Changes in the secretome of *Vitis vinifera* cv. Monastrell cell cultures treated with cyclodextrins and methyl jasmonate

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ABSTRACT

Elicitors induce defense responses that resemble those triggered by pathogen attack, such as the synthesis of phytoalexins and pathogen-related proteins which are accumulated in the extracellular space. In this work we have analyzed the changes in the secretome of *Vitis vinifera* cv. Monastrell cell cultures, which is refers to secreted proteome obtained from cell suspension cultures, in response to treatment with cyclodextrins and methyl jasmonate, separately or in combination using label free quantitative approaches. Among all the proteins found thirty-three did not show significant differences among the different treatments carried out indicating that these proteins were expressed in a constitutive way in both control and elicited-grapevine cell cultures. These proteins include pathogenesis-related proteins 4 and 5, class III peroxidases, NtPRp-27, chitinases and class IV endochitinases, among others. Moreover, eleven proteins were differentially expressed in presence of cyclodextrins and/or methyl jasmonate: three different peroxidases, two pathogenesis related protein 1, LysM domain-containing GPI-anchored protein 1, glycerophosphoryl diester phosphodiesterase, reticulin oxidase, heparanase, β-1,3-glucanase and xyloglucan endotransglycosylase.

The treatment with cyclodextrins reinforces defensive arsenal and induces the accumulation of peroxidase V and xyloglucan endotransglycosylase. However, the elicitation with methyl jasmonate decreased the levels of several proteins such as pathogenesis related protein 1, LysM domain-containing GPI-anchored protein 1, cationic peroxidase, and glycerophosphoryl diester phosphodiesterase whereas this

signal molecule increased the levels of new gene products such as heparanase, β-1,3 glucanase, reticulin oxidase, and peroxidase IV which could be used as potential biomarkers in the grapevine defense responses.

KEYWORDS: cyclodextrins, methyl jasmonate, pathogenesis- related protein, secretome, *Vitis vinifera* cv. Monastrell.

1. INTRODUCTION

Plant strategies to fight against external attack include constitutive and inducible mechanisms. Structural barriers or reservoirs of antimicrobial compounds represent constitutive defenses against tissue colonization. However, induced defense responses require the activation of defense-related genes to produce phytoalexins and pathogenesis related protein (PR-proteins) (Belchí-Navarro et al., 2012). Most PR-proteins are induced by the action of pathogen-derived compounds, plant-derived molecules or elicitors. These proteins have shown antimicrobial activities through their hydrolytic activities on cell walls and they are involved in plant defense reactions (Kawano and Furuichi, 2007). In some cases, these defense responses are mediated by signaling molecules such as jasmonates which include jasmonic acid and its more active derivative methyl jasmonate (MJ) (Zhao et al., 2005). These signaling molecules take part in plant defense reactions increasing secondary metabolite production in grapevine (Tassoni et al., 2005). Application of MJ to the surface of grapevine leaves provoked the formation of lesions that mimic the typical hypersensitive response, and the initiation of defense responses such as an increase of phenolic compound production and expression of defence-related genes (Faurie et al., 2009). In fact, the presence of MJ resulted in stilbene accumulation in *V. vinifera* cell cultures (Tassoni et al., 2005). This signal molecule was also able to enhance the gene expression involved in *trans*-resveratrol biosynthetic pathway in *V. vinifera* cell cultures cv. Monastrell (Lijavetzky et al., 2008, Almagro et al., 2014), and

induced the accumulation of PR-proteins in grapevine (Wang et al., 2011; Martínez-Esteso et al., 2009). In fact, the treatment with MJ enhanced the levels of PR-1 in leaves of *Vitis quinquangularis* and the accumulation of peroxidases and class III and IV chitinases in *V. vinifera* cell cultures cv. Gamay (Wang et al., 2011; Martínez-Esteso et al., 2009).

On the other hand, cyclodextrins (CD) which are cyclic oligosaccharides consisting of seven to eight α-d-glucopyranose residues linked by α(1→4) glucosidic bonds act as elicitors inducing defense responses in *V. vinifera* cell cultures since they induced the production of *trans*-resveratrol and the expression of *phenylalanine ammonia lyase*, *cinnamate 4-hydroxylase*, *4-coumarate CoA ligase* and *stilbene synthase* genes (Almagro et al., , 2014). Moreover, CD also increased the abundance of different proteins involved in grapevine defense reactions such as subtilisin-like protease, β-1,3-glucanase and peroxidase (Martínez-Esteso et al., 2009). The way in which CD act could be due to their chemical similarity with the alkyl-derived oligosaccharides that are released from the cell walls during a fungal attack (Bru et al., 2006). Moreover, the effect of combined treatment with MJ and CD on both the production of *trans*-resveratrol and expression profile of the genes involved in its biosynthetic pathway in *V. vinifera* cv. Monastrell cell cultures has been well characterized (Lijavetzky et al., 2008; Belchí-Navarro et al., 2012; Almagro et al., 2014). However, the defense responses triggered by these two elicitors on secretome in *V. vinifera* cv. Monastrell cell cultures is poorly known.

