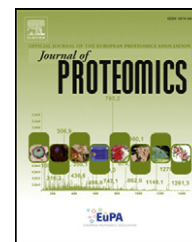


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Characterizing the proteome and oxi-proteome of apple in response to a host (*Penicillium expansum*) and a non-host (*Penicillium digitatum*) pathogen

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ABSTRACT

Apples are subjected to both abiotic and biotic stresses during the postharvest period, which lead to large economic losses worldwide. To obtain biochemical insights into apple defense response, we monitored the protein abundance changes (proteome), as well as the protein carbonyls (oxi-proteome) formed by reactive oxygen species (ROS) in 'Golden Smoothee' apple in response to wounding, *Penicillium expansum* (host) and *Penicillium digitatum* (non-host) pathogens with select transcriptional studies. To examine the biological relevance of the results, we described quantitative and oxidative protein changes into the gene ontology functional categories, as well as into the KEGG pathways.

We identified 26 proteins that differentially changed in abundance in response to wounding, *P. expansum* or *P. digitatum* infection. While these changes showed some similarities between the apple responses and abiotic and biotic stresses, Mal d 1.03A case, other proteins as Mal d 1.03E and EF-Tu were specifically induced in response to *P. digitatum* infection.

Using a protein carbonyl detection method based on fluorescent Bodipy, we detected and identified 27 oxidized proteins as sensitive ROS targets. These ROS target proteins were related to metabolism processes, suggesting that this process plays a leading role in apple fruit defense response against abiotic and biotic stresses. ACC oxidase and two glutamine synthetases showed the highest protein oxidation level in response to *P. digitatum* infection. Documenting changes in the proteome and, specifically in oxi-proteome of apple can provide information that can be used to better understand how impaired protein functions may affect apple defense mechanisms. Possible mechanisms by which these modified proteins are involved in fruit defense response are discussed.

Biological significance

Mechanical damage in apple fruits is linked annually to large economic losses due to opportunistic infection by postharvest pathogens, such as *P. expansum*. Despite the current

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use of chemical fungicides and the implementation of new alternative strategies, blue mold remains a critical disease of these stored fruits worldwide. Actual trends are focused on acquiring the knowledge of the host–pathogen interactions because it may help on finding new rational and environmentally friendly control alternatives. Despite the economic importance of some postharvest diseases, proteomics has only been applied in a few cases to study fruit–pathogen interactions. On the one hand, this is the first study that monitored changes at the proteome and oxi-proteome level in ‘Golden Smoothee’ apple fruits in response to *P. expansum* (compatible) and *P. digitatum* (non-host) pathogens. On the other hand, the main technological innovation of the reported research is the detection and quantification of oxidized (carbonylated) proteins to assess protein oxidative damage, avoiding the immunoblotting technique.

The importance of the biological process investigated lies in the different mechanisms induced in fruit in response to *P. expansum* and *P. digitatum*. Results revealed that fruit recognizes and reacts to *P. expansum* in a similar manner to wounding, while its response to *P. digitatum* exhibits few differences in the protein profile. Documenting changes in the proteome and, specifically in oxi-proteome of apple can provide information that can be used to better understand how impaired protein functions may affect apple defense mechanisms. It also provides new biomarkers for oxidative damage mainly caused by the oxidative response occurring in fruit tissue in response to a host and a non-host pathogen.

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1. Introduction

During the postharvest period, apples are subjected to both abiotic and biotic stresses. The mechanical injury makes the fruit more susceptible to opportunistic infection by postharvest wound pathogens, such as *Penicillium expansum*, the causal agent of blue mold [1]. Among the various postharvest pathogens of apple, *P. expansum* is the most devastating, being responsible for significant economic losses wherever apples are produced and stored. Whereas some postharvest pathogens of fruits, such as *Penicillium digitatum* are highly specialized, affecting only one or two types of fruit such as citrus fruits, *P. expansum*, has the ability to infect a broader range of fruit species, including apples, pears, strawberries and tomatoes. While *P. expansum* may be categorized as a host–pathogen of apple, *P. digitatum* may be considered a non-host pathogen because it has a very limited ability to cause infection in apples [2,3].

Despite the use of commercial fungicides and the implementation of new alternative strategies, blue mold remains a critical disease of pome and other stored fruits worldwide. Therefore, studies designed to characterize host–pathogen interactions are essential for understanding host resistance in apple, but also for the development of novel, safe and more effective control strategies.

Recently, a transcriptional approach of apple gene expression in response to a host (*P. expansum*) and a non-host (*P. digitatum*) pathogen was conducted to characterize disease resistance mechanisms [4]. Although transcriptomic analyses have provided insights into host resistance responses in apple, a number of defense-associated proteins, such as kinases, may not be responsive to defense-related signals at the transcriptional level. Thus the potential role of these proteins/genes cannot be fully characterized in transcriptomic studies. A complementary approach to transcriptome-based defense gene discovery is the use of proteomics to identify pathogen-induced proteins [5]. Proteomic studies also can provide insight into mechanisms that involve the post translational modifications (PTMs) of proteins.

While changes in the proteome of the vegetative portions of a plant in response to a broad range of abiotic stress factors, such as cold, heat, drought, salinity, flooding, ozone treatment and mechanical wounding, have been extensively described [6,7], the response of fruit to abiotic stresses is in need of further research. In this regard, changes in the apple fruit proteome in response to wounding have been recently published [8].

Despite the economic importance of some postharvest diseases, however, proteomics has only been applied in a few cases to study fruit–pathogen interactions. This includes studies in peach [9], sweet cherry [10], jujube [11] and tomatoes [12], designed to characterize the biochemical interactions between fruits and pathogens subjected to different environmental conditions [13].

One of the earliest and most rapid defense responses of fruit is the oxidative burst, which involves the production of reactive oxygen species (ROS) [14]. The association of ROS production with the induction of host defense responses has been demonstrated in many plant–pathogen interactions [15], where hydrogen peroxide (H_2O_2) produced in response to wounding and/or pathogen attack was shown to be integral to the induction of defense mechanisms in fruit [16–22]. The imbalance between production and destruction of ROS can affect cellular components and structures. ROS can oxidize various biomolecules including proteins and nucleic acids. Protein oxidation (carbonylation), a deleterious posttranslational modification (PTM), can alter protein structure leading to partial or total loss of their function [23,24]. The role of PTMs in the adaptation of plants to different abiotic and biotic stimuli is considered to play an important role in the operation and functioning of many host–pathogen interactions [25,26]. The level of protein oxidation has been used to assess protein oxidative damage in peach [27] and in sweet cherry fruit [28,29] using two dimensional gel electrophoresis coupled with immunoblotting. Different methods for the detection and quantification of oxidized proteins, including the use of fluorescent probes, such as BodipyFL-Hz have also been

developed. Using Bodipy, western blot and immunodetection are no longer needed, shortening the procedure and increasing accuracy being able to detect a lower level of oxidized proteins [30]. This method also provides the timely detection and excision of the protein spot after two-dimensional gel electrophoresis for further identification [23].

In the present study, a proteomic analysis was conducted to examine temporal changes in the total proteome and oxi-proteome of 'Golden Smoothee' apples in response to a host (*P. expansum*) and a non-host (*P. digitatum*) pathogen, and whether specific proteomic changes are correlated with related changes in transcription with the objective of developing a better understanding of disease resistance in apple fruit. Additionally, changes in the total proteome and oxi-proteome were integrated into functional networks in order to identify their potential biological significance in the host and in the non-host pathogen interaction.

2. Materials and methods

2.1. Fruit source

Apples (*Malus x domestica* L. cv 'Golden Smoothee') were harvested on September 7th, 2011 from a commercial orchard in Mollerussa (Catalonia, Spain) and used immediately after harvest. Fruits were selected for uniform size, absence of physical defects or apparent infections, washed thoroughly with tap water, and dried at room temperature. Prior to inoculation, apples were artificially wounded with a sterilized nail (3 mm wide and 3 mm deep). Wounds were inoculated with 10 μ l of sterile water (mock inoculation) or a conidial suspension (pathogens) and then incubated at 20 °C and 85–90% relative humidity (RH).

2.2. Fungal cultures

P. digitatum (strain PDM-1) and *P. expansum* Link (strain CMP-1) were isolated from decayed citrus and pome fruits, respectively. These isolates were the most aggressive in our fungal collection (Pathology Laboratory, IRTA, Lleida) in regard to their ability to infect citrus and pome fruits, respectively. *P. digitatum* and *P. expansum* were grown on Petri dishes containing Potato Dextrose Agar medium (PDA: 200 mL/L boiled potato extract; 20 g/L dextrose, 20 g/L agar, pH 5.5) in the dark at 25 °C for 7–10 days to achieve conidia production. Conidial suspensions were prepared by adding 10 mL of sterile distilled water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA, and by rubbing the surface of the agar with a sterile glass rod. The final conidia concentration was adjusted using a haemocytometer and diluted to different concentrations depending on each fungus.

2.3. Fruit inoculation and sample preparation

Apples wounded (mock inoculation) or wounded/inoculated with either a host (*P. expansum*) or a non-host (*P. digitatum*) pathogen were sampled at different time-points and used to identify changes in protein abundance and the level of protein oxidative damage (carbonylation). *P. expansum* or *P. digitatum*

were inoculated at 10^5 and 10^7 conidia mL⁻¹, respectively. Wounded tissue (mock inoculation) was removed at 24 h (W24), 48 h (W48) and 7 days (W7). Tissue from unwounded (UW) and wounded (W0) apples were removed at time 0 h. Wounded tissue, inoculated with *P. digitatum*, was removed at 24 h (PD24), 48 h (PD48) and 7 days (PD7). Likewise, wounded tissue, inoculated with *P. expansum* was removed at 24 h (PE24) and 48 h (PE48). Fifteen cylinders of peel and pulp tissue (8 mm diameter and 3 mm deep) encompassing the wounds from five individual fruits were removed using a cork borer. The experiment was repeated to obtain three biological replicates. All cylinders were pooled per time point, immediately frozen with liquid nitrogen and stored at –80 °C before processing. For the proteomic analysis, tissue was removed at 24 and 48 h. For the quantification of relative mRNA levels, tissue was removed at 24 h, 48 h, and 7 days. Samples used in the Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR) assay were lyophilized for 72 h prior to RNA extraction and stored at –80 °C until used.

2.4. Protein extraction and solubilization

Total protein was extracted from pooled samples according to the phenol-based protocol described by Buron-Moles et al. [8], but in the presence of a protease inhibitor cocktail (1 $\times$ Complete Tablet, Roche Applied Science, Mannheim, Germany) in the extraction buffer. The final protein precipitate was dried under vacuum for 3 min before being solubilized in 150 μ l of Tris-HCl 20 mM pH 7.5 and 2% SDS. Protein concentrations were determined with a 2D Quant Kit (GE Healthcare), using Bovine Serum Albumin (BSA) as a standard. Three technical repetitions were used for each treatment.

2.5. Protein oxidation analysis

Protein oxidation in a total protein extract was determined using an ascorbate/iron system. The inactivation of the enzyme was performed in opened 1.5 ml polypropylene tubes in a Thermomixer Comfort (Eppendorf) for 30 min at 20 °C and 1000 rpm. The reaction mixture (20 μ l) contained 9 pmol of pyruvate kinase, 50 μ M FeCl₃ and 10 mM ascorbate in 50 mM MES-KOH buffer pH 6.2. The fluorescent probe Bodipy-FL-hydrazide (Bodipy-Hz) was used to derivatize protein carbonyls, following Tamarit et al.'s method [30].

2.6. Two dimensional gel electrophoresis

Proteins were first separated by isoelectric focussing (IEF) using a Protean IEF Cell (Bio-Rad) and ReadyStrip™ IPG strip gels (18 cm) (BioRad) with a linear pH gradient 4–7, according to the manufacturer's instructions. For analytical gels, IPG strips were loaded with 120 μ g of total protein and passively rehydrated overnight in a final volume of 340 μ l of Protein Extraction Reagent type 4 [8].

2.7. Image acquisition and analysis

A total of twenty-one gels were analyzed. Gel images of carbonylated proteins were acquired in a VersaDoc MP4000

imager (BioRad), with an excitation wavelength of 468 nm and an emission filter of 530BP for Bodipy-Hz. After obtaining these images, gels were fixed for 2 h in a fixing solution containing 40% ethanol, 10% acetic acid, (v/v) and total protein staining was performed with Flamingo (BioRad). Images of total protein were acquired using the same imager system, setting the excitation wavelength to 468 nm and using an emission filter of 605BP for Flamingo.

Image analysis was performed using the PDQuest software (version 8.0, Bio-Rad) for spot quantification, normalization, and matching. Image analysis was first conducted separately on Flamingo stained gels. Spot detection and matching among gels were performed automatically, and then manually, in order to verify the accuracy of automated gel matching and to correct for any errors or missing spots that occurred in the automatic procedure, prior to final data analysis. The normalized spot volumes (individual spot intensity/normalization factor), calculated for each gel based on the total quantity of valid spots, were determined and used for statistical calculation of protein expression. Total proteome analysis of Flamingo gels was performed using spots that were reproducible and showed a quantitative volume variation by a factor of at least 2-fold increase/decrease, as well as being statistically significant (Student's *t* test, $P < 0.05$).

Oxi-proteome analysis of Bodipy gels was performed according to published method [30], with some modification. Briefly, Bodipy gel images were used for automatic spot detection and matching (analysis was also checked manually), to select the oxidized protein spots in each condition. Then, Flamingo images were added to the analysis and their spots matched to the selected spots in their corresponding Bodipy images. For each spot, Bodipy signal was normalized to that of the protein signal (Flamingo gels). Spots displaying a Bodipy-fluorescence/protein signal ratio greater than 1.5 were selected for further analysis, and considered a relative measure of the carbonyl content of each spot [31].

2.8. In-gel protein digestion and MS analysis

Protein spots were excised from gels and subjected to in-gel digestion with trypsin on a perforated microtiter plate (Proxeon Biosystems). Following digestion, as described by Buron-Moles et al. [8], protein identification was performed by peptide mass fingerprinting (PMF) or MS/MS mass spectrometry in an AutoflexSpeed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bruker Española, S.A., Madrid, Spain). Mass spectra (mode reflectron, MH+) were acquired by Flex Control version 3.0 software (Bruker Daltonics), recording in the range 800–4500 Da, and the MS/MS information was obtained in LIFT (laser-induced forward transfer) mode. MS spectra were externally calibrated using Peptide Calibration Standard II (Bruker Daltonics), post processed with FlexAnalysis version 3.3 software (Bruker Daltonics) and, when possible, internal calibration with peptides from the autodigestion of trypsin was also applied. BioTools version 3.1 protein analysis package (Bruker Daltonics) was used for protein database searching, including MS and MS/MS combined spectra. The peak lists obtained were compared against the Swiss-Prot, non-redundant and Plant EST databases of the NCBI, using the MASCOT version 2.3 software package (Matrix Sciences, UK;

www.matrixscience.com). The search parameters were set as monoisotopic peptide masses, carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modifications, respectively, one trypsin missed cleavage and a maximum of ± 100 ppm for PMF peptide tolerance and ± 0.4 Da for MS/MS tolerance, where the charge (*Z*) of the precursor ion was +1. MASCOT protein scores and peptide ion scores greater than 52 were considered statistically significant ($P < 0.05$).

2.9. Bioinformatic analysis

Identified protein sequences were functionally annotated using Blast2GO software v2.7.0 (<http://www.blast2go.org>, [32]).

To examine whether some specific gene ontology (GO) terms are over-represented in our experimental conditions, we used the Ontologizer software v2.0 [33]. In particular, the reference GO annotation set for all the *Malus x domestica* proteins was downloaded from the GDR website (<http://www.rosaceae.org/>). To determine the over-representation of GO terms, a Fisher's exact test was performed. The corresponding *P*-values were corrected for multiple-testing via the Benjamini–Hochberg approach. GO categories with an adjusted *P*-value less than 0.05 were considered significantly over-represented.

Likewise, a test for over-representation of particular metabolic pathways was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG — <http://www.genome.jp/kegg/>) [34]. The reference KEGG annotation set for all the *Malus x domestica* proteins was also downloaded from the GDR website. The hypergeometric test ($P < 0.05$) was used to determine the significantly over-representation of the KEGG pathways.

2.10. RNA extraction and RT-qPCR

In brief, 1 g of lyophilized apple tissue (pulp and peel) powder was added to a preheated mixture of 5 mL of extraction buffer (100 mM Tris-HCl, pH 8.0, 2 M NaCl, 25 mM EDTA, pH 8.0, 2% (w/v) cetyltrimethylammonium (CTAB), 2% (w/v), polyvinylpyrrolidone 40, 0.5 g L⁻¹ spermidine). 2% β -mercaptoethanol and 2% sarkosyl were added just before use. After homogenization with a Polytron PT 10/55 (Kinematica AG, Lucerne, Switzerland) for 30 s, the extract was incubated at 70 °C for 15 min and 3.75 mL of chloroform was added. The homogenate was centrifuged at 10,000 *g* for 15 min at 4 °C and the supernatant was transferred to different Eppendorf tube. A half volume of 95% ethanol was added and the homogenate was transferred onto a Qiagen RNeasy Mini spin column (Qiagen). RNA was purified according to the manufacturer's instructions and eluted in 100 μ L of RNase-free water. RNA concentration was quantified using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, DE, USA) and the RNA integrity was verified by agarose gel electrophoresis stained with ethidium bromide. Extracted RNA was treated and purified with turbo DNA-free kit (Ambion, TX, USA) according to the manufacturer's instructions and diluted to 6.25 ng μ L⁻¹. RT-qPCR analysis was performed using Power SYBR Green RNA-to-C_T™ 1-step kit (Applied Biosystems, CA, USA). Gene-specific primers, listed in Table 1, were used at an optimal concentration of 2.0 pmol of each primer per reaction. The ABI 7900 (Applied Biosystems, CA, USA) was set to cycle as

follows: cDNA synthesis at 48.0 °C for 30 min; 95.0 °C denaturation for 10 min; 40 cycles of 95.0 °C for 15 s followed by 57–58 °C (depending on primers used, Table 1) for 1 min; followed by ABI-specified hold and melt curve stages. Primers were verified for specificity by using apple genomic DNA template and assessing the resulting amplicon by agarose gel electrophoresis and qPCR with genomic DNA on the ABI 7900; all primers had a single band and single peak. Non-amplification of cDNA derived from *P. expansum* and *P. digitatum* DNA was also verified. Primer efficiency was also determined for all primer sets by qPCR analysis of a standard curve, constructed by serially diluting RNAs from the sample set starting at some concentration above what was used in unknown samples and ending at a concentration well below it. Three technical repetitions were used for each biological replicate for both the test and the reference gene. The standard curve method was used to calculate transcript abundance relative to EF1 α (AJ223969.1) which was used as reference gene (user bulletin no. 2; Applied Biosystems). The ANOVA test was used to assess significant differences in gene expression between the experimental conditions.

