Identification of bitter pit protein markers in *Malus domestica* using differential in-gel electrophoresis (DIGE) and LC-MS/MS

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Keywords: bitter pit, apple, pathogenesis-related (PR) proteins, DIGE, LTQ-Orbitrap

47 **Abstract**

48 Bitter pit is a physiological disorder that occurs in apple, pear and quince and has long
49 been associated with calcium uptake or lack thereof. In the present study, pooled
50 biological *Malus domestica* proteins were collected from healthy and naturally
51 occurring bitter pit fruit. Protein samples (bitter pit and healthy) were analyzed with
52 differential in-gel electrophoresis (DIGE) and SameSpots software was used to
53 compare gel spots by intensity. Identified spots ($p < 0.05$) were spot picked and trypsin
54 digested. Peptides were separated by liquid chromatography (LC) and submitted to
55 LTQ-Orbitrap mass spectrometer to infer protein identification. A total of 200 ± 5
56 protein spots were detected, 41 spots classified as having $p < 0.05$ and were
57 successfully identified by their peptide sequence listed in an online *Malus domestica*
58 database. Thirteen spots were identified as having $p < 0.05$ and a minimum 2-fold
59 change. Several pathogenesis-related (PR) proteins belonging to three PR families,
60 PR-5 (10.3-fold), PR-8/chitinase (35.5-fold) and PR-10 (6.8-fold) were upregulated in
61 bitter pit sampled tissues. Three proteins involved in several metabolic processes
62 including ethylene biosynthesis (3.3-fold), glycosyltransferase reactions in
63 metabolism (2.3-fold) and metal binding (11.1-fold) were found to be overexpressed
64 in healthy sampled tissues. This research provides a significant advance in the
65 knowledge of protein expression alterations occurring in bitter pit in comparison with
66 sound tissues.

67

1. Introduction

Bitter pit, a physiological disorder occurring in apple, pear, and quince, has been long associated with calcium uptake or lack thereof. Symptoms are known to develop in storage, appearing several weeks to months after harvest. Symptoms have also been seen on young, vigorously growing, nitrogen fertilized trees, with partial fruit maturity (Ferguson & Watkins, 1989). The severity of bitter pit is often associated with nutrient concentration (N, K, Mg, Ca), seasonal rainfall, and temperatures, which are known to enhance the development of the disorder (Retamales et al., 2000).

Bitter pit development has been described as the result of low Ca^{2+} concentrations in fruit tissue (Fuller, 1980). Calcium uptake is directly influenced by climate, water availability, nutrition, vigor, and crop production. Uneven calcium distribution in fruit tissue has been thought to be one of several factors that cause bitter pit (Wilkinson, 1964). Previous calcium studies have suggested the majority of apple Ca^{2+} is located in the vacuole and cell wall of fruit (Freitas, 2012, Harker & Venis, 1991), reporting large concentrations of Ca^{2+} in vacuolar and cell wall regions responsible for cell regulation. Freitas (2012) suggested Metabolic changes in Ca^{2+} content could weaken plasma membrane structures, eventually causing cell death and bitter pit symptoms. It has also been reported that the skin, seeds, and core tissue of bitter pit affected fruits have higher calcium concentrations than the flesh (Wilkinson, 1961, Wilkinson, 1964).

Spray treatments (utilizing calcium chloride or calcium nitrate) have been studied as a key approach in reducing bitter pit occurrence (Ferguson & Watkins, 1989). However, no treatment has been completely successful to control bitter pit, and reducing fruit loss has become a priority for fruit growers.

93 Despite many years of research, the mechanisms involved in bitter pit development
94 are still not well understood. A deep knowledge of the factors that trigger this disorder
95 is needed to devise more effective control strategies during breeding programs.

96 At the genomic level, predictive quantitative trait locus (QTLs) DNA tests have been
97 developed to predict and breed out bitter pit alleles. The BP16, BP13, and LG16ma
98 alleles are thought to be partially responsible for bitter pit, and were discovered while
99 selective breeding for shelf life, color, acidity, and flavor (Lezzoni et al., 2010).

100 RosBREED has developed BP16-indel and BP13-SSR tests to validate QTL alleles.

101 However, in the quest to understand biological processes, the study of proteins cannot
102 be avoided. Proteomics provides us with a variety of exciting new strategies to
103 address biological questions.