In *V. vinifera* cv. Monastrell cell cultures the extracellular medium is vital for cell life because it is considered a dynamic cell wall compartment which plays a key role in interacting with environmental factors, including those required for defense responses and nutrition (Sabater-Jara et al., 2014). In this sense, the study of differentially expressed proteins in the secretome of *V. vinifera* cv. Monastrell cell

cultures could improve our understanding of the role played by this cell compartment in grapevine defense responses. Among extracellular proteins, it is important to highlight the presence of PR-proteins, which are found constitutively in both plant tissues and cell cultures, and the fact that their levels increases in the presence of biotic and abiotic elicitors (Sabater-Jara et al., 2014). In fact, some PR-proteins such as peroxidases, endochitinases and chitinases are often found in the secretome of cell cultures of *Taxus baccata*, *Zinnia elegans*, *Capsicum annuum*, among other (Novo-Uzal et al., 2009; Sabater-Jara et al., 2010).

Label-free quantitative approaches have gained significance in the proteomic field due to rapid, high resolution, very high reproducibility, easy, sensitivity large scale data processing and low-cost measurements of protein abundance in complex biological samples (Wang et al., 2011). For these reasons, the aim of this work was to investigate the effect of MJ and CD separately or in combination on the secretome of *V. vinifera* cv Monastrell cell cultures using label-free quantitative approaches.

2. MATERIAL AND METHODS

2.1 Plant material

Vitis vinifera cv. Monastrell calli were established in 1990 as described by Calderón et al., (1993). Grapevine cell cultures derived from them have been routinely maintained by periodical subcultures as described by Belchí-Navarro et al. (2012).

2.2 Elicitation of Vitis vinifera cv. Monastrell cell cultures

V. vinifera cv. Monastrell cell cultures were elicited with 50 mM CD alone or in combination with 100 µM MJ. Elicitation experiments were performed in triplicate using 14 days old grapevine cell cultures. For this, 4 g of washed cells (fresh weight) were transferred to 100 mL flasks and suspended in 20 mL of sterile fresh culture medium which contained 50 mM CD alone or in combination with 100 µM MJ followed by 96 h of incubation at 25 °C in darkness in a rotary shaker (110 rpm).

Control treatments without elicitors were always run in parallel. After elicitation, cells were separated from the culture medium under a gentle vacuum and the spent medium was used for measuring the *trans*-resveratrol content and protein isolation.

2.3 Protein extraction

Proteins from *V. vinifera* cv. Monastrell cell cultures were extracted as described in Martínez-Esteso et al. (2009). Previously, four ml of cell-free medium from either the control or elicited cultures was frozen, thawed and then centrifuged. The supernatants were extracted with 25% ethylacetate. After removing the organic phase, the aqueous phase was supplemented with 2% (w/v) polyvinylpolypyrrolidone, and after 60 min incubation with shaking, the slurry was centrifuged at 8000×*g* for 15 min. The proteins in the supernatant were precipitated by adding trichloroacetic acid to a final concentration of 8% (w/v) and pelleted at 5000×*g* for 10 min. Pellets were washed three times in ice-cold methanol and three times in ice-cold acetone. The final dried precipitates were solubilized in buffer containing 7 M urea, 2 M thiourea, 50 mM DTT, 2% (w/v) CHAPS and 0.5% (v/v) IPG buffer. Protein amount was quantified by the Bradford method. Then, 30 µg of protein extract was digested with trypsin overnight. The peptides were desalted using a PepClean C-18 Spin Column (Agilent Technologies, USA) following the recommendations of the manufacturer. The extracted peptides were dried in vacuum and were resuspended in 0.1% formic acid to a final concentration of 1µg/µL.

2.4 MS and MS/MS spectra acquisition

LC-MS/MS analyses were performed using an Agilent 1100 HPLC-ChipCube/MS Interface coupled with an Agilent XCTplus Ion Trap mass spectrometer equipped with a nano-ESI source. Peptide separation was achieved using the ProtID-Chip-150 (II) which assembles a 4 mm, 40 nL enrichment column, and an analytical separation column 150 mm x 75 µm (5 µm, ZORBAX 300SB-C18). Chromatography runs

consisted of a 85 min linear gradient of 5-60% acetonitrile containing 0.1% (v/v) formic acid at a constant flow rate of 0.3 µL min^{-1}.

MS and MS/MS spectra were acquired in the standard enhanced mode (26000 *m*/*z*/s) and the ultrascan mode (8100 *m*/*z*/s), respectively. Mass spectrometer settings: ionization potential of 1.8 kV and an ICC smart target of 400000 or 150 ms of accumulation. MS/MS scans were performed using automated switching with a preference for doubly charged ions and a threshold of 105 counts and a 1.3 V fragmentation amplitude.

2.5 Label free analysis of LC-MS

The LC-MS raw machine output files were converted to mzXML files and loaded in Progenesis LC-MS (Nonlinear Dynamics) v2.5 label-free analysis software. Quantification was done based on peak intensity. The Mascot search result files were used for protein identification. Proteins identified and quantified were filtered using a Mascot Score of 60 and ANOVA *p*-value< 0.009. The data file that yielded most features (peaks) was used as reference, toward that the retention time of all other measurements were aligned and intensities (area under the peak) normalized. Correction for experimental variations was done by calculating the robust distribution of all ratios. The features once converted into an intensity list data were filtered to include only features with charge state two or three, and appearing in a retention time window 20 to 60 min. The intensity lists were clustered according to the experimental groups (Control, MJ, CD and CD+MJ), and the average intensity ratios of the matched features were automatically calculated. To identify the proteins from which features come from, all the MS/MS spectra collected were launched in a unique search by Mascot against the NCBInr database using the following settings: 1 missed cleavage allowed; Viridiplantae as taxonomy; Cys carbamidomethylation as fixed modification; deamidation of asparagine or glutamine, oxidation of methionine and

pyroglutamate formation of N-terminal glutamine or glutamate, as variable modifications; peptide tolerance 1.2 Da; fragment ion tolerance 0.6 Da; peptide charge 2+ and 3+. The database search result XML files were imported in Progenesis LC-MS which assigned peptide and protein identities to the corresponding features. The features were filtered by search score and by species, applying a MASCOT score above 25 and "Vitis", respectively. Once peptides were assembled into proteins, assignment conflicts were resolved according to MASCOT score, the highest the winner, or were left unresolved in case of having the same score, that is the same sequence. Protein quantitative data were calculated as the average of their constitutive features quantitative normalized data. Quantitation quality is determined according to an ANOVA test p value calculated on the features normalized data.

2.6 Bioinformatic functional analysis

Gene ontology analysis of identified proteins was carried out using Blast2GO v2.4.0[37]. A file of FASTA format sequences of the identified and/or quantified protein set was batch retrieved from NCBI website. Blast2GO was fed with the FASTA file and run first to incorporate sequence description by performing a BLASTp search against NCBInr (e-value cutoff 1 x 10^{-50}, 100 for retrieved number of BLAST hits, 33 for HSP (highest scoring pair) length cutoff), second to map GO, EC and Interpro terms and then to annotate the sequences (E-Value Hit-Filter of 1 x 10^{-6}, a Hsp-Hit Coverage Cutoff of 0, an Annotation Cutoff of 55, and a GO Weight of 5). Automatic annotation performed by Blast2GO was manually revised to guarantee accurate assignment.

3. RESULTS AND DISCUSSION

Proteomic analysis of secretome of *Vitis vinifera* cv Monastrell cell cultures elicited with cyclodextrins and/or methyl jasmonate

V. vinifera cell cultures exhibited a high capacity to produce *trans*-resveratrol extracellular accumulation when these cultures were elicited with CD and MJ (Belchí-Navarro et al., 2012; Almagro et al., 2014). For this reason, we analyzed the production of *trans*-resveratrol in the presence of these elicitors in *V. vinifera* cv. Monastrell. The most striking *trans*-resveratrol accumulation was observed in grapevine cell cultures elicited with CD and MJ (data not shown). This effect was due to an increased expression of the genes encoding enzymes involved in *trans*-resveratrol biosynthetic pathway (Lijavetzky et al., 2008). Taking into account that both the production of *trans*-resveratrol and the expression of the genes involved in its biosynthetic pathway have been studied in *V. vinifera* cv. Monastrell cell cultures treated with CD and MJ (Lijavetzky et al., 2008, Belchí-Navarro et al., 2012; Almagro et al., 2014), we propose to study the secretome of these *V. vinifera* cv. Monastrell cell cultures under elicitation conditions.

In this work, label-free approach has been used for the identification of extracellular proteins of grapevine cell cultures elicited with CD and/or MJ. For the analysis of the data, Progenesis LC-MS software (NonLinear Dynamics), which allows the quantification of proteins based on the intensity signals of the precursors and their identification by using their corresponding scans MS/MS was used.

Selection of 2266 possible peptide precursors (features) was based in their charge (+2 or +3) and their retention time (20-60 min). The peptide precursors which had a value $p<0.05$ and $p<0.01$ in an ANOVA test were specifically labeled and analyzed. The searches were performed against NCBInr database. The search was limited to proteins from species of *Vitis* and performed using the built-in decoy search option of Mascot. In addition to those proteins that are expressed differentially in the different treatments (p-value<0,009), other proteins with a score higher than 60 and a p-value>0.05 were identified. These proteins were expressed constitutively, and their

levels did not change significantly in the presence of the different treatments. Amongst these proteins highlight PR-4, class III peroxidases, PR-5, NtPRp-27, chitinases and class IV endochitinases (Table 1).

The PR-4 are proteins classified as endochitinases (van Loon et al., 2006) because they have a weak chitinase activity (Hawkins et al., 2015). These proteins are induced by attacks of pathogens such as *Botryosphaeria dothidea* in *Malus domestica* (Bai et al., 2013) as well as by holaphyllamine, a steroid, in *Arabidopsis* plants (Rao et al., 2002). PR-4 has been also found in the extrafloral nectar of acacia where it had a protective effect against fungal infections (González-Teuber et al., 2009). The presence of this protein in the grapevine culture medium agrees with the results obtained by Nogueira et al. (2007) who detected a PR-4 in embryogenic cell suspensions of cowpea and they suggested that this protein could have a role in the histodifferentiation of pro-embryogenic masses into somatic embryos.

Moreover, we found that the presence of osmotin (PR-5) was constitutive since it was expressed both in the control and elicited culture media in our grapevine cell cultures. Therefore, osmotin-like proteins not only were correlated with grapevine defense responses against elicitors but also, they could be expressed in non-elicited cell cultures due to the mechanical stress suffered by cell cultures when they are grown in flasks in continuous agitation. In this way, osmotin proteins play an important role in the defense against pathogenic fungi (Misra et al., 2016; Gond et al., 2015) since they act permeabilizing fungal cell membranes (Ullah et al., 2017). Osmotin-like proteins can also be induced by several hormonal signals, including abscisic acid or auxin, and environmental signals such as dehydration, salinity, and fungal infection (Ahmed et al., 2013). In addition, PR-5 proteins are also involved in growth and development processes so they can be found constitutively (Zhu et al., 1995; Kim & Hwang, 2000) as occurs in our grapevine cell cultures where thaumatin was found in

control and elicited-grapevine cell cultures (Table 1). This protein has been also found in *C. annuum* and *C. chinense* cell cultures (Sabater-Jara et al., 2010 and 2011) as well as wheat and tobacco plants (Alam et al., 2014; Okushima et al., 2000). In agreement with our results, NtPRp-27 was constitutively expressed in tobacco roots although this protein can also be induced by tobacco mosaic virus, wounding, drought, and by the application of ethylene, MJ, salicylic acid, and abscisic acid (Okushima et al., 2000; Elvira et al., 2008; Sabater-Jara et al., 2010).

Likewise, an endochitinase was detected in control and elicited grapevine cell cultures at 96 h of treatment (Table 1) probably due to the *in vitro* conditions where grapevine cell cultures are grown. Plant chitinases are monomeric proteins which have a molecular weight between 25-40 KDa. Class I, II and IV chitinases have homologous catalytic domains. This domain is homologous to wheat germ agglutinin and it is responsible for binding of the enzyme to chitin (Pusztahelyi et al., 2018). The different types of chitinases are differentiated by their structure, enzymatic properties, spatial and temporal localization, regulation and function (Kasprzewska, 2003). These enzymes are involved in plant defense responses as well as plant growth and development (Grover et al., 2012). Thus, the presence of chitinases in the secretome of non-elicited cell cultures from *Z. elegans*, *C. revoluta*, *T. baccata* cell cultures (Novo-Uzal et al., 2009), *Capsicum* sp cell cultures (Sabater-Jara et al., 2010, 2011) and *N. tabacum* cell cultures (Lippmann et al., 2009) has been described and it agree to our finding in grapevine cell cultures. Moreover, endochitinases are enzymes able to hydrolyze N-acetyl glucosamine (chitin) polymers from the cell wall of fungi (Hamid et al., 2013). Harfouche et al. (2008) observed that an endochitinase was expressed in chestnut control *in vitro* cultures and its abundance increased when they added salicylic acid to cell cultures. The expression of endochitinase genes in untreated cells can be explained mainly by either *in vitro* conditions, which to some extent may

influence gene expression or the production of ethylene which is accumulated at low concentrations in closed *in vitro* containers.

On the other hand, Fig. 1 represents the distribution of all proteins classified in different categories such as cellular compartment, molecular functions and biological processes. As can be observed in the classification of the identified proteins based on its cellular compartment (Fig. 1A), most of the annotations corresponds to proteins which are localized in endomembrane systems such as cytoplasmic vesicles delimited by membrane (37%) and proteins present in the vacuole (14%) as well as other proteins anchored in the plasma membrane (5%). Another group of proteins are localized in the cell wall (8%), mitochondria (6%), and in the apoplast (20%) (Fig. 1A). The presence of annotations which corresponds to proteins localized in the vacuoles, cytoplasmic vesicles and mitochondria can be explained by the multiple localization of these proteins during their biogenesis or by the existence of isoforms located in different cellular compartments whereas proteins detected in the extracellular medium of *Vitis* cell cultures mainly come from the apoplast. In fact, the spent medium of *Vitis* cell cultures provides a convenient, continuous, and unique source of extracellular proteins, easily obtained without cell disruption and so, without any cytosolic contamination (Delaunois et al. 2014). Indeed, the extracellular proteome, is refers to secretome, which is the spent medium since it contains secreted proteins from cell cultures as described by Delaunois et al. (2014).

Delaunois et al. (2013) performed a proteomic analysis from the grapevine leaf apoplastic fluid. These authors described that most of the proteins found in the apoplastic fluid were related to plant defense mechanism and cell wall metabolism representing 50.7% PR-proteins, 16% proteases, 11.9% cell wall modifying enzymes and 2.4% peroxidases. They described that the main PR-proteins detected in grapevine leaf apoplastic fluid were osmotin, chitinases, glucanases, thaumatin-like

proteins and peroxidases as occurs in our secretome obtained from elicited spent medium (Table 1).

As regards the classification of the identified proteins based on their molecular functions (Fig. 1B), most of the annotations correspond to proteins of peroxidase type (15%), calcium- and hemo-binding proteins (29%), as well as electron transport proteins (15%). Other annotations correspond to PR-proteins induced as defense responses against pathogens as well as abiotic stresses such as cold, drought, osmotic stress, UV light, among other (Agarwal and Agarwal, 2010; Tian et al., 2015). Among these proteins, it is worth noting those which have chitinase (10%), endopeptidase (6%), transferase (6%) and hydrolase activities (6%) as well as chitin-binding proteins (10%).

Moreover, the annotations correspond to the biological processes (Fig. 1C) showed the presence of proteins related to plant defense responses against abiotic stimuli (6.41%), innate immune responses (8.33%) and oxidative stress (7.69%). Other group of annotations are those related to cell and carbohydrate catabolism (8.33 and 6.41%, respectively), as well as other metabolic processes which occur in the cell wall (7.05%), and polymers (7.05%) and polysaccharides metabolism (9.61%). The last group of annotations corresponds to plant processes related to development (8.33%) (Fig. 1C).

In order to study the differential levels of proteins, we selected those peptides whose ANOVA had a *p value*<0.05 (Figs. 2-6). The most abundant protein was peroxidase V from *V. vinifera* (Fig. 2A; gi|225455195) in *V. vinifera* cv Monastrell cell cultures treated with CD alone or in combination with MJ, being the abundance 35 and 14 times higher than in control treatments. Moreover, we also found a protein like cationic peroxidase of *A. thaliana* (Figure 2B; gi|225439625), which was strongly expressed in control treatments, and in a lesser extent, in CD-treated cells (2.7 times

less than in control treatments). However, the abundance of this peroxidase was significantly reduced in the treatment with MJ. In addition, peroxidase IV (gi|225434381) was identified in the treatment with MJ alone or in combination with CD, and its abundance were 8 times higher in the combined treatment than in MJ-treated grapevine cells (Fig. 2C). These types of peroxidases belong to class III peroxidases and they are induced in response to stress, injuries or pathogens, and belong to PR-9 protein subfamily according to the classification of van Loon et al. (2006), being their functions related to limit the spread of infection by developing structural barriers or by producing ROS and/or reactive nitrogen species (Passardi et al., 2004). In addition, these peroxidases can produce the oxidation of *trans*-resveratrol in the presence of H_2O_2 exogenous. In fact, an extracellular medium obtained from elicited *Vitis* cell culture was able to convert all *trans*-resveratrol into *trans*-resveratrol dimers when exogenous H_2O_2 was added (Martínez-Esteso et al., 2009).

On the other hand, the results showed that the treatment with MJ provoked a decrease in the levels of some proteins while the elicitation with CD alone or in combination with MJ caused protein accumulation. This phenomenon was also observed in culture media obtained from elicited *V. vinifera* cv Gamay analyzed with 2D-electrophoresis gels (Martínez-Esteso et al., 2009). In fact, MJ decreased the abundance of PR-1 proteins (Fig. 3, gi|225429117 and gi|163914225), but their content was also constitutive since these proteins were also detected in control treatments, and in a lesser extent, in CD-treated cells. These PR-1 proteins have been expressed constitutively in tomato (Tornero et al., 1997) and they have also been described in Arabidopsis plants in the presence of salicylic acid and *Trichoderma* sp. (Wu et al., 2012; Brotman et al., 2013).

The inhibitory effect of MJ on the levels of some apoplastic proteins such as LysM domain-containing GPI-anchored protein 1 from *V. vinifera* was also observed (Fig. 4A; gi|225459538). In fact, MJ decreased its abundance by 67 times with respect to control treatments (Fig. 4A). In the plant kingdom, proteins containing LysM domains act as receptors that bind chitin. Chitin is the major component of the cell wall of fungi, so these proteins with LysM domains have been associated with plant defense responses against fungi (Zhang et al., 2009). In our grapevine cell cultures, LysM domain-containing GPI-anchored protein 1 can be considered as constitutive because they are present in both control- and CD-treated cells. Likewise, a glycerophosphoryl diester phosphodiesterase was also down-regulated in the presence of MJ (Fig. 4B; gi|147811111) but its content was high in control and CD-treated cells. Glycerophosphoryl diester phosphodiesterase is found in a wide variety of organisms and contain highly conserved domains, which are essential for their enzymatic activity (van der Rest et al., 2004). These proteins seem to be involved in the metabolism of phospholipids, and they are located on the cell surface participating in the cell wall organization and the cell signaling pathway (Hayashi et al., 2008; Lino et al., 2016). Therefore, our results indicated that glycerophosphoryl diester phosphodiesterase was expressed constitutively and it would be involved in the cell wall organization as well as in the cell signaling pathway lead by CD and MJ in grapevine cell cultures.

On the other hand, there are two proteins strongly induced in the presence of MJ, a reticulin oxidase (gi|147846526) and a protein similar to heparanase (gi|147862366) (Fig. 5A and Fig. 5B, respectively). Reticulin oxidase catalyzes the formation of (S)-esculerin from (S)-reticulin, which is involved in the production of benzophenanthridine alkaloids. These compounds have cytotoxic activity and their production increase in response to pathogenic attacks (Liu et al., 2015). In this

sense, Custers et al. (2004) observed that the addition of salycilic acid induced the accumulation of reticulin oxidase in leaves of lettuce and sunflower. We also found sequences which had homologies with the protein 3 similar to a heparanase of *A. thaliana* (Fig. 5B). This protein is synthesized in the Golgi Apparatus in form of proenzyme, and then, it is transferred to the endosomes for its transport to the cell surface where it exerts its glucuronidase activity, participating in the metabolism and remodelling of the cell wall. These results indicated that MJ led to the activation of plant defense responses in grapevine increasing the production of PR-proteins, specifically, reticulin oxidase and heparanase.

Among the proteins that were activated in response to stress in *Vitis*, a β-1,3-glucanase (Fig. 6A; gi|37992763) was strongly induced by MJ or CD, although its content was reduced by half when both elicitors added to grapevine cell cultures. Martínez-Esteso et al. (2009) also found a β-1,3-glucanase in *V. vinifera* cv Gamay cell suspensions in both control and CD treatments. These PR-proteins were also detected in *C. annuum* cell cultures elicited with MJ (Sabater-Jara et al., 2010) as well as in cell cultures of *Zinnia elegans*, *Cycas revoluta* and *Taxus baccata* (Novo-Uzal et al., 2009). Linthorst et al. (1991) also showed that genes encoding β-1,3-glucanases, which were constitutively found in healthy tobacco plants, were strongly induced after tobacco mosaic virus infection or salicylate treatment. These results are agreeing with our results since the levels of β-1,3-glucanase (gi|37992763) increased in the presence of MJ and CD indicating that this protein is clearly involved in grapevine defense responses.

Finally, a xyloglucan endotransglycosylase of *Populus tremula* x *Populus tremuloides* (gi| 225462505) was found when *V. vinifera* cv Monastrell cell cultures were elicited with MJ or CD alone (Fig. 6B). The activity of xyloglucan endotransglycosylases are often well-correlated with cell elongation (Braidwood et al. 2014; Lee et al. 2018). In

fact, overexpression of xyloglucan endotransglycosylase from *Brassica campestris* in Arabidopsis, revealed that the transgenic plant had enlarged organ and elongated stem length (Shin et al., 2006). In addition, these proteins have also been associated with defense responses since Albert et al. (2004) demonstrated that the accumulation and activity of xyloglucan endotransglycosylases were correlated with the tomato-parasite interaction. In line with these findings, an increase in xyloglucan endotransglycosylase protein was registered at 96 h of treatment with CD or MJ, suggesting that this protein could be involved in the plant defense mechanism of grapevine plants against different types of elicitors.

4. Conclusion

In conclusion, the secretome of *V. vinifera* cv. Monastrell cell cultures contain constitutive defense related-proteins, however the elicitation with CD reinforces the defensive arsenal, and remarkably induces the accumulation of peroxidases. Moreover, the elicitation with MJ decreased the levels of PR-1, LysM domain-containing GPI-anchored protein 1, cationic peroxidase, and glycerophosphoryl diester phosphodiesterase whereas increased the abundance of new gene products such as heparanase, β-1,3 glucanase and reticulin oxidase, which could be used as potential biomarkers.

5. Author Contribution Statement

SBN, RBM and MAP conceived and designed research. SBN conducted experiments. SBN and LA analyzed data. SBN and LA wrote the manuscript. All authors read and approved the manuscript.

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Figure captions

Fig 1. Classification of identified proteins based on A) cellular compartment, B) molecular functions and C) biological processes.

Fig 2. Effect of cyclodextrins (CD) and/or methyl jasmonate (MJ) on peroxidase differentially expressed in elicited grapevine cell culture (cv. Monastrell). A) Peroxidase V from *V. vinifera* (gi|225455195), B) cationic peroxidase 1 from *A. thaliana* (gi|225439625), and peroxidase 4 from *V. vinifera* (gi|225434381).

Fig 3. Effect of cyclodextrins (CD) and/or methyl jasmonate (MJ) on PR-1 differentially expressed in elicited grapevine cell culture (cv. Monastrell). A) PR-1 from *V. vinifera* (gi|163914225) B) PR-1 from *V. vinifera* (gi|225429117).

Fig 4. Effect of cyclodextrins (CD) and/or methyl jasmonate (MJ) on A) LysM domain-containing GPI-anchored protein 1 from *V. vinifera* (gi|225459538) and B) glycerophosphoryl diester phosphodiesterase (gi|147811111) differentially expressed in elicited grapevine cell culture (cv. Monastrell).

Fig 5. Effect of cyclodextrins (CD) and methyl jasmonate (MJ) on A) reticulin oxidase from *V. vinifera* (gi|147846526) and B) heparanase (gi|147862366) differentially expressed in elicited grapevine cell culture (cv. Monastrell).

Fig 6. Effect of cyclodextrins (CD) and methyl jasmonate (MJ) on A) β-1,3-glucanase from *V. riparia* (gi|37992763) and B) xyloglucan endotransglycosylase of *Populus tremula* x *Populus tremuloides* (gi| 225462505) differentially expressed in elicited grapevine cell culture (cv. Monastrell).

Table 1. Proteins expressed in the secretome of grapevine cell cultures at 96 h of treatment with a score higher than 60.

ACCESSION	PEPTIDE COUNT	SCORE	DESCRIPTION[1]
gi|225459180	18	1064.61	Cationic peroxidase [*Arabidopsis thaliana*]
gi|225439623	11	486.13	Cationic peroxidase [*Arabidopsis thaliana*]
gi|225453022	9	421.87	PR-4 [*Vitis vinifera*]
gi|147838866	8	382.11	Class III Peroxidase [*Vitis vinifera*]
gi|225430555	8	325.23	Aspartyl protease family protein At5g10770 [*Arabidopsis thaliana*]
gi|225434078	5	291.98	Class IV Chitinase [*Vitis vinifera*]
gi|3511147	4	280.44	PR-4 [*Vitis vinifera*]
gi|225441373	8	260.67	β-1,3-glucanase [*Vitis riparia*]
gi|147814943	3	206.84	α-amilase [*Vitis vinifera*]
gi|2306811	6	182.45	Class IV Chitinase [*Vitis vinifera*]
gi|6273385	3	178.63	Thaumatin 1SCULTL2 [*Vitis vinifera*]
gi|225434050	3	172.20	Class IV Chitinase [*Vitis vinifera*]
gi|225432965	5	167.36	Protease inhibitor [*Vitis vinifera*]
gi|1839046	3	156.66	Osmotin-type protein [*Vitis vinifera*]
gi|147860594	2	155.29	Class III peroxidase [*Vitis vinifera*]
gi|147823287	2	142.92	Trypsin inhibitor [*Vitis vinifera*]
gi|225432971	2	137.06	Protease inhibitor [*Vitis berlandieri* x *Vitis riparia*]
gi|2306813	6	133.60	Class IV Endochitinase [*Vitis vinifera*]
gi|147784683	2	132.07	Pathogenesis-related protein NtPRp27 [*Vitis vinifera*]
gi|225457600	3	131.48	β-1,3-glucanase [*Vitis vinifera*]
gi|225439627	3	127.74	Cationic peroxidase 1 [*Vitis vinifera*]
gi|225427492	2	123.57	Acyl-CoA-binding protein [*Vitis vinifera*]
gi|225428879	3	112.3	Cysteine proteinase RD21A [*Vitis vinifera*]
gi|225453525	2	111.77	Putative expansin-B2 [*Vitis vinifera*]
gi|225470066	3	106.52	2S Albumin storage proteins [*Vitis vinifera*]

gi|147805839	2	98.57	Class I Chitinase [*Vitis vinifera*]
gi|225441645	3	85.32	β-1,3-glucanase [*Vitis vinifera*]
gi|225451467	1	72.91	Peroxidase 55 [*Arabidopsis thaliana*]
gi|225447360	2	72.69	Cysteine-rich repeat secretory protein 38 [*Vitis vinifera*]
gi|225456525	2	70.15	Similar to MtN19-like protein [*Vitis vinifera*]
gi|225445051	1	64.87	Acid phosphatase 1-like [*Arabidopsis lyrata* subsp. lyrata]
gi|1549400	1	64.59	β-1,3-glucanase [*Vitis vinifera*]
gi|225448120	2	62.27	Serine-type endopeptidase inhibitor [*Vitis vinifera*]

[1]This description is that captured in B2GO[37]; the accesion numbers corresponds to Vitis species.

Figure 1.

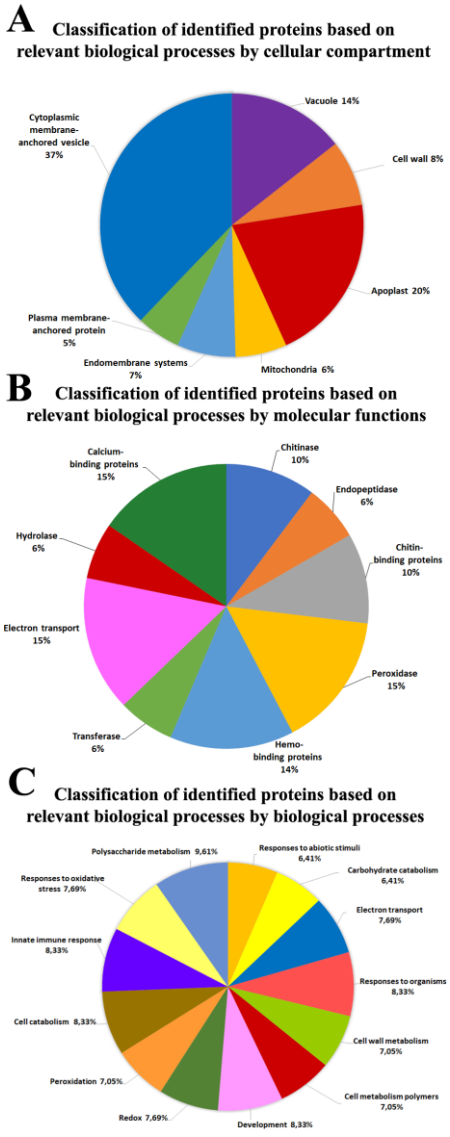

Figure 2.

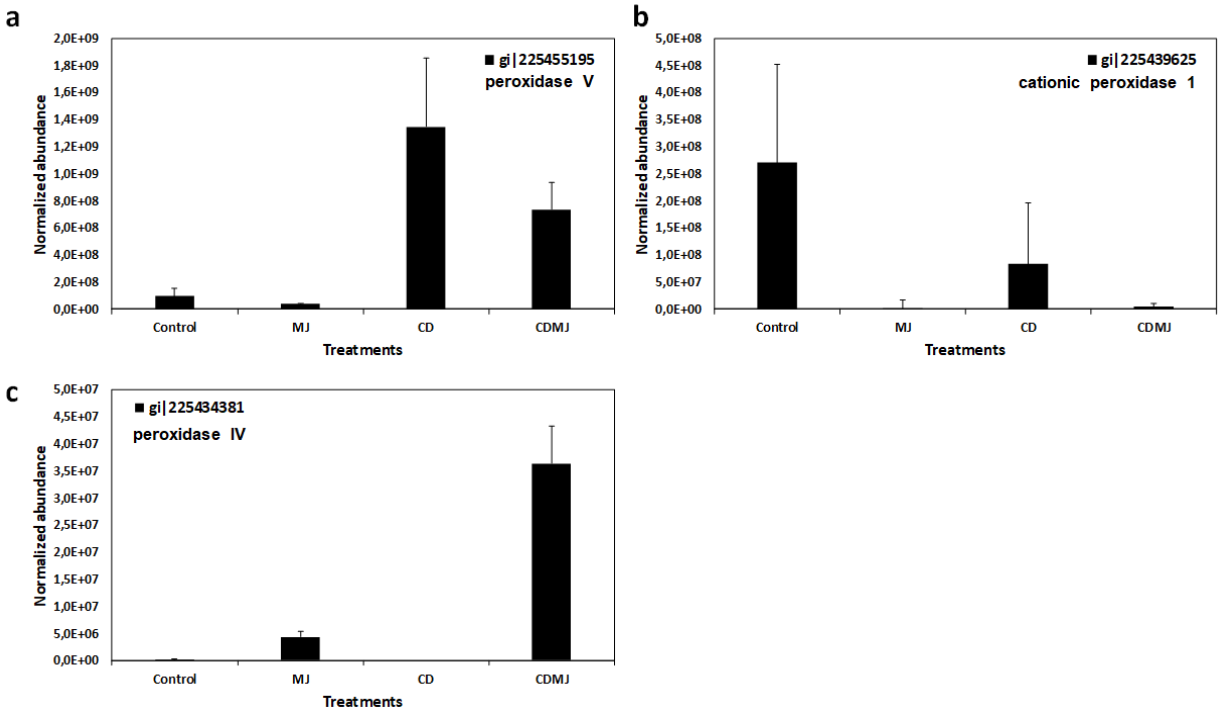

Figure 3.

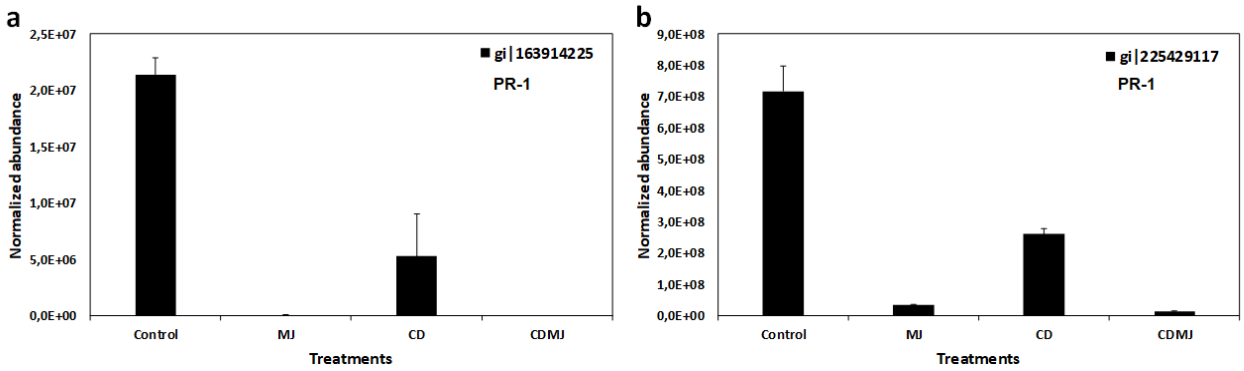

Figure 4.

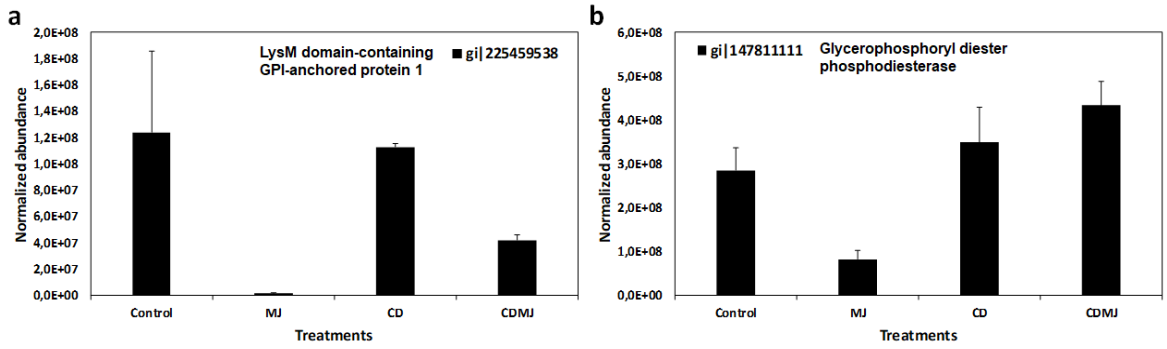

Figure 5.

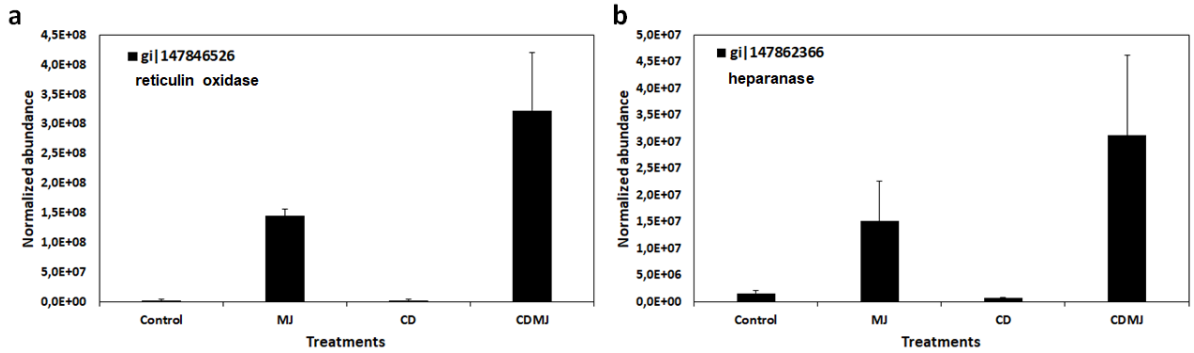

Figure 6.