To investigate whether gene expression is correlated with protein expression, the relative quantitation in RT-qPCR for apple genes encoding ACO, APX, Mal d 1.03E, HSP, NAC and EF-Tu was determined.

3. Results

Approximately 350 protein spots were detected on each gel stained with Flamingo (Fig. 1A–D), whereas approximately 87 protein spots were detected on each gel stained with Bodipy-Hz (Fig. 1E–H).

Thirty-four out of the 350 spots detected in Flamingo gels showed significant changes in different samples, compared with their respective control. Eight spots, however, could not be identified by MALDI-TOF/TOF. The location of the 26 spots that exhibited significant changes in relative intensity among the treatments is shown in Fig. 2. The identified proteins are reported in Table 2. Detailed information on each of the peptide sequences and their homology to matching proteins can be found in Supplemental Table 2.

Twenty-seven out of the 87 spots detected in Bodipy gels displayed a significant increase in oxidation (carbonylation) among treatments (Fig. 3). All were successfully identified and are listed in Table 3. Detailed information on each of the peptide

sequences and their homology to matching proteins can be found in Supplemental Table 3. Proteins were categorized according to their biological process, encompassing mainly ‘metabolism’, ‘response to stress’ and ‘oxidation-reduction processes’ (Tables 2 and 3).

3.1. Identification of wound-responsive proteins (abiotic stress)

To analyze wound-responsive proteins in ‘Golden Smoothee’ apple, unwounded tissue served as a control in the time-course study. Representative 2-DE gels of total and oxidized protein extracts from peel (exocarp) and flesh (mesocarp) tissues of unwounded and wounded apple tissue at 24 h are shown in Fig. 1A–B and Fig. 1E–F, respectively. Eighteen spots displayed a significant difference in abundance after wounding. Sixteen of the 18 spots were successfully identified and grouped according to their biological process (Table 2, Supplemental Table 2). In general, most of the proteins whose abundance increased are involved in response to stress, whereas the proteins whose abundance decreased are involved in metabolism process. For instance, Mal d 1.06C (spot 2001), Mal d 1 (spot 4005) and Mal d 1.03A (spot 5005) proteins were identified. Levels of Mal d 1 protein increased in abundance 2.1 fold at W24, whereas Mal d 1.06C and Mal d 1.03A increased in abundance 3.0 and 4.5 fold, respectively at W48 (Supplemental Fig. 2A).

Ten protein spots exhibited a significant increase in carbonylation after wounding, all were successfully identified and mainly grouped in the category ‘metabolism processes’ (Table 3, Supplemental Table 3). Eight out of the ten proteins were already highly carbonylated at W0. Two of them were glycolytic enzymes: pyruvate dehydrogenase E1 component subunit beta-1 mitochondrial-like (spot 2201) and phosphoglucosyltransferase cytoplasmic (spot 5704); and two were chloroplastic: ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit partial (spot 6505) and phosphoglycerate kinase (spot 4305), involved in the Calvin cycle. Two isoforms of cytosolic malate dehydrogenase (spots 6205 and 7201) are involved in glyoxylate metabolism. Oxidation of these proteins indicates that apple defense response may be impaired due to decreased metabolism. Actin 7 (spot 1403) and Actin partial (spot 2404), carbonylated only at W0, are a cytoskeletal protein involved in cell vesicle formation and movement, cell motility and signaling. Actin partial showed the highest increase in oxidation (5.1-fold) at W0 (Supplemental Fig. 2B).

Table 1 – Primer sequences used to analyze expression of the *M. domestica* selected genes: ACC oxidase 1 (ACO), ascorbate peroxidase (APX), major allergen Mal d 1.03E (MalD1.03E), class I heat shock protein (HSP), translation elongation factor EF-Tu precursor, chloroplast (EF-Tu) and NAC domain class transcription factor (NAC). EF1 α used as reference gene.

Gene	Forward (5'–3')	Reverse (5'–3')
ACO	GTGTGCTTAGAGGGGAAAT	TTGTTTCCGACCCTCTT
APX	GACGAGGGTTGAAGTTCT	CCATCCCAAACCAAACCA
Mal d 1.03E	CCAGCCACTACCATACCA	CCAATTACAAGCCAAAACAC
HSP	GTGCCGAAAGAGGTGAAGA	AAACCCAAAAGTAGCCGA
EF-Tu	CAGAGAAGGAGGAAGAC	GGCACCAAATTGCAAAAAC
NAC	CCATATCAACTGCCTGGAA	ATCCTCTATCATCCCCCAA
EF1 α	GACATTGCCCTGTGGAAGTT	GGTCTGACCATCCTTGAAA

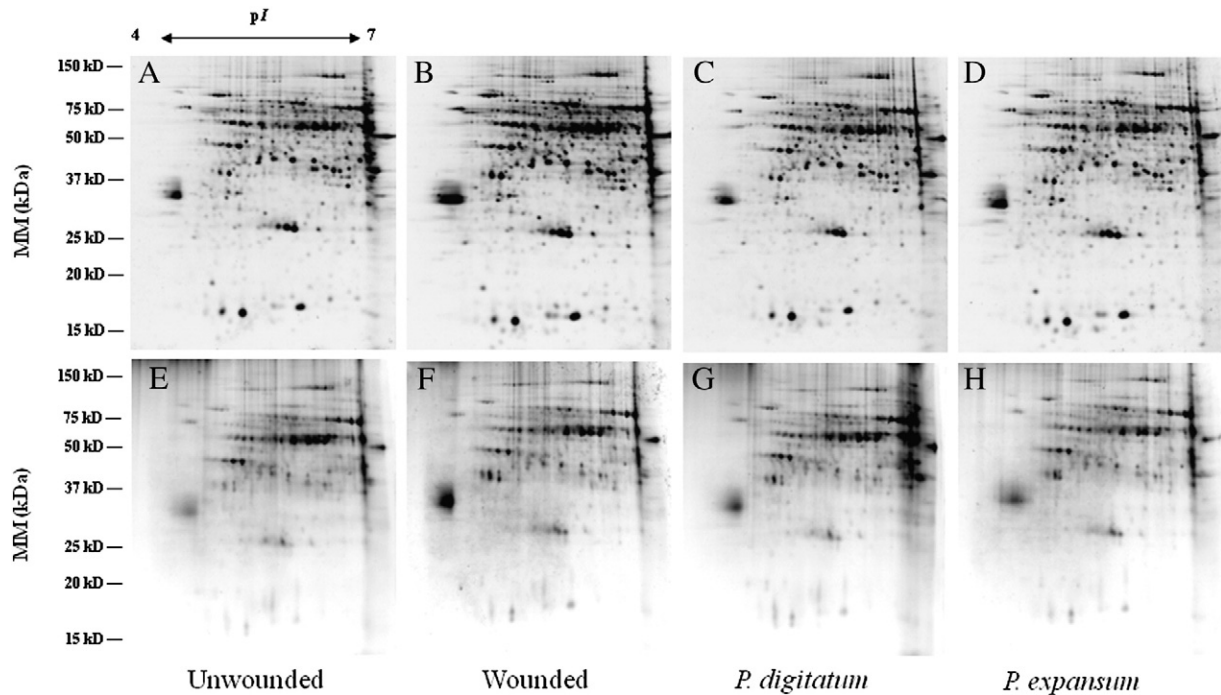


Fig. 1 – The images shown correspond to total protein stain by Flamingo (A–D) and Bodipy (E–H) in unwounded (A and E), wounded (B and F) and wounded/inoculated with *P. digitatum* (C and G) or *P. expansum* (D and H) samples of ‘Golden Smoothee’ apples at 24 h. Protein separation was realized on 18 cm strips pI 4–7. Images corresponding to 48 h are shown in Supplemental Fig. 1.

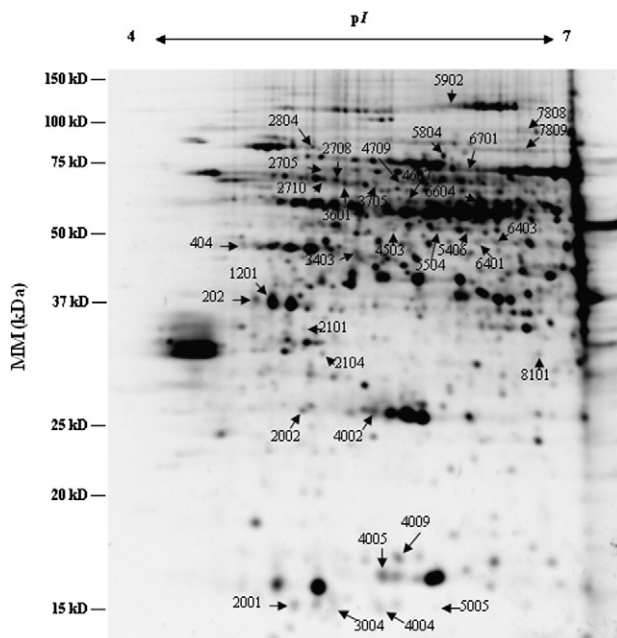


Fig. 2 – Representative 2-DE gel of total protein stain by Flamingo from ‘Golden Smoothee’ wounded tissue at 24 h. Proteins were separated over the pI range 4–7 in the first dimension and on 11% SDS-polyacrylamide gels in the second dimension. Spot numbering refers to Table 2 and Supplemental Table 2 shows protein identification obtained by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS) analysis.

3.2. Identification of pathogen (*P. expansum*) and non-host pathogen (*P. digitatum*) responsive proteins (biotic stress)

Temporal changes in apple protein expression in response to *P. expansum* (host pathogen) and *P. digitatum* (non-host pathogen) were analyzed, with wounded tissue serving as a control. In particular, special attention was placed on a comparison of temporal changes in protein expression that were specific to the non-host pathogen, *P. digitatum*, and those that were specific to the host pathogen, *P. expansum*.

3.2.1. *P. digitatum*-responsive proteins

Apple protein changes in response to *P. digitatum* inoculation at 24 h (PD24) and 48 h (PD48) were analyzed using wounded samples from the same time periods as a control.

Seven proteins displayed a significant difference in abundance during the time-course of the study and were successfully identified (Table 2, Supplemental Table 2). Results indicated that the category with considerable enrichment was response to stress.

Few differences in total proteins were observed at 24 h, whereas six of the seven identified proteins were markedly more abundant at 48 h. Mal d 1.03E (spot 3004), Mal d 1.03D (spot 4004) and Mal d 1.03A (spot 5005) proteins were identified. Whereas Mal d 1.03D (4.0-fold) and Mal d 1.03A (7.3-fold) exhibited increased abundance at 24 h, all three Mal d 1 proteins remained high (4.3, 3.2, 4.5-fold, respectively) at 48 h. Other proteins also displayed increased abundance in *P. digitatum*-inoculated tissue at 48 h: polyphenol

Table 2 – Proteins with significant abundance changes after wounding and pathogen inoculation. Proteins were excised from the 2D PAGE gel and digested with trypsin. The peptides were extracted and their masses were measured by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS). Spot number, protein description, accession number, organism, database, method of identification, MASCOT score and finally the theoretical molecular mass (MM) and isoelectric point (pI) are listed.

Spot ^a	Protein description ^b	Accession number ^c	Organisms	Database	Identification method	Mascot score	MM (kDa)	pI	Responsive to	Functional category ^d
202	ACC oxidase 1	O24063	<i>Malus domestica</i>	SWP	PMF	142	35.6	5.2	Wound	Metabolic process/Response to stress/Oxidation–reduction process
1201	ACC oxidase 1	O24063	<i>Malus domestica</i>	SWP	PMF	164	35.6	5.2	Wound	Metabolic process/Response to stress/Oxidation–reduction process
2001	Major allergen Mal d 1.06C	gij60280825	<i>Malus domestica</i>	nrNCBI	PMF	167	17.4	5.5	Wound	Response to stress
2101	Short-chain dehydrogenase/reductase 2b-like	EB123506	<i>Malus domestica</i>	Plant EST	MS/MS	194	20.0	8.9	PE	Metabolic process
2104	Thiazole biosynthetic enzyme	AT000365	<i>Malus domestica</i>	Plant EST	MS/MS	85	7.3	8.1	PE	Metabolic process/Response to stress
2708	Polyphenol oxidase 2 precursor	gij14194273	<i>Malus domestica</i>	nrNCBI	PMF	224	65.8	6.1	Wound/PD/Int.	Metabolic process/Oxidation–reduction process
2710	Mitochondrial HSO70 2 isoform 1	CN908186	<i>Malus domestica</i>	Plant EST	MS/MS	71	12.4	4.5	Wound	Response to stress
2804	High molecular weight heat shock protein	gij6969976	<i>Malus domestica</i>	nrNCBI	PMF	242	71.6	5.2	Wound	Response to stress/Oxidation–reduction process
3004	Major allergen Mal d 1.03E	gij60280855	<i>Malus domestica</i>	nrNCBI	PMF	234	17.6	5.4	PD	Response to stress
3403	Probable sarcosine oxidase-like	CN849922	<i>Malus domestica</i>	Plant EST	MS/MS	72	19.6	5.8	PE	Metabolic process/Oxidation–reduction process
3601	Polyphenol oxidase 2 precursor	gij14194273	<i>Malus domestica</i>	nrNCBI	PMF	222	65.8	6.1	Wound/Int.	Metabolic process/Oxidation–reduction process
3705	Polyphenol oxidase 2 precursor	gij14194273	<i>Malus domestica</i>	nrNCBI	PMF	258	65.8	6.1	Wound	Metabolic process/Oxidation–reduction process
4004	Major allergen Mal d 1.03D	gij60280849	<i>Malus domestica</i>	nrNCBI	PMF	143	17.6	5.5	PD/PE	Response to stress
4005	Major allergen mal d 1	gij4590378	<i>Malus domestica</i>	nrNCBI	PMF	174	17.7	5.7	Wound	Response to stress
4009	Class I heat shock protein	CO723224	<i>Malus domestica</i>	Plant EST	PMF	143	18.6	6.1	Wound	Response to stress/Oxidation–reduction process
4503	4-Hydroxyphenylpyruvate dioxygenase-like	EB136504	<i>Malus domestica</i>	Plant EST	PMF	118	16.4	9.1	PD/PE	Metabolic process/Oxidation–reduction process
4607	Enolase	EB131141	<i>Malus domestica</i>	Plant EST	MS/MS	162	21.5	7.7	Wound	Metabolic process
4709	Apkm	DY256418	<i>Malus domestica</i>	Plant EST	MS/MS	167	18.5	5.9	Wound	Metabolic process/Response to stress
5005	Major allergen Mal d 1.03A	gij60280841	<i>Malus domestica</i>	nrNCBI	PMF	152	17.6	5.7	Wound/PD/PE	Response to stress
5504	Aminoacylase, putative	DT001087	<i>Malus domestica</i>	Plant EST	MS/MS	101	24.0	9.0	Wound	Metabolic process
5804	Heat shock protein STI-like	EB135855	<i>Malus domestica</i>	Plant EST	MS/MS	251	21.9	4.8	PE	Metabolic process/Response to stress
5902	Cytosolic aconitase	CN900040	<i>Malus domestica</i>	Plant EST	PMF	111	21.5	6.5	Wound	Metabolic process
6401	Translation elongation factor EF-Tu precursor, chloroplast	DT000463	<i>Malus domestica</i>	Plant EST	MS/MS	71	20.6	9.8	PD/Int.	Metabolic process
6604	ATP1 (mitochondrion)	gij404481694	<i>Malus domestica</i>	nrNCBI	PMF	188	55.6	6.2	Wound	Metabolic process
6701	NADP-dependent malic enzyme-like	EB136452	<i>Malus domestica</i>	Plant EST	PMF	121	23.8	8.9	Wound	Metabolic process/Oxidation–reduction process
8101	Pyridoxine biosynthesis protein-like	GO510968	<i>Malus domestica</i>	Plant EST	PMF	187	23.5	6.0	PD	Metabolic process/Response to stress

^a Spot identification number as assigned in Fig. 2.

^b Description of proteins identified from the Plant EST, nrNCBI or SwissProt database.

^c Accession numbers according to Viridiplantae SwissProt, nrNCBI or Plant EST Database of NCBI.

^d Functional categories were inferred using the Blast2GO software.

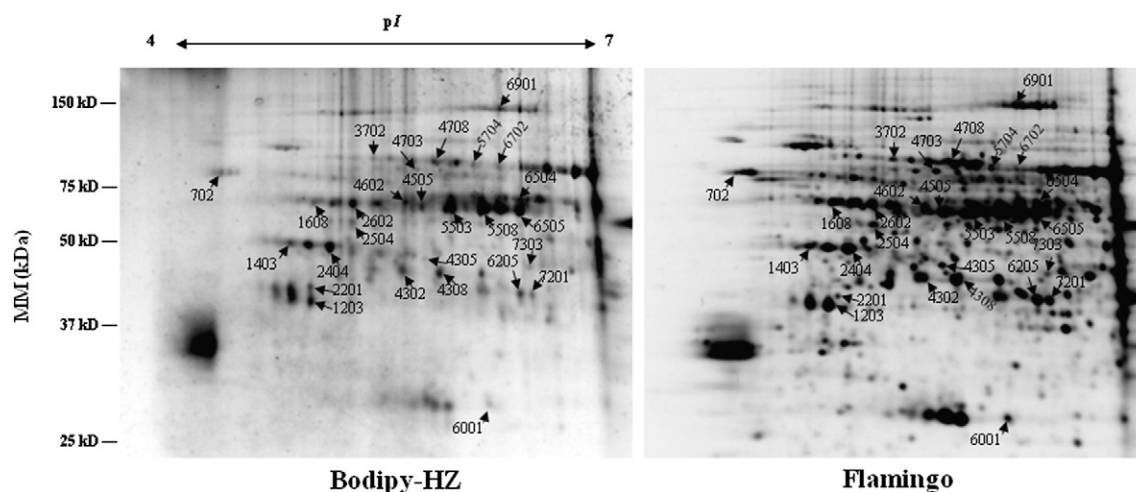


Fig. 3 – Representative 2-DE gel of oxidized protein stain by Bodipy and total protein stain by Flamingo of the ‘Golden Smoothee’ wounded tissue at 24 h. Proteins were separated over the pI range 4–7 in the first dimension and on 11% SDS-polyacrylamide gels in the second dimension. Spot numbering refers to Table 3 and Supplemental Table 3 shows protein identification obtained by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS) analysis.

oxidase 2 precursor (spot 2708), 4-hydroxyphenylpyruvate dioxygenase-like (spot 4503), and a translation elongation factor EF-Tu precursor (spot 6401) (Fig. 4A). No proteins were observed that had a significant decrease in abundance in response to the non-host pathogen.

Eight protein spots had a significant increase in carbonylation in response to *P. digitatum* inoculation during the time-course of the study and all were successfully identified (Table 3, Supplemental Table 3). Most of the carbonylated proteins were categorized as being involved in ‘metabolism processes’. Four of the eight modified proteins exhibited increased carbonylation at 24 h. These four spots were identified as ACC oxidase 1 (spot 1203), regulatory particle triple-A ATPase 3 isoform 2, partial (spot 2504), polyphenol oxidase IIa (spot 4708) and cytosolic malate dehydrogenase (spot 7201). Two of the proteins, ACC oxidase 1 (16.8-fold) and polyphenol oxidase IIa (1.7 fold) are involved in the biosynthesis of secondary metabolites, whereas a cytosolic protein, malate dehydrogenase (1.7-fold), is involved in the tricarboxylic acid cycle. Regulatory particle triple-A ATPase 3 isoform 2, partial or 26S protease regulatory subunit 6B homolog (1.6-fold) is involved in the ATP-dependent degradation of ubiquitinated proteins. Lastly, a Vacuolar H⁺-ATPase (spot 3702), that exhibited increased carbonylation at 48 h (1.7-fold), is implicated in the transport of protons against an electrochemical gradient using energy from ATP hydrolysis (Fig. 4B).

3.2.2. *P. expansum*-responsive proteins

Temporal changes in the abundance of proteins in response to *P. expansum* inoculation at 24 (PE24) and 48 h (PE48) were analyzed using wounded samples from the same time periods as a control.

Eleven proteins displayed significant changes in abundance during the time-course of the study. Seven out of the eleven differentially expressed proteins were successfully identified (Table 2, Supplemental Table 2). Proteins that increased in abundance at 48 h are involved in ‘response to stress’, whereas

proteins that decreased in abundance are involved in ‘metabolism process’.

Mal d 1.03D (spot 4004) and Mal d 1.03A (spot 5005) proteins were among the identified proteins. High levels of these two Mal d 1 proteins were observed during the entire time-course analyzed. At 24 h, the abundance of Mal d 1.03D and Mal d 1.03A increased 3.1-fold and 3.8-fold, respectively. Similarly, at 48 h, the abundance of Mal d 1.03D and Mal d 1.03A were 2.7-fold and 3.7-fold, respectively. Other proteins also exhibited increased abundance in *P. expansum*-inoculated tissue at 48 h. These proteins included: a short-chain dehydrogenase/reductase 2b-like (spot 2101), a thiazole biosynthetic enzyme (spot 2104), and a 4-hydroxyphenylpyruvate dioxygenase-like protein (spot 4503). Proteins with significantly reduced abundance were also found in this host-pathogen interaction at 48 h, where probable sarcosine oxidase-like (spot 3403) and heat shock protein STI-like (spot 5804) decreased 0.5-fold and 0.4-fold, respectively (Fig. 4C).

In regard to the oxi-proteome, eight protein spots displayed a significant increase in carbonylation at 24 h in response to *P. expansum* inoculation. These proteins were successfully identified and mainly grouped in ‘metabolism processes’ (Table 3, Supplemental Table 3). The eight proteins that were highly carbonylated at 24 h were an actin 7 (spot 1403), an ATP synthase subunit beta, mitochondrial-like (spot 1608), an actin, partial (spot 2404), an apple phosphoglyceromutase (apgm) (spot 4703), a triosephosphate isomerase, cytosolic-like (spot 6001), a ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) (spot 6504), a bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic-like (spot 6702), and a cytosolic aconitase (spot 6901). The actin 7 (2.3-fold), RUBISCO (2.2-fold), and the bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic-like (2.1-fold) were the proteins with the highest level of carbonylation in response to *P. expansum* at 24 h after inoculation (Fig. 4D).

Table 3 – Proteins oxidatively modified after wounding and pathogen inoculation. Proteins were excised from the 2D PAGE gel and digested with trypsin. The peptides were extracted and their masses were measured by MALDI-TOF fingerprint (PMF) or MALDI-TOF/TOF (MS/MS). Spot number, protein description, accession number, organism, database, method of identification, MASCOT score and finally the theoretical molecular mass (MM) and isoelectric point (pI) are listed.

Spot ^a	Protein description ^b	Accession number ^c	Organisms	Database	Identification method	Mascot score	MM (kDa)	pI	Responsive to	Functional category ^d
702	Putative cytosolic NADP-malic enzyme	EB117833	<i>Malus domestica</i>	Plant EST	MS/MS	146	19.6	6.4	Wound	Metabolic process/Oxidation–reduction process
1203	ACC oxidase 1	O24063	<i>Malus domestica</i>	SWP	PMF	189	35.6	5.2	PD/Int.	Metabolic process/Response to stress/Oxidation–reduction process
1403	Actin 7	CN896143	<i>Malus domestica</i>	Plant EST	PMF	140	57.5	5.8	Wound/PE	Metabolic process/Response to stress
1608	ATP synthase subunit beta, mitochondrial-like	CN898384	<i>Malus domestica</i>	Plant EST	PMF	218	22.1	6.2	PE/Int.	Metabolic process/Response to stress
2201	Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial-like	ES790079	<i>Malus domestica</i>	Plant EST	PMF	202	62.3	9.0	Wound	Metabolic process/Oxidation–reduction process
2404	Actin, partial	gi 355329944	<i>Malus domestica</i>	nrNCBI	PMF	202	40.4	5.7	Wound/PE	Metabolic process/Response to stress
2504	Regulatory particle triple-A ATPase 3 isoform 2, partial	CN888682	<i>Malus domestica</i>	Plant EST	PMF	223	24.4	6.5	PD	Metabolic process/Response to stress
2602	ATP synthase beta chain, mitochondrial precursor	DR991435	<i>Malus domestica</i>	Plant EST	MS/MS	279	17.8	4.7	Int.	Metabolic process
3702	Vacuolar H ⁺ -ATPase	gi 131573315	<i>Malus domestica</i>	nrNCBI	PMF	408	69.2	5.4	PD	Metabolic process
4302	Glutamine synthetase cytosolic isozyme 1	DT003241	<i>Malus domestica</i>	Plant EST	MS/MS	120	27.7	9.5	Int.	Metabolic process
4305	Phosphoglycerate kinase, chloroplastic-like	CN893843	<i>Malus domestica</i>	Plant EST	PMF	149	21.1	8.8	Wound	Metabolic process/Response to stress
4308	Glutamine synthetase nodule isozyme-like	CN894859	<i>Malus domestica</i>	Plant EST	PMF	113	16.7	5.8	Int.	Metabolic process
4505	Enolase	GO520405	<i>Malus domestica</i>	Plant EST	PMF	94	22.1	8.9	Int.	Metabolic process/Response to stress
4602	Enolase	CV081922	<i>Malus domestica</i>	Plant EST	PMF	107	18.7	7.8	PD	Metabolic process
4703	Apgm	DY256418	<i>Malus domestica</i>	Plant EST	MS/MS	134	18.5	5.9	PE	Metabolic process/Response to stress
4708	Polyphenol oxidase IIa	gi 384563679	<i>Malus domestica</i>	nrNCBI	PMF	217	67.8	6.2	PD/Int.	Metabolic process/Oxidation–reduction process
5503	Enolase	GO520405	<i>Malus domestica</i>	Plant EST	PMF	106	22.1	8.9	PD/Int.	Metabolic process/Response to stress
5508	Enolase-like	EB151863	<i>Malus domestica</i>	Plant EST	PMF	154	22.8	5.3	Int.	Metabolic process
5704	Phosphoglucomutase, cytoplasmic	DT041983	<i>M. domestica</i> x <i>M. sieversii</i>	Plant EST	MS/MS	121	26.8	8.8	Wound	Metabolic process
6001	Triosephosphate isomerase, cytosolic-like	CN908475	<i>Malus domestica</i>	Plant EST	PMF	147	24.0	6.4	PD/PE	Metabolic process/Response to stress
6205	Cytosolic malate dehydrogenase	gi 78216493	<i>Malus domestica</i>	nrNCBI	PMF	256	36.0	6.0	Wound	Metabolic process
6504	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	CN872362	<i>Malus domestica</i>	Plant EST	PMF	141	22.5	8.8	PE	Metabolic process
6505	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	CN872362	<i>Malus domestica</i>	Plant EST	PMF	223	22.5	8.8	Wound	Metabolic process
6702	Bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic-like	gi 462403009	<i>Prunus persica</i>	nrNCBI	MS/MS	46	54.5	6.2	PE	Metabolic process/Oxidation–reduction process
6901	Cytosolic aconitase	EB122770	<i>Malus domestica</i>	Plant EST	MS/MS	131	17.9	8.9	PE	Metabolic process
7201	Cytosolic malate dehydrogenase	gi 78216493	<i>Malus domestica</i>	nrNCBI	PMF	187	36.0	6.0	Wound/PD/Int.	Metabolic process
7303	Phosphoglycerate kinase, cytosolic-like	CN938321	<i>Malus domestica</i>	Plant EST	PMF	200	22.1	9.2	Wound	Metabolic process

^a Spot identification number as assigned in Fig. 3.

^b Description of proteins identified from the Plant EST, nrNCBI or SwissProt database.

^c Accession numbers according to Viridiplantae SwissProt, nrNCBI or Plant EST Database of NCBI.

^d Functional categories were inferred using the Blast2GO software.

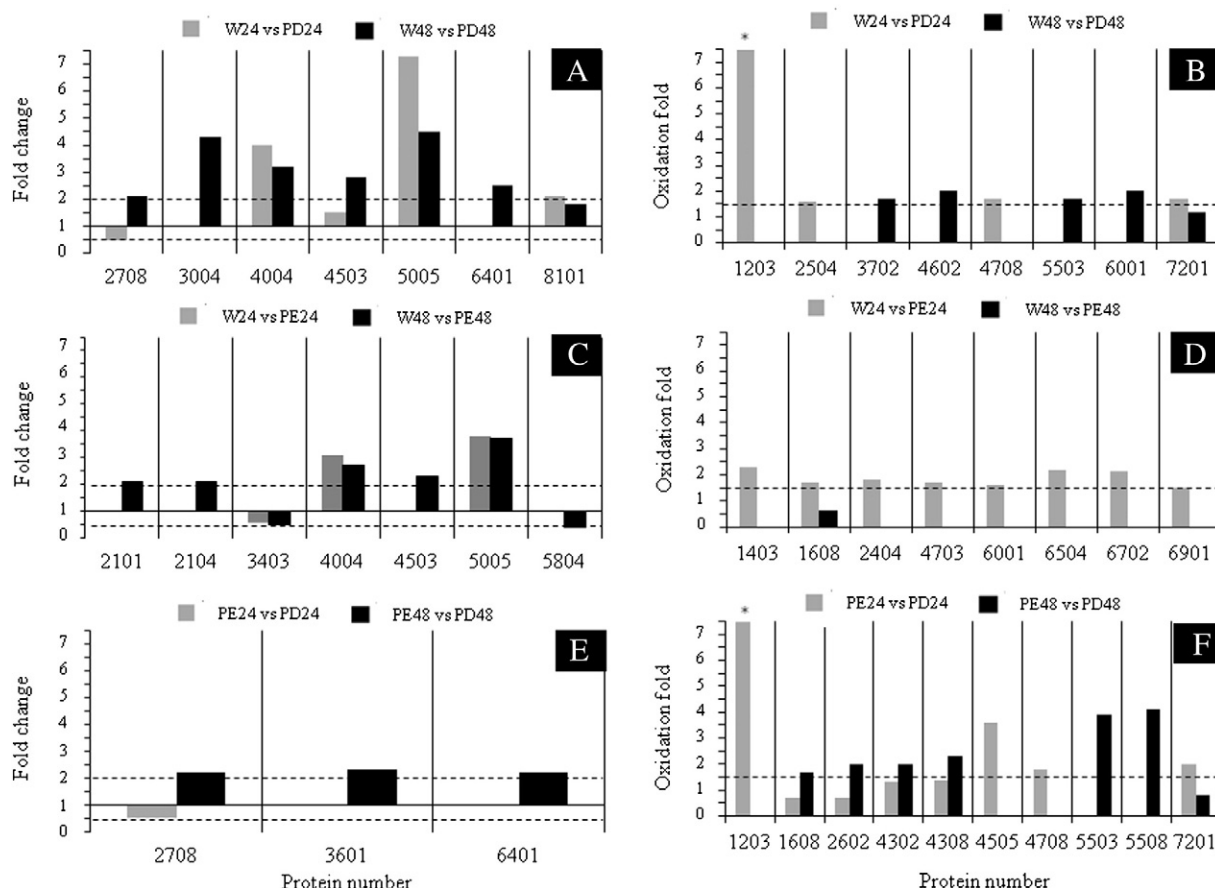


Fig. 4 – Temporal changes in the proteome and oxi-proteome of ‘Golden Smoothee’ apples in response to a compatible (*P. expansum*) and a non-host (*P. digitatum*) pathogen inoculation. In A, C and E, columns represent the fold changes (2-fold) of protein abundance between inoculated and control conditions. Values ≥ 2 or ≤ 0.5 (— threshold) are considered an increase or a decrease in protein abundance. In B, D and F, columns represent the oxidation fold (1.5-fold) ratio [Bodipy signal/protein signal] of each spot between inoculated and control conditions. Values ≥ 1.5 (— threshold) are considered oxidized. *: Protein with an oxidation fold greater than 7. Data are the mean of three independent gels. W = wounded control (mock inoculated), PD = *P. digitatum*, PE = *P. expansum*. Column absence indicates a spot absence at the specific condition.

3.2.3. Differences between *P. expansum* and *P. digitatum*-responsive proteins

Changes in protein abundance of apple fruit in response to *P. digitatum*- and *P. expansum*-inoculation at 24 and 48 h were analyzed using *P. expansum*-inoculated tissue as the control at time point.

Among total proteins, seven exhibited significant differences in abundance during the time-course of the study. Three of the seven proteins were successfully identified and mainly belonged to metabolism and oxidation–reduction processes (Table 2, Supplemental Table 2). The three proteins that increased in abundance at 48 h were polyphenol oxidase 2 precursor (spot 2708 and 3601) and a translation elongation factor EF-Tu precursor (6401). The two spots identified as the same protein most likely indicate the presence of multiple isoforms. The abundance of these three proteins at 48 h increased by 2.2, 2.3 and 2.2-fold, respectively (Fig. 4E).

Ten protein spots displayed a significant increase in carbonylation in response to *P. digitatum* compared with

P. expansum inoculation during the time-course of this study. These proteins were successfully identified and associated with the category ‘metabolism processes’ (Table 3, Supplemental Table 3). At 24 h, four proteins exhibited an increased level of carbonylation and were identified as ACC oxidase 1 (spot 1203), enolase (spot 4505), polyphenol oxidase IIa (spot 4708), and cytosolic malate dehydrogenase (spot 7201). ACC oxidase 1 (28.2-fold) and polyphenol oxidase IIa (1.8 fold) are involved in the biosynthesis of secondary metabolites. Enolase (3.6-fold) is involved in glycolysis, and malate dehydrogenase (2.0-fold) is involved in the tricarboxylic acid cycle. Both of the later proteins are cytosolic proteins. The six carbonylated proteins identified at 48 h were ATP synthase subunit beta, mitochondrial-like (spot 1608), ATP synthase beta chain, mitochondrial precursor (spot 2602), glutamine synthetase cytosolic isozyme 1 (spot 4302), glutamine synthetase nodule isozyme-like (spot 4308), enolase (spot 5503) and enolase-like (spot 5508). The enolase (3.9-fold), enolase-like (4.1-fold) and glutamine synthetase nodule isozyme-like (2.3-fold) proteins exhibited the highest level of carbonylation in response to

P. digitatum inoculation compared with *P. expansum* inoculation at 48 h after inoculation (Fig. 4F).

3.3. GO functional analysis, enrichment, and integration of proteomic data into biological interaction networks

Sequenced and identified proteins with quantitative changes in abundance and levels of oxidation were classified into functional categories of the gene ontology (GO). Concerning the biological process, the categories with considerable enrichment of the proteins whose abundance and levels of oxidation change after wounding and inoculation were related to 'response to stress' and 'metabolism processes'.

GO terms that were significantly over-represented in wounded and inoculated apples in relation to their respective controls were evaluated by Ontologizer. For wound-responsive proteins the GO categories that were over-represented were 'response to biotic stimulus' (GO:0009607), 'aminoacylase activity' (GO:0004046), 'phosphoglycerate mutase activity' (GO:0004619) and 'glucose catabolic process' (GO:0006007). For *P. digitatum* responsive proteins the over-represented categories were 'response to biotic stimulus' (GO:0009607), 'defense response' (GO:0006952) and '4-hydroxyphenylpyruvate dioxygenase activity' (GO:0003868). Whereas, for *P. expansum* responsive proteins were 'response to biotic stimulus' (GO:0009607), 'sarcosine oxidase activity' (GO:0008115), 'tetrahydrofolate metabolic process' (GO:0046653), '4-hydroxyphenylpyruvate dioxygenase activity' (GO:0003868) and 'thiamine biosynthetic process' (GO:0009228). Results of the analysis did not indicate any significant over-representation of any GO categories between *P. expansum* and *P. digitatum* responsive proteins. The same statistical analysis was applied to the GO terms over-represented in changes in the apple oxi-proteome. For wound-responsive proteins the GO categories over-represented included 'phosphoglycerate kinase activity' (GO:0004618), 'malate metabolic process' (GO:0006108), 'glycolysis' (GO:0006096), 'ribulose-bisphosphate carboxylase activity' (GO:0016984), 'malate dehydrogenase (decarboxylating) (NAD⁺) activity' (GO:0004471), 'L-malate dehydrogenase activity' (GO:0030060), 'carbon fixation' (GO:0015977), and 'intramolecular transferase activity, phosphotransferases' (GO:0016868). Results of the analysis did not reveal a significant over-representation of any GO categories in response to *P. digitatum* inoculation, whereas for *P. expansum* responsive proteins the over-represented categories were 'ribulose-bisphosphate carboxylase activity' (GO:0016984), 'hydrogen-exporting ATPase activity, phosphorylative mechanism' (GO:0008553), 'phosphoglycerate mutase activity' (GO:0004619), 'mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)' (GO:0000275) and 'shikimate 3-dehydrogenase (NADP⁺) activity' (GO:0004764). GO categories over-represented between *P. expansum* and *P. digitatum* responsive proteins were 'glutamate-ammonia ligase activity' (GO:0004356), 'glutamine biosynthetic process' (GO:0006542), 'hydrogen-exporting ATPase activity, phosphorylative mechanism' (GO:0008553) and 'mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)' (GO:0000275).

The KEGG metabolic pathways significantly over-represented for proteins whose abundance change in response to the experimental treatments were 'RNA degradation' (ko03018),

'methane metabolism' (ko00680), 'vitamin B6 metabolism' (ko00750) and 'glycolysis/gluconeogenesis' (ko00010). The same statistical analysis was used to identify KEGG metabolic pathways over-represented for proteins whose oxidation level change and were 'carbon fixation in photosynthetic organisms' (ko00710), 'glycolysis / gluconeogenesis' (ko00010), 'glyoxylate and dicarboxylate metabolism' (ko00630), 'citrate cycle (TCA cycle)' (ko00020), 'pyruvate metabolism' (ko00620) and 'proximal tubule bicarbonate reclamation' (ko04964).

3.4. Correlation between gene transcription and protein expression

RT-qPCR was performed to examine the transcript levels of a select number of proteins identified in the proteomic studies (Tables 2 and 3). The transcriptional levels of six *M. domestica* genes—corresponding to the identified *M. domestica* proteins ACC oxidase 1 (ACO), ascorbate peroxidase (APX), major allergen Mal d 1.03E (MalD1.03E), class I heat shock protein (HSP), translation elongation factor EF-Tu precursor, chloroplast (EF-Tu) and NAC domain class transcription factor (NAC) were assessed at 0, 24, 48 h and 7 d in wounded, wounded/inoculated and unwounded tissue (Fig. 5). The correlation between mRNA and protein expression was also examined for proteins, such as APX and NAC, whose abundance or oxidation level did not change in response to the experimental treatments. In general, changes in mRNA abundance at the three time points were not well correlated with changes in the abundance of their corresponding proteins, except for MalD1.03E, where the calculated Pearson correlation value was 0.93 (P-value < 0.001).

4. Discussion

In apples, mechanical damage provides an optimal infection site for wound pathogens such as *Penicillium* spp., which require damaged tissue to germinate and colonize the tissue. Therefore, a greater understanding of how apple fruit responds to fungal pathogens is essential for developing and implementing effective strategies for postharvest disease control that do not lie on synthetic chemical fungicides.

The current study provided evidence on the induction of several defense-related proteins, such as pathogenesis-related (PR) proteins in response to abiotic and biotic stresses. The major allergen Mal d 1 belongs to the PR-10 class of PR proteins, and its induction has been described under a range of abiotic and biotic stresses [35]. The three Mal d 1 proteins that increased in abundance in response to wounding, predominantly at 48 h, were Mal d 1 (MDP0000103627), Mal d 1.03A (MDP0000295540) and Mal d 1.06C (MDP0000216907). The wound-induced Mal d 1.03A (4.5-fold), was also induced in response to the host (3.7-fold) and non-host pathogen (4.5-fold) at 48 h. However, the highest level of Mal d 1.03A was exhibited in response to *P. digitatum* at 24 h (7.3-fold). Overall it suggests that there are some similarities between the apple defense responses to biotic and abiotic stresses. For instance, Mal d 1.03D (MDP0000827820) increased in abundance specifically in response to both pathogens. This result indicated that some Mal d 1 proteins are commonly induced by *P. expansum* and *P. digitatum* suggesting

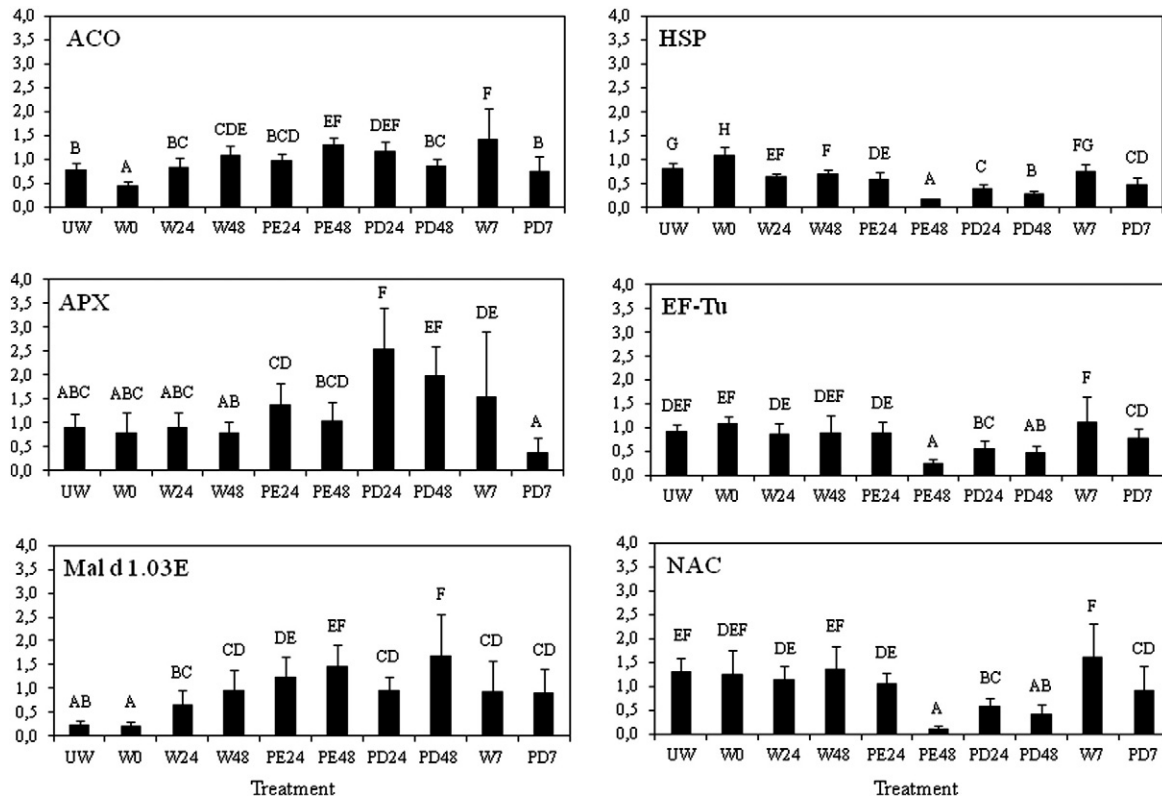


Fig. 5 – RT-qPCR analysis of genes encoding proteins in spots: 1203 (ACO), 3009 (APX), 3004 (Mal d 1.03E), 4009 (HSP), 6401 (EF-Tu) and 7305 (NAC), in unwounded (UW), wounded (W) and pathogen (PE for *P. expansum* and PD for *P. digitatum*) inoculated samples. Gene expression data were normalized against expression of EF. Values were analyzed by Student's t-test. Different letters indicates statistically significant difference ($P < 0.05$).

that this protein may be associated with an early resistance response. Interestingly, Mal d 1.03E (MDP0000295543) was specifically induced in response to *P. digitatum* at 48 h after inoculation. This indicates that Mal d 1.03E may be specifically associated with apple response to a non-host pathogen and could be associated to disease resistance to *P. digitatum*. None of the Mal d 1 proteins exhibited oxidative modifications in response to the experimental treatments. The expression of the Mal d 1.03E gene characterized by RT-qPCR indicated that this gene was induced after wounding and in response to *P. expansum* and *P. digitatum* inoculation. Although, significant differences were found between transcript levels of *P. expansum* and *P. digitatum* with their respective wounded controls at 48 h, *P. digitatum* exhibit the highest transcript level. Transcript and protein levels of the Mal d 1.03E protein correlated in our experimental conditions. Our data showed that for unwounded and wounded tissues at 0 h the Mal d 1.03E gene expression was very low. These results are in agreement with those obtained by Pagliarani et al. [36], where no transcript expression for Mal d 1.03E was observed by qPCR in apple fruit cv Florina. Mal d 1 has been described by other authors as an apple defense-associated protein that is induced in response to abiotic and biotic stresses [8,37–40]. In particular, Beuning et al. [37] and Paris et al. [41] reported an induction in Mal d 1 gene expression in apple leaves in response to *Venturia inaequalis* inoculation. Our group previously reported that only a portion of the Mal d 1 proteins (such as Mal d 1.03G) increase in abundance in over-mature 'Golden delicious' apple fruit after wounding [8]. Those results suggest

that there is a functional specialization present in the different Mal d 1 protein family members and that this may explain why only a portion of the Mal d 1 proteins are wound-induced and others are specifically induced in response to a host or a non-host pathogen.

In apple, Mal d 1 is considered a complex, multigene family, which includes at least 20 different genes, mainly clustered on two linkage groups (LGs) 13 and 16 [36,38,39]. Mal d 1.03G has been recently mapped to LG 16 [42]. Mal d 1 genes have been classified into four subfamilies based on the presence or absence of introns and their length. Our results encompass members from two out of four of the groups, specifically Mal d 1.06C a member of subfamily III, which contains introns, and Mal d 1.03A, Mal d 1.03D and Mal d 1.03E, members of subfamily IV, which is intronless. Mal d 1.06C is located on LG 16, whereas Mal d 1.03A, Mal d 1.03D and Mal d 1.03E are located on LG 13, suggesting that the Mal d 1 clusters on these two LGs evolved differently [38]. These data support the hypothesis that the presence of many homologous Mal d 1 genes in the apple genome has been conserved during evolution due to their role in signal transduction or in pathogen mediated-selection, considering that the most rapidly evolving families have been associated with pathogen defense [43]. The role of each Mal d 1 protein in apple response to abiotic and biotic stresses deserves further studies in order to understand their involvement in apple disease resistance and the molecular mechanisms by which Mal d 1 regulates host defense responses.

Heat shock proteins (HSPs) play important roles in maintaining plants' function when they are exposed to a variety of abiotic and biotic stresses [44]. Four HSPs exhibited significant changes in abundance in response to the experimental treatments, although none of them showed evidence of oxidation. Mitochondrial HSO70 2 isoform 1 (MDP0000416692) and a high molecular weight HSP (MDP0000322220) decreased in abundance, while a Class I HSP (MDP0000323296) increased in abundance after wounding. The results also demonstrated that only Class I HSP increased in abundance after wounding, suggesting that it may be involved in wound response in apple. HSP STI-like (MDP0000422652) reduce specifically its abundance in response to *P. expansum* at 48 h after inoculation. The expression level of the Class I HSP gene was characterized using RT-qPCR to further validate its association with apple response to abiotic stress. The resulting data demonstrated that the Class I HSP gene was up-regulated after wounding but down-regulated in response to both pathogens. A recent report documenting apple gene expression showed that an HSP101 gene was up-regulated in response to *P. expansum* and an HSP70 gene was induced by both *P. expansum* and *P. digitatum* [4]. In contrast, our proteomic data indicated a functional specificity for the corresponding Class I HSP protein since it was induced specifically after wounding. Overall results showed that HSP's role in abiotic and biotic stress-related responses has yet to be elucidated.

Elongation Factor-Tu (EF-Tu) plays a pivotal role in protein biosynthesis and response to both abiotic and biotic stresses in plants. Furthermore, EF-Tu proteins possess chaperone activity and also play an important role in plant cells in inducing innate immunity [45]. Our data revealed a 2.5-fold increase in the abundance of EF-Tu (MDP0000291397) in response to *P. digitatum* inoculation compared to wounded control at 48 h. More interestingly, this protein also increased 2.2-fold in response to *P. digitatum* compared to *P. expansum* inoculation at 48 h. Transcript expression of the EF-Tu gene was similar at all-time points after wounding and was not significantly different to transcript levels in unwounded tissue. In contrast, a significant decrease in transcript abundance was observed in response to *P. expansum* inoculation compared to wounded control at 48 h. The reduction in the mRNA level of EF-Tu in response to host pathogen has not been previously reported. A significant, but less marked, decrease in transcript levels of EF-Tu was found in response to *P. digitatum* at 24 and 48 h after inoculation, relative to transcript levels in wounded controls at 24 and 48 h. Although EF-Tu expression in response to different environmental stresses has been studied in several plant species, in the present study, no evidence was found of abiotic stress-related EF-Tu expression at the proteomic level.

Since transcription factors play an important role both in abiotic and biotic stress responses in plants [46], additional attempts were made to analyze their response to wounding and wounding/inoculation. Another transcription factor, specifically a NAC domain class protein (MDP0000868419) – without significant changes in abundance or levels of oxidation in our experimental conditions – was also evaluated at the transcriptomic level using RT-qPCR. NAC gene exhibited a very similar pattern of expression to EF-Tu. Although NAC genes have been reported to be induced in response to several postharvest abiotic stresses, including wounding, anoxia, low

temperature, and ethylene [47], their role in the regulation of plant defense response against pathogens is less understood [48].

Oxidative burst is one of the earliest and most rapid defense responses that occur in many plant–pathogen interactions [14,15] and so a significant attention has been placed on characterizing oxidative metabolism. In plants, the cause–effect relationship between ROS and tissue injury under environmental stress is obvious, but the biochemical processes connecting them has not been elucidated [49]. Superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) are among the most important proteins responsible for ameliorating oxidative stress [50]. It is noteworthy that none of these proteins showed significant changes in abundance or levels of oxidation in our experimental conditions. However, the list of target proteins obtained in this study enables a discussion of possible mechanism of ROS-mediated biochemical processes in apple defense response. Due to the importance of these proteins in the modulation of ROS levels in fruit, the APX gene was examined at transcriptional level. APX transcript abundance was observed at any of the time points after wounding. Transcript abundance in response to the non-host pathogen was significantly higher than in response to the host pathogen at 24 and 48 h. Although changes in transcript abundance are good indicators of fruit response to abiotic and biotic stresses, they may not always correlate with protein abundance [51,52], as demonstrated the results of our study for 5 out of 6 genes analyzed.

Glutamine synthetases (GS) are enzymes involved in the primary assimilation of inorganic nitrogen, as well as in the reassimilation of free photorespiratory ammonium. Two GS were found to be significantly oxidized at 48 h in response to *P. digitatum* compared to *P. expansum*. The oxidized proteins were identified as glutamine synthetase cytosolic isozyme 1 (MDP0000196909) and glutamine synthetase nodule isozyme-like (MDP0000151581).

The genome sequences of two Spanish strains of *P. digitatum* have recently become available, revealing that *P. digitatum* has a remarkable reduction in gene content compared with its close relative but non-phytopathogenic, *P. chrysogenum* [53]. Functional classes of the genes lost in *P. digitatum* have important physiological implications including a loss in the ability to use nitrate as a nitrogen source. Therefore, taking into account that *P. digitatum* has lost its ability to use nitrate as a nitrogen source, we hypothesize that apple fruit might have evolved a defense response that takes advantage of this lack and has developed the ability to oxidize both GS proteins to avoid utilization of nitrate by this fungus. This hypothesis requires further studies to verify that *P. digitatum* has lost homologues of these GS genes and to demonstrate the involvement of apple GS genes in defense mechanisms associated with resistance to this non-host pathogen of apples.

Oxidative burst and ethylene production are part of the innate immunity response of plant cells and which forms the basis for resistance to infection [45]. Two proteins, both encoding ACC oxidase (ACO) (MDP0000195885) were among the wound-responsive proteins identified to increase in abundance at 24 h. Wound-induced ethylene production, regulated by ACC synthase, has been previously reported to

be involved in the generation of ROS [54]. ACO catalyzes the last step of ethylene biosynthesis. In this regard, apple ACO genes are part of a multigene family [55], and are regulated by a complex network of developmental and environmental signals responding to both internal and external stimuli [56]. In the present study, ACO (spot 1203) was observed to be highly oxidized (16.8 and 28.2-fold) in response to *P. digitatum* at 24 h after inoculation, relative to the levels evident in either wounded tissue and apple tissue that had been wounded and then inoculated with *P. expansum*, respectively. Oxidation was absent after either wounding or *P. expansum* inoculation. RT-qPCR results indicated slight but statistically significant changes in the ACO expression level. ACO transcripts increased in response to wounding and in response to *P. expansum* at 48 h after inoculation, but decreased in response to *P. digitatum*. The results obtained on the oxi-proteome indicated that, in regards to ethylene biosynthesis, ACO was strongly oxidized in response to *P. digitatum* at 24 h after inoculation. This result suggests that early oxidation of ACO may reduce or inhibit ethylene production and respiration rates in apple fruit and consequently decrease metabolic activities, such as protein synthesis.

Two other metabolic proteins were found to be oxidized in response to *P. digitatum* at 48 h after inoculation, relative to levels of oxidation observed in response to *P. expansum*. Both were identified as ATP synthase. One of the ATP synthases exhibited increased levels of oxidation in response to *P. expansum* at 24 h after inoculation. These results suggest that the oxidation of ATP synthase could affect proton movement more severely in response to the non-host pathogen than in response to the host pathogen. Modulation of plasma membrane H^+ -ATPase activity has been previously suggested as a possible molecular switch in several plant resistance responses [57]. In this regard, the reduction or inactivation of apple proteins related to energy metabolism due to their oxidation, may contribute to an energy deficiency that it will differentially impact the ability of *P. expansum* or *P. digitatum* to acquire nitrogen, carbon, and proteins from injured tissue. These results suggest that the prevention of free nutrient movement is an important apple defense mechanism against both host and non-host pathogens. Therefore, the specific role of the oxidized proteins as part of an innate immunity response, pathogenicity, and host resistance needs to be further explored.

5. Conclusions

To the best of our knowledge, this is the first study conducted in apple fruit that has examined global changes in the apple proteome and oxi-proteome in response to wounding and host (*P. expansum*) and non-host (*P. digitatum*) pathogens. The results of the proteomic study indicate a strong interaction between the non-host pathogen and apple relation to both defense responses and metabolic pathways. In general, the relationship between some of the identified proteins and their transcripts was low. Only the Mal d 1.03E protein was correlated with the level of transcripts of its corresponding gene in our experimental conditions, supporting the general observation that levels of transcription and protein

expression usually do not correspond. However, it must be kept in mind that the transcript levels of only a small number of genes were examined.

The additional information provided on protein oxidation in response to a host and a non-host pathogen indicate that oxidation of apple proteins related to energy metabolism may contribute to an energy deficiency that may differentially impact the ability of *P. expansum* and *P. digitatum* to acquire nitrogen, carbon and proteins. Therefore, the oxidation of apple proteins in response to wounding and both pathogens which affects energy metabolism arises as an important defense mechanism that needs to be further explored.

Documenting changes in the oxi-proteome of apple can provide information that can be used to better understand how impaired protein functions may affect apple defense mechanisms. It also provides new biomarkers for oxidative damage mainly caused by the oxidative response occurring in fruit tissue in response to host and non-host pathogens. The molecular and bioinformatics data presented represent the first step towards both a functional genomics and systems biology approach to understanding gene function and the regulatory networks involved in apple innate immunity responses.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2014.11.007>.

Conflict of interest

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