104 With the influx in genomic data and improvements in analytical technology,
105 proteomics has become increasingly important to study the many different aspects of
106 plant functions. While proteins serve as important components of major signaling and
107 biochemical pathways, studies at protein levels are essential to reveal molecular
108 mechanisms underlying plant growth, development, and interactions with the
109 environment (Chen & Harmon, 2006).

110 Besides the importance of the proteomic approach in the advance of understanding of
111 diseases in plants, to the best of our knowledge, no scientific publication dealing with
112 this issue in bitter pit material can be found in the literature. Our research group
113 addressed this subject for the first time in a recent study (unpublished data). By means
114 of one dimensional (1D) denaturing sodium dodecyl sulphate polyacrylamide gel
115 electrophoresis (SDS-PAGE) followed by MALDI/TOF/TOF analysis of selected
116 proteins, we succeeded in identifying two pathogenesis-related (PR) proteins as
117 markers of bitter pit tissues, Mal d 1 and Mal d 2. However, due to the complexity of

the samples, the methodology employed presented some limitations like migration problems and saturation of several protein bands within the single dimension, which compromised further accurate identification in some protein bands potentially upregulated in bitter pit tissues.

A major challenge in proteomics research and biomarker discovery is the analysis of complex samples. The global strategy to address proteomic research must integrate a series of comprehensive and accurate separative and analytical technologies to deal with the immense complexity involved. Historically, two-dimension polyacrylamide gel electrophoresis (2D-PAGE) has been the protein separation technique most commonly associated with proteomics. It relies on a first separation of proteins by isoelectric focusing (IEF) and then by molecular mass in the second dimension on application of an electrical potential across a solid-based gel. However, difference gel electrophoresis (DIGE) is a technology that has emerged in the last decade as a very valuable technique, as it allows for accurate quantification with statistical confidence while controlling for non-biological variation, increasing the dynamic range and sensitivity of traditional 2D PAGE (Lilley & Friedman, 2004). Difference gel electrophoresis 2D-DIGE is based on direct labeling of lysine groups on proteins with cyanine CyDye DIGE Fluor minimal dyes before isoelectric focusing, enabling the labeling of three samples with different dyes and electrophoresis of all the samples on the same 2D gel. This capability minimizes spot pattern variability and the number of gels in an experiment while providing simple, accurate and reproducible spot matching (Tannu & Hemby, 2006).

The main objective of the present study was to identify proteins markers (significantly up or down-regulated) in sound and bitter pit affected tissues from apple (*Malus domestica*), and to compare their relative abundance by means of the global strategy

143 explained above; 2D-DIGE followed by in-gel digestion and LC/MS analysis of
144 peptides to infer protein identity. As a case study, the Golden Delicious Smoothee
145 cultivar was selected due to its production value and known sensitivity to bitter pit
146 development.

147 Given the lack of proteomic studies focused in bitter pit, it is expected that this
148 research contributes to characterize for the first time alterations in the expression of
149 proteins linked to bitter pit development and helps to better understand the inner
150 mechanisms involved in this disorder.

151

2. Materials and Methods

2.1.Acquired Fruit

Malus domestica cv. Golden Delicious Smoothee fruit were purchased in November 2011, from a commercial grower in Aragon, Spain. Fruit were stored in a temperature-controlled (1-4°C), low light room for six months before being processed. Fruit were arranged in plastic trays (18 fruit per container), weighing between 10 and 20 kg. At time of purchase, less than 1% of purchased apples exhibited bitter pit symptoms.

2.2.Sampling

Apples were grouped according to visual symptoms (bitter pit/healthy). After six months of storage, approximately 35 % of each container (6-8 fruit) expressed bitter pit symptoms.

Bitter pit apples expressing symptoms (i.e. sunken spots, patches, or discoloring) were sampled starting from the calyx end, removing localized subcutaneous tissue at random. Healthy tissue was sampled similarly. No more than 5 tissue samples were taken from each apple (0.5 g dry weight). A total of 150 sampled bitter pit subcutaneous tissue (sampled from 30 apples, 5 spots/apple) and 50 healthy tissue extractions (10 apples, 5 spots/apple) were collected and allocated into tubes. Tissues were collected in 5 mL plastic tubes on ice and stored at -20°C. Two bitter pit pool samples and eight healthy pool samples were analyzed

2.3.Lyophilization

Sample tubes were placed inside a Cryodos 50 laboratory freeze-dryer (Telstar lifesciences, Terrassa, Spain) maintained at -0.062 mbar, -50.5 °C and lyophilized for 7 days to remove sample moisture (water content = 0%). Lyophilized samples were

ground to a fine powder (1-100 μ M). Samples were stored in a freezer (-80°C), until further analysis.

2.4. Protein Extraction

Proteins were extracted according to the methods described by Bestel-Corre et al (2002). Approximately 0.5 g apple lyophilizate were added to 2.5 mL phenol extraction buffer [0.5 M Tris-HCL pH 7.5, 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 10 mM thiourea, 2 mM PMSF, and 2% B-mercapethanol (1:5, v/v)]. Samples were agitated for several minutes, and then 2.5 mL of saturated phenol pH 8 (Aqua phenol, MP Biomedicals, Santa Ana, CA, USA) were added. The solution was vigorously shaken for 30 minutes then centrifuged at 12000 x g. The upper phase was transferred into a new 30 mL tube and 10 mL of extraction buffer were added. After 30 minutes of agitation, the phenol phase was transferred into a new 30 mL tube and 5 volumes of ice-cold methanol containing 0.1 M ammonium acetate were added. Proteins precipitated overnight at -20°C.

The following day tubes were centrifuged at 10,000 x g for 30 minutes, producing a protein pellet at the bottom. The supernatant was discarded. The pellet was washed twice with ice-cold methanol followed by acetone and speedvaced to dry.

2.5.2D-DIGE electrophoretic analyses

The precipitated pellets were re-dissolved in 0.5 mL DIGE-compatible sample (lysis) solution [30 mM Tris (pH 8.5), 7 M Urea, 2 M Thiourea, 4% CHAPS, pH 9.5]. Proteins were quantified using RC DC protein assay kit (Bio-Rad Life Science, Hercules, CA) using BSA as a standard. 50 μ g were labeled with 200 pmol (1 l) of Cy protein, incubated on ice for 30 min in the dark, and quenched with 1 μ l of 10 mM lysine; each sample was then incubated on ice for 10 min in the dark (GE Lifesciences). Two healthy and two bitter pit samples (50 μ g of protein each) were

202 labeled separately with either Cy3 or Cy5, and the internal standard (50 µg of protein
203 comprising 25 µg from each of the 2 samples) was labeled with Cy2. One internal
204 standard, healthy, and bitter pit sample forming a set of Cy2-, Cy3-, and Cy5-labeled
205 samples were combined (1:1:1). IPG strips (pH 3–7, 24 cm, GE Healthcare) were
206 rehydrated using 450 µl of Destreak solution (GE) with IPG buffer (0.05% v/v)
207 overnight. The IPG strips were cup-loaded with 50 µg of each Cy2-, Cy3- and Cy5-
208 labeled sample in a buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65
209 mM DTT, and 1% (v/v) IPG buffer. Isoelectric focusing was carried out in a Protean
210 IEF cell (Bio-Rad Life Science, Hercules, CA) at 62 kV h in different phases as
211 follows: 10 min at 50 V, 1-h ramp up to 500 V, 1 h at 500 V, 2-h ramp up to 1000 V,
212 10-h ramp up to 10,000 V, and 2 h at 10,000 V. Prior to second dimension SDS-
213 PAGE, the strips were equilibrated for 15 min in DTT buffer (375 mM Tris–HCl pH
214 8.8, 6 M urea, 20% v/v glycerol, 2% w/w SDS and 130 mM DTT), followed by
215 another 15 min incubation in iodoacetamide (375 mM Tris–HCl pH 8.8, 6 M urea,
216 20% v/v glycerol, 2% w/w SDS and 135 mM iodoacetamide). For the second
217 dimension, strips were applied directly to 16% SDS-polyacrylamide gels, and 4 gels
218 were run simultaneously on a Protean Plus Dodeca Cell (Bio-Rad Life Science,
219 Hercules, CA) at 1W/gel until the bromophenol blue tracking front reaches the end of
220 the gel. Immediately after 2D-DIGE, gels were individually scanned in a Typhoon
221 Trio scanner (GE Healthcare) and the Photo Multiplier Tube voltage was adjusted for
222 maximum image quality with minimal signal saturation. Images were checked for
223 saturation during the acquisition process using ImageQuant TM TL Software (GE
224 Healthcare). Cy2, Cy3, and Cy5 images for each gel were scanned at 488/510-,
225 532/530-, and 633/500-nm excitation/emission wavelengths, respectively at 100-µm
226 resolution, obtaining a total of 12 images (4×3). Determination of protein spot

intensity and correlation was performed using Progenesis SameSpots v 4.0 software (Nonlinear Dynamics, U.K.). Statistically significant changes in protein spots were determined using ