongation factor 1B subunit beta eEF-1Bb	A0A4P6XV53	−0.33
Translationally controlled tumor protein homolog	A0A4P6XLH6	−0.49
Cell structure		
1,3-β-glucan synthase	A0A4P6XL35	1.10
Cell division control protein 24	A0A4P6XHM7	0.72
Protein MPUL0B10660	A0A4V1AE37	0.57
Altered inheritance of mitochondria protein 24	A0A4P6XE51	0.55
Protein 8	A0A4P6XLM8	0.49
Lectin, mannose-binding 1	A0A4P6XXT3	0.39
Plastin-1	A0A4P6XVW6	0.35
Carbohydrate metabolism, glycolysis, TCA cycle, pentose phosphate pathway		
Glycolysis		
Glycogen phosphorylase	A0A4P6XNE9	0.64
Pyruvate carboxylase	A0A4P6XRS0	0.52
Alcohol dehydrogenase	A0A4P6XIG9	0.51
Alpha-aminoadipate reductase	A0A4P6XPL5	0.48
Glycogen synthase	A0A4P6XNZ3	0.33
Dihydroxyacetone kinase	A0A4P6XJI7	−0.34
Phosphoglycerate mutase	A0A4P6XQV5	−0.42
Inositol-3-phosphate synthase	A0A4P6XU52	−0.59
TCA cycle		
Isocitrate dehydrogenase [NAD] subunit mitochondrial	A0A4P6XJU1	−0.37
Pentose phosphate pathway		
6-Phosphogluconolactonase-like protein	A0A4P6XT89	−0.35
Ribose-5-phosphate isomerase	A0A4P6XM88	−0.35
Lipid metabolism		
Fatty acid, phospholipid synthesis and degradation		
Acetyl CoA carboxylase	A0A4P6XP86	0.69
Phospholipid: diacylglycerol acyltransferase	A0A4P6XWE0	0.65
Acyl-CoA synthetase (AMP-forming)	A0A4P6XT21	0.62
Acetyl esterase/lipase	A0A4P6XGX7	0.57
Phosphatidylserine synthase	A0A4P6XKJ6	0.55
Fatty acid synthase subunit alpha	A0A4P6XDU9	0.49
Acyl carrier protein	A0A4P6XJD6	−0.52
Sterol synthesis		
Squalene synthase	A0A4P6XUN0	0.50
Erg28 like protein	A0A4P6XJQ1	0.50
Squalene monooxygenase	A0A4P6XGK4	0.34
Diphosphomevalonate decarboxylase	A0A4V1AEY9	−0.36
Energetic metabolism		
Oxidative phosphorylation		
Cytochrome *c* oxidase assembly protein subunit 11	A0A4P6XPN9	1.69
F-type H + -transporting ATPase subunit f	A0A4P6XQ83	0.51
ATP synthase subunit gamma	A0A4P6XHK8	−0.35
ATP synthase subunit beta	A0A4P6XRF0	−0.35
Holocytochrome c-type synthase	A0A4P6XFE6	−2.28
Ubiquinol-cytochrome *C* reductase hinge protein	A0A4P6XFG5	−2.53

Protein name	Accession	Fold change (log_2)

(*continued on next page*)

Table 1 (*continued*)

Protein name	Accession	Fold change (log_2)
Cellular metabolism		
Amino acid biosynthesis and degradation		
Amino-acid acetyltransferase, mitochondrial	A0A4P6XLJ1	0.60
Phenylpyruvate decarboxylase	A0A4P6XS03	0.54
Phenylpyruvate decarboxylase	A0A4P6XUY8	0.38
Aspartate transaminase	A0A4P6XIJ8	−0.39
Amidase	A0A4P6XGJ3	−0.40
Alanine-glyoxylate aminotransferase	A0A4V1AEF0	−0.41
Amidase, hydantoinase/carbamoylase family	A0A4P6XT41	−0.41
Homocysteine S-methyltransferase	A0A4P6XH98	−0.57
Ornithine aminotransferase	A0A4P6XRD9	−0.58
Protein modification, maturation and degradation		
Alpha 1,2-mannosyltransferase	A0A4P6XRC6	1.51
Di-and tripeptidase	A0A4P6XGX0	1.40
Alpha 1,2-mannosyltransferase	A0A4P6XQ59	0.82
Mannosyl-oligosaccharide glucosidase	A0A4P6XEM6	0.61
Cullin 1	A0A4P6XIE7	0.59
Peptidase inhibitor I9	A0A4P6XJP1	0.57
Peptide hydrolase	A0A4P6XNG5	0.53
26S proteasome regulatory subunit RPN2	A0A4V1AEC9	0.40
HAT family C-terminal dimerization region	A0A4P6XR54	0.34
Alpha-mannosidase	A0A4P6XG79	0.30
Candidapepsin	A0A4V1AEX1	0.28
E3 ubiquitin-protein ligase	A0A4P6XT76	0.28
Proteasome subunit alpha 4	A0A4P6XIW4	−0.32
DnaJ family protein B member 4	A0A4V1AE11	−0.34
Peptidase_M16 domain-containing protein	A0A4P6XVL2	−0.34
26S proteasome regulatory particle non-ATPase Rpn10p	A0A4P6XT53	−0.36
E3 ubiquitin protein ligase	A0A4P6XVH1	−0.39
Xaa-Pro dipeptidase	A0A4P6XQ87	−0.41
Prefoldin subunit 3	A0A4P6XEX3	−0.44
Trafficking and vesicle-mediated transport		
AP-2 complex subunit alpha	A0A4P6XUC0	3.43
Nucleoporin	QFZ26283.1 *	2.98
AP-1 complex subunit	A0A4P6XNZ7	0.68
Solute carrier family 25 (2-oxodicarboxylate transporter)	A0A4P6XPP2	0.62
Translocation protein SEC63	A0A4P6XJA7	0.61
Golgi apparatus membrane protein TVP18	A0A4P6XKW6	0.56
TPR_REGION domain-containing protein	A0A4V1AEH4	0.54
Ras-related protein Rab-11B	A0A4P6XL32	0.53
Vacuolar protein sorting-associated protein	A0A4P6XXG4	0.50
Ca2 + -dependent lipid-binding protein	A0A4P6XSJ8	0.47
Coatomer subunit gamma	A0A4P6XK21	0.46
Ca2 + -dependent lipid-binding protein	A0A4V1AET2	0.45
Purine nucleoside permease	A0A4P6XK35	0.45
PH domain-containing protein	A0A4P6XLG8	0.45
Emp24/gp25L/p24 family/GOLD	A0A4P6XR33	0.40
Sorting nexin-4	A0A4P6XPB1	0.39
Coatomer subunit alpha	A0A4P6XIV4	0.37
Synaptobrevin family protein YKT6	A0A4P6XMH8	0.36
Putative arabinose efflux permease, MFS family	A0A4P6XR40	0.30
Solute carrier family 25 (Aspartate/glutamate transporter)	A0A4P6XN26	0.27
GRIP domain-containing protein	A0A4V1AF00	−0.38
Transport factor 2 (NTF2) domain-containing protein	A0A4P6XKZ2	−0.49
Clathrin light chain	A0A4P6XL13	−0.50

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Table 1 (*continued*)

Protein name	Accession	Fold change (log_2)
Solute carrier family 25 (Phosphate transporter)	A0A4P6XTW4	−1.98
Import inner membrane translocase subunit TIM12	A0A4P6XLY6	−2.37
Redox regulation		
1-Acylglycerone phosphate reductase	A0A4P6XND3	0.68
Glutamate-cysteine ligase	A0A4V1ADU0	0.35
Glutathione reductase	A0A4P6XQZ2	−0.32
Thioredoxin	A0A4P6XKV0	−0.33
Protein disulfide-isomerase	A0A4P6XR67	−0.35
Multifunctional beta-oxidation protein	A0A4P6XLH3	−0.47
Aldehyde dehydrogenase acceptor	A0A4V1AEE0	−1.27
DOPA 4,5-dioxygenase	A0A4P6XHV1	−1.90
Signal transduction		
Serine/threonine protein kinase	A0A4P6XUQ2	1.57
ATP pyrophosphate-lyase	A0A4P6XX81	0.78
Protein kinase A	A0A4P6XJS6	0.65
Protein kinase domain-containing protein	A0A4P6XWH9	0.45
AarF domain-containing kinase	A0A4P6XKT5	0.41
Choline kinase	A0A4P6XCJ1	0.40
Protein-serine/threonine kinase	A0A4P6XSF6	0.37
Casein kinase II subunit alpha	A0A4P6XQQ8	0.36
Serum/glucocorticoid-regulated kinase 2	A0A4P6XR63	0.36
Serine/threonine-protein phosphatase	A0A4P6XNH5	0.35
NADPH-dependent methylglyoxal reductase	A0A4V1AE64	−0.67
Sulfur metabolism		
Cystathionine gamma-synthase	A0A4V1AEX5	−0.32
Rhodanese-related sulfurtransferase	A0A4P6XPR1	−0.33
Iron-sulfur cluster assembly protein	A0A4P6XTA4	−0.51
Biosynthesis of cofactors and secondary metabolites		
Thiamine pyrophosphokinase	A0A4P6XT93	1.33
Phosphatidylinositol 4-phosphate phosphatase	A0A4P6XNC7	0.47
Kynureninase	A0A4P6XP53	0.31
Phosphopantothenate-cysteine ligase	A0A4P6XWI8	0.26
Pyridoxal 5′-phosphate synthase	A0A4P6XJ92	−0.44
Miscellaneous and unknown function		
Uncharacterized protein	A0A4P6XIV5	0.78
Uncharacterized protein	A0A4P6XU76	0.48
Uncharacterized protein	A0A4P6XG51	0.41
Uncharacterized protein	A0A4P6XKB8	0.37
Cupin-like domain-containing protein	A0A4P6XJ63	−0.33
Uncharacterized protein	A0A4P6XPJ9	−0.35
DUF1752 domain-containing protein	A0A4P6XIB0	−0.57
Uncharacterized protein	A0A4P6XQC0	−0.61
Aminotransferase class-III	A0A4P6XTR4	−0.63

Protein names and accession numbers correspond to the UniProt database. Protein codes belong to *Metschnikowia pulcherrima* except for proteins marked with an asterisk, which belong to *Candida lusitaniae*. All proteins are differentially accumulated at p-val < 0.05. Positive and negative fold change (log_2) numbers mean upregulation and downregulation, respectively. Functional annotation of the differential proteins was performed using UniProt, KEGG, GO, DAVID and related published references.

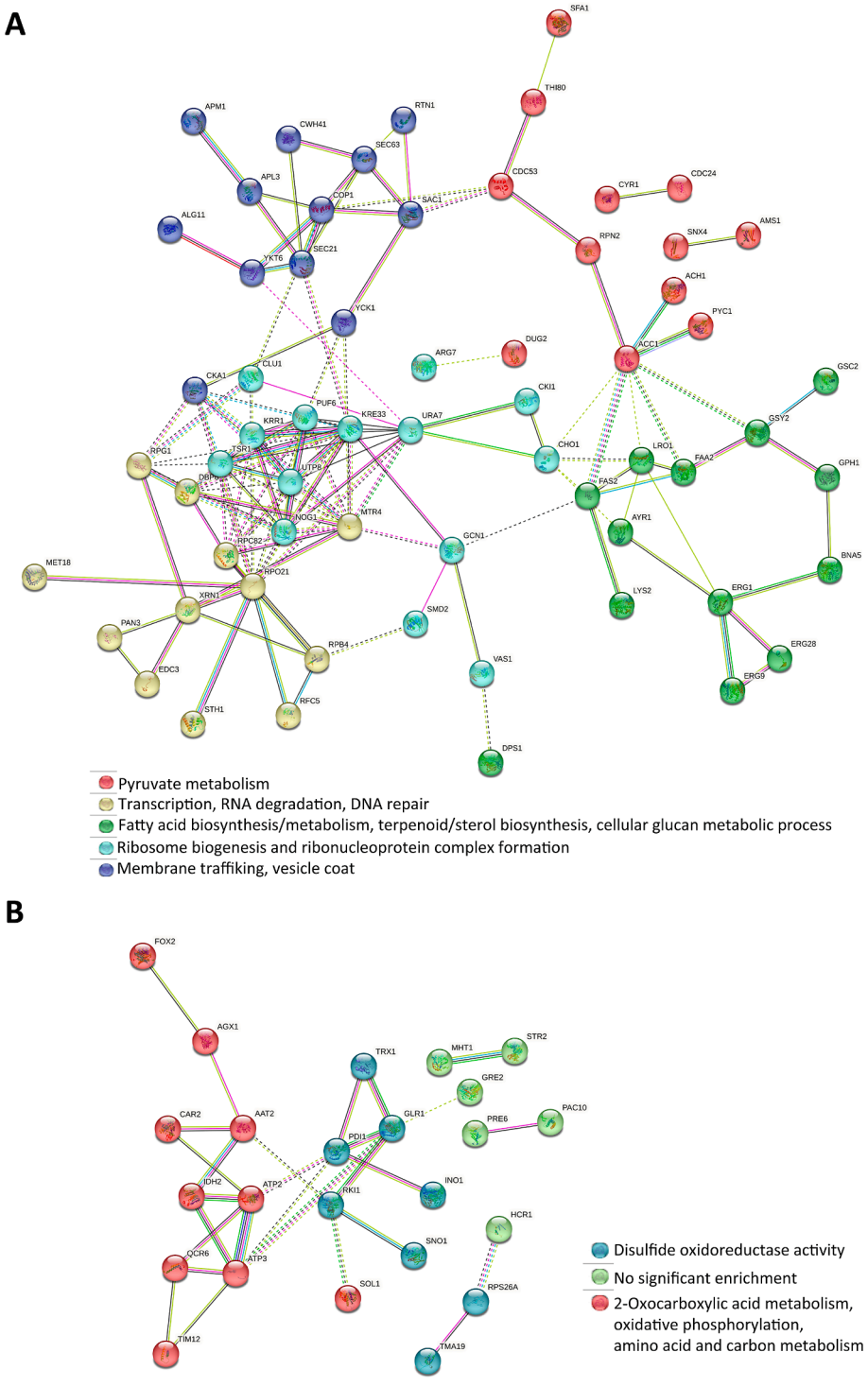

Fig. 5. Protein interactome network for the increased (A) and decreased (B) proteins. Network analysis was performed by submitting the corresponding protein IDs to STRING (Search Tool for the Retrieval of Interacting Genes) software (v.11.5) (https://string.org/). Proteins are represented with nodes and the interactions with continuous lines to indicate direct interactions (physical), while indirect ones (functional) are represented by dashed lines. All edges were supported by at least a reference from the literature or from canonical information stored in the STRING database. K means clustering was applied.

four RNA polymerases that were differentially enhanced, **RNA polymerase II subunit (RPB4)** was the third protein with the highest fold change value. It is part of the subcomplex that is necessary for transcription initiation, elongation and termination. It has been noted that this polymerase is not required in normal growth conditions, but is essential for cell viability in stress environments (Choder, 2004). We have also observed that the RNA degradation pathway was enriched in proteins. Among them, **helicases** and a **small nuclear ribonucleoprotein (Sm-D2)** are of crucial importance. Sm-D2, along with other ribonucleoproteins, plays important roles in pre-mRNA splicing (Scofield and Lynch, 2008). Indeed, RNA degradation is a key mechanism for maintaining RNA homeostasis and involves monitoring, updating and RNA processing in eukaryotic cells (Scofield and Lynch, 2008).

In concert with an enhanced gene expression of Mp-30 cells, an increase in tRNA synthesis and processing along with ribosome biogenesis-related proteins and translation elongation/activator factors indicated a positive regulation of protein synthesis in the yeast in the presence of *B. cinerea*. Therefore, transcription of DNA was regulated to generate mRNA that encoded functional proteins and ribosomal RNAs.

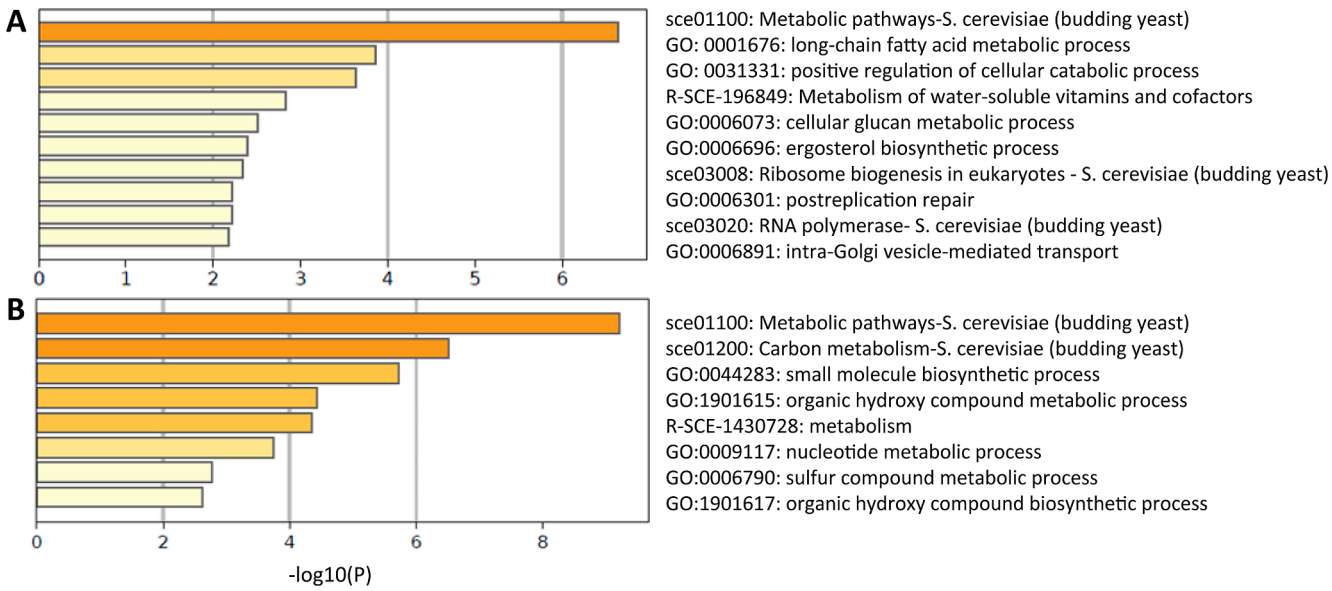

Fig. 6. Functional analysis of the differentially accumulated proteins using Metascape. Significantly enriched pathways related to increased (A) and decreased (B) proteins are shown.

Subsequently, there was a general activation of mRNA translation and, finally, the RNA degradation system maintained the stability and quality control of the gene expression process. Li et al. (2021) observed a similar pattern of upregulated proteins and processes related to gene expression when *S. cerevisiae* was grown under high levels of Cu^{2+}.

In concordance with an enhancement of gene expression and protein translation, 25 differential proteins related to membrane trafficking throughout the cell were significantly increased, suggesting an activation of the movement of compounds inside and outside the cell. For example, it is remarkable that the accumulation of a **nucleoporin**, which is part of the nuclear pore complex that mediates the nucleocytoplasmic transport of macromolecules (Akey et al., 2022), was increased 7.9-fold. In addition, two proteins related to mitochondrial membrane trafficking and 12 proteins of the endoplasmic reticulum (ER)-Golgi-vacuole-plasma membrane (PM) route were upregulated. The Golgi apparatus is a sorting node from which proteins are delivered to their final destinations following a retrograde route to the ER, or anterograde routes to the cell surface and to the vacuole through the endosomal system (Hoya et al., 2017). Different proteins facilitate trafficking between cell compartments and a variety of membranous carriers mediate the transport of proteins, metabolites and lipids (De Matteis and Luini, 2008). Among these, the **assembly polypeptide (AP) adaptor AP-2** was of significant importance due to it being the most highly increased in this study (10.8). While **clathrin** protein and **AP-1** (increased and decreased, respectively) are required for the packaging of some cargoes into carriers, AP-2 participates in endocytosis, delivering proteins, lipids and other molecules from the PM to the endosomes (Arora and Damme, 2021). Indeed, surface proteins of eukaryotes are regulated by intracellular vesicle trafficking along the secretory pathway to the PM or through endocytosis and trafficking to the vacuole (Laidlaw et al., 2022; Stalder and Gershlick, 2020). In addition, another enhanced protein that was observed, **sorting nexin Snx4**, belongs to a family of proteins that play a role in PM recycling. This control of PM composition is related to several physiological functions, including regulation of nutrient homeostasis and lipid and protein quality control (Hanley and Cooper, 2021). In addition, dynamic actin filaments are required for the formation and internalization of endocytic vesicles, serving as a track for their translocation to early endosomes (Galletta and Cooper, 2009). **Plastin-1** (increased in Mp-30) is involved in the organization of actin networks (Giganti et al., 2005; Gimona et al., 2002) and could be related to an enhancement of vesicle trafficking in the yeast cell.

4.2. *The yeast cell wall plays an important role in the antagonistic interaction with B. cinerea*

Significant proteins related to cell wall structure were increased, such as **1,3-β-glucan synthase** and three **mannosyl transferases** (Fig. 7). 1,3-β-Glucan synthase catalyzes the generation of β-glucan, the major structural component of the yeast cell wall, and is involved in synthesis, maintenance and cell wall remodeling. On the other hand, while the inner surface of the cell wall is composed of chitins and glucans, glycosylated mannoproteins are major components of the yeast cell wall outer layer (Klis et al., 2006; Schiavone et al., 2014). The proteins of this layer are involved in several functions often related to cell-to-cell interactions such as flocculation, mating or formation of biofilms (Klis et al., 2002). Carbohydrate moieties determine characteristics that are crucial for cell wall structure and biogenesis and regulate cell wall permeability (De Groot et al., 2005; Lesage and Bussey, 2006). Indeed, upregulation of genes involved in cell wall biosynthesis is activated in response to cell wall stress, leading to a remodeling of the cell wall architecture to be more robust (Udom et al., 2019). Therefore, the change in cell wall composition of Mp-30 cells in the presence of *B. cinerea* seems reasonable.

Another increased protein, **mannose-binding lectin 1** (MBL), fits perfectly in this hypothesis. Enhancement of MBL could be directly related to the yeast-*B. cinerea* interaction and Mp-30 biocontrol ability. Lectins are carbohydrate binding proteins appearing in all domains of life and, in yeasts, they are mostly localized on the cell surface (Singh et al., 2011). These proteins are very important for the interaction of cells with their environment as they recognize specific carbohydrate structures on the cell surface of other neighboring cells (Berg et al., 2002; Chettri et al., 2021). That is why MBL is described as playing a dominant role in first-line defense in eukaryotes (Kuipers and Dijk, 2003). In fact, some lectins are used as antimicrobial agents because binding to carbohydrates on the cell surface may result in changes in cell permeability, pore formation, interference in the quorum sensing mechanism and biofilm development (Breitenbach Barroso Coelho et al., 2018; Coelho et al., 2018). For example, lectins from cyanobacteria or *Penicillium* spp. were active against some pathogenic fungal species like

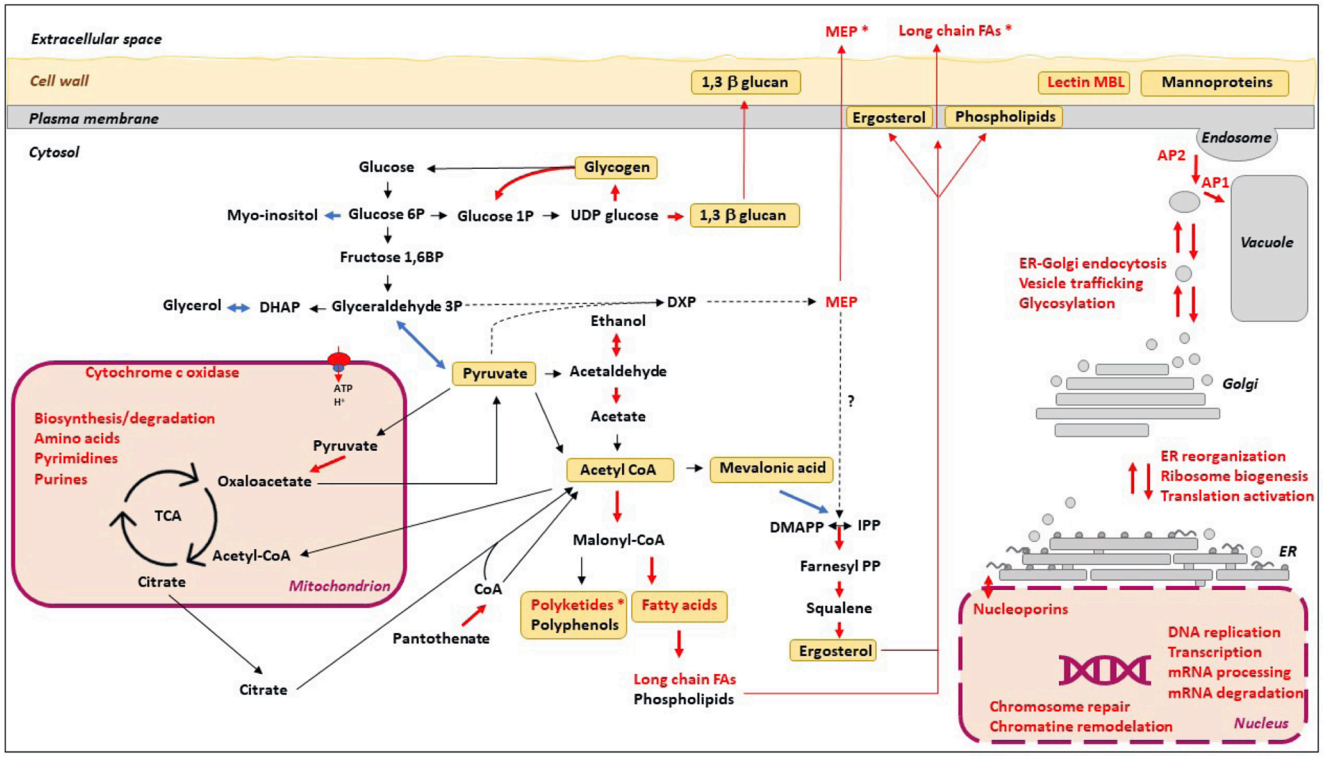

Fig. 7. Schematic representation of Mp-30 cell pathways involved in the antagonistic interaction with *B. cinerea* in wounded apples. The represented pathways are based on differentially accumulated proteins and have been simplified to highlight key end-products. (1) The presence of *B. cinerea* spores in the surrounding environment promotes enhancement of yeast gene expression, including an increase in DNA, RNA and protein biogenesis-related proteins. (2) Vesicle-mediated trafficking in the endoplasmic reticulum (ER)-Golgi-plasma membrane pathway, endocytosis and protein glycosylation are upregulated. An increase in mannosylation activity will probably result in a higher presence of mannoproteins in the cell wall. In addition, a higher presence of mannose binding lectin 1 (MBL) in the cell wall might be involved in *B. cinerea* sensing and the yeast antagonistic response. (3) The presence of *B. cinerea* causes higher activity in the glycogen synthesis and degradation pathway with a subsequent enhancement of 1,3-β-glucan production for cell wall restructuration. (4) Enhancement of pyruvate conversion to oxaloacetate and through to higher acetyl-CoA synthesis would serve to boost the tricarboxylic acid cycle (TCA) and energy production in the mitochondria. (5) An increase in acetyl-CoA supports the upregulation of malonyl-CoA conversion to produce more polyketides, long chain fatty acids (FAs) and phospholipids for the plasma membrane. (6) The synthesis of ergosterol is enhanced, and this sterol may be redirected to the plasma membrane. We propose that downregulation of the first step of the mevalonic acid pathway towards ergosterol synthesis might be compensated by a hypothetically active methyl erythritol phosphate (MEP) pathway. Arrows, proteins or processes in red represent increase; blue arrows represent decrease. Dotted arrows correspond to hypothetical steps. Red asterisks indicate compounds that have been demonstrated to be differentially secreted in the presence of *B. cinerea* through metabolomics (Fernandez-San Millan et al., 2021). Detailed information of proteins involved in these pathways is listed in Table 1. AP: assembly polypeptide adaptor; CoA: coenzyme A; DHAP: dihydroxyacetone phosphate; DMAPP: dimethylallyl pyrophosphate; DXP: deoxylulose5-phosphate; IPP: isopentenyl pyrophosphate; MEP: 2- C- methyl-D-erythritol-4-phosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cryptococcus or *Aspergillus* spp. among others (Jones et al., 2017; Singh et al., 2013). Moreover, other studies suggest that there might be some indirect consequences caused by lectin-carbohydrate linkages on the fungal cell wall surface (Chettri et al., 2021). Indeed, several physiological roles have been proposed for fungal lectins related to growth, development, morphogenesis, defense, pathogenicity, symbiosis or yeast flocculation and parasitism (Berg et al., 2002; Chettri et al., 2021; Singh et al., 2011; Varrot et al., 2013).

We also have observed that the presence of *B. cinerea* seems to alter the carbohydrate metabolism of yeast cells, promoting the glycogen biosynthesis and degradation pathway (Fig. 7). The net rate of glycogen accumulation is determined by the opposing actions of **glycogen synthase** and **glycogen phosphorylase** (Feng et al., 1991). In yeast, glycogen accumulation is initiated by nutrient-limiting conditions, and serves as a storage form of both energy and carbon (Lillie and Pringle, 1980; Wilson et al., 2010). In our case, the increased of both enzymes suggested the use of glycogen as a source of compounds for production of more glucans for the cell wall. Considering that there is no differential growth pattern of Mp-30 growing alone or in the presence of *B. cinerea* and no nutrient limitation, it is plausible that this pathway diverts resources so that more glucans are deposited in the cell wall of the yeast cells in contact with fungal spores. This is in concordance with the observed enhancement of 1,3-β-glucan synthase.

Although three proteins of the glycolytic pathway that produce pyruvate decreased (**dihydroxyacetone kinase, inositol-3-phosphate synthase** and **phosphoglycerate mutase**), other pathways derived from pyruvate were enhanced (Fig. 7). Oxaloacetate formation from pyruvate through **pyruvate carboxylase** increase replenishes the TCA cycle intermediates. Higher availability of oxaloacetate and acetyl-CoA for the TCA cycle is in concordance with changes in mitochondrial activity for production of energy because seven proteins of the electron chain were differentially accumulated. Among them, **cytochrome *c* oxidase** increased, and several **ATP synthase subunits** were increased or decreased. In addition, proteins of the purine/pyrimidine, amino acid biosynthesis, and mitochondrial degradation pathways were also differentially accumulated in the presence/absence of the fungus.

On the other hand, acetyl-CoA seems to be a central component in the adaptation of yeast cells to the presence of *B. cinerea* (Fig. 7). Three proteins of its biosynthetic pathway were increased, encompassing CoA production from pantothenate (**phosphopantothenate-cysteine ligase**) to its conversion from ethanol and acetaldehyde via **alcohol dehydrogenase** and **α-aminoadipate reductase**. Next, enhanced

acetyl-CoA conversion to malonyl-CoA through upregulation of **acetyl CoA carboxylase** is the first step of increasing fatty acid (FA) biosynthesis (Fakas, 2016). Malonyl-CoA serves as a substrate for the FA synthetase complex, but may also be used for the synthesis of many bioactive compounds (Wang et al., 2014). Two major groups of such compounds are polyphenols and polyketides (Johnson et al., 2017). Indeed, in a previous metabolomic study we observed that Mp-30 differentially secreted two polyketides (rhodomycin and 88a-deoxyoleandolide) into the extracellular medium when cocultured with *B. cinerea* (Fernandez-San Millan et al., 2022a, 2022b). However, malonyl-CoA as a building block for secondary metabolite synthesis has only a minor role in cellular metabolism. It primarily serves as an extender unit for the synthesis of FAs that constitute the membrane lipids (Cronan and Thomas, 2009; Milke and Marienhagen, 2020). Pathways for the synthesis of long chain FAs and phospholipids were clearly enhanced in our study, with five main proteins being increased: **acyl-CoA synthetase (ACS), FA synthase (FAS), diacylglycerol acyltransferase, acetyl esterase** and **phosphatidylserine synthase** (Fig. 7). Among them, ACS increase is of great interest, since it participates in several cellular pathways including energy production, lipid synthesis and degradation, membrane biogenesis, post-translational modification of proteins, regulation of gene expression, and vesicular trafficking (Black and DiRusso, 2007). FAS is an essential enzymatic complex responsible for the entire synthesis of C16- and C18-fatty acids (Lomakin et al., 2007). Hexadecanoic acid and octadecenoic acid are two of the end points of the biosynthetic route that metabolizes acetyl-CoA via malonyl-CoA. Indeed, hexadecanoic acid was secreted into the extracellular medium by Mp-30 when cultured in the presence of *B. cinerea* mycelium and its *in vitro* antifungal effect was demonstrated (Fernandez-San Millan et al., 2022a, 2022b).

In this context, enhanced levels of phospholipids following increases of the proteins involved in their synthesis also supports the hypothesis of yeast remodeling because these proteins are major structural components of cellular membranes and are essential for vital processes (van Meer et al., 2008; Voelker, 2009).

Besides readjustments in phospholipidic patterns and β-glucan composition, as well as mannosylation of proteins and increases in MBL, additional changes also seem to affect the lipidic composition of the cell membrane due to the increase of three proteins of the ergosterol biosynthetic pathways comprising **squalene synthase, squalene monooxygenase** and **ergosterol biosynthesis protein Erg28**. Enhancement of this route is interesting because ergosterol is the main fungal sterol and an important component of the yeast cell membrane. It plays an important role in ensuring membrane fluidity and integrity, cell viability and cellular transport (Ke et al., 2018; Kołaczkowska et al., 2012). In addition, ergosterol abundance is critical for yeast adaptation to changes in the surrounding environment and increased ergosterol levels are associated with better resistance to low temperature, sugar deficiency and oxidative stress (Jordá and Puig, 2020).

However, the pattern of regulation in the acetyl-CoA pathway was somewhat inconsistent. Increases of proteins in the biosynthesis of ergosterol contrasted with decrease of **diphosphomevalonate decarboxylase** (Fig. 7), which is an enzyme that should provide increased isopentenyl diphosphate (IPP) from mevalonic acid to enhance ergosterol production. Isoprenoids are derived from both IPP and dimethylallyl diphosphate (DMAPP) (McGarvey and Croteau, 1995). Two pathways lead to IPP and DMAPP in nature: the methylerythritol phosphate (MEP) and the mevalonic acid (MVA) pathways (Rohmer, 1999a, 1999b; Vranová et al., 2013). While the MVA pathway is present in a few eubacteria, archaebacteria and in the cytosol of almost all eukaryotic cells, the MEP pathway has been described in many apicomplexan parasites, bacteria, cyanobacteria, algae and plant chloroplasts (Kuzuyama and Seto, 2003). Recently, a study with the yeast *Yarrowia lipolytica* suggested for the first time that both pathways coexist and nitrogen-limiting conditions trigger utilization of the MEP pathway (Dissook et al., 2021). Interestingly, we reported in a previous study the presence of MEP as an Mp-30 secreted metabolite when cocultured with *B. cinerea* (Fernandez-San Millan et al., 2022a). We hypothesize that Mp-30 is another species that could have a MEP pathway present, which supports the source of the extra IPP necessary for ergosterol production in the presence of *B. cinerea*. Experiments are underway to investigate this hypothesis.

Overall, the results point to the relevance of cell wall and membrane remodeling in this antagonistic yeast-fungal pathogen interaction. In fact, it has been reported before that yeast's adaptation to various abiotic stresses includes changes in FA synthesis and the degree of their unsaturation, the phospholipid components present in the membranes, and mitochondrial activity (Qiu et al., 2019).

4.3. Signaling pathways change during the adaptation of yeast metabolism to the presence of B. cinerea

Under the conditions studied, proteins related to redox regulation and sulfur metabolism were decreased. Among them were **glutathione reductase, thioredoxin, protein disulfide isomerase (PDI), rhodanese** and **cystathionine gamma-synthase**. Indeed, oxidative modification of proteins leads to protein function impairment or activation of specific pathways involved in signaling and regulation of key cellular functions (Herrero et al., 2008). In yeast cells, the glutaredoxin and thioredoxin systems are part of such redox regulation in different compartments of the cell and there is an interplay between both systems (Herrero et al., 2008). Glutathione is present in large amounts in yeasts and plays an important role in redox equilibrium, forming disulfide bonds or scavenging free radicals for oxidative stress protection (Chakravarthi et al., 2006; Penninckx, 2002). Both glutathione reductase and glutathione peroxidase mediate these reactions (Rahman et al., 2006).

In this context, PDIs are part of the thioredoxin superfamily with wide-ranging functions in proteostasis of the secretory pathway (Lu and Holmgren, 2014). Rhodanase is a mitochondrial enzyme that catalyzes the sulfur transfer in several pathways related to sulfur metabolism and the reduction of glutathione and thioredoxin (Cipollone et al., 2007).

By contrast, other yeast proteomic studies have observed that the enhancement of redox proteins and sulfur metabolism processes is related to an adaptive response of the cells to oxidative stress. For example, upregulation of the thioredoxin system was observed when cells were grown at an elevated temperature (Sekova et al., 2021). In our study, although decreases of these proteins will probably have had an impact on the redox status or the yeast cells, the results seem to indicate that the generation of ROS is not a key event in the Mp-30/*B. cinerea* interaction. A more probable hypothesis is that the observed down-regulation is related to the control of the central cellular pathways, since redox conditions constantly regulate metabolism according to the oxidation status and, hence, the activity of multiple proteins (Brandes et al., 2011).

In this regulatory context, it is not surprising to find eight kinases and a phosphatase among the increased proteins. Protein kinases are signaling molecules that catalyze phosphotransfer reactions. Only a few kinases are constitutively active in yeasts and they are precisely controlled for the fine-tuning of cell functions (Manning et al., 2002). Signal transduction pathways play a major role in the response to environmental changes by controlling enzyme cascades that promote transcriptome and proteome remodeling (Creamer et al., 2022). The enhancement of signal transduction proteins in our study underscores the ability of yeast cells to sense and rapidly respond to the changes in their external environment in the presence of other microorganisms

such as *B. cinerea*.

5. Conclusions

In this study, we have demonstrated that Mp-30 strain is a good biocontrol agent of *B. cinerea* in apples, which could be of great agronomic and commercial interest. Total control of the infection caused by *B. cinerea* was only achieved by yeast living cells and *in vitro* and *in vivo* assays have shown a variety of antifungal mechanisms involved in the process. Interestingly, the proteomic analysis has shed light on the main Mp-30 mechanisms, underlying the increase of transcription- and translation-related proteins, cell wall and plasma membrane rearrangements and increase of proteins related to long chain fatty acid biosynthesis and signal transduction. These changes are not related to yeast growth differences in the presence/absence of *B. cinerea* and emphasize the ability of the yeast to sense and adapt to diverse environments via a global response that elicits coordinated changes in the cell. Our results have confirmed the complex metabolic regulation of this yeast strain in such antagonistic interactions with *B. cinerea* and they also reflect the major role of the cell wall and plasma membrane in the first line of antifungal mechanisms. Indeed, we have demonstrated that several pathways cooperate and tightly regulate the organization, composition and biophysical properties of the cell envelope. This study points to a multifactorial nature of the antagonistic mechanisms in yeasts and will help to identify critical proteins and pathways involved in biocontrol responses.

CRediT authorship contribution statement

Alicia Fernandez-San Millan: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Joaquin Fernandez-Irigoyen:** Formal analysis, Investigation. **Enrique Santamaria:** Formal analysis, Investigation. **Luis Larraya:** Formal analysis, Methodology. **Inmaculada Farran:** Methodology, Funding acquisition. **Jon Veramendi:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105266.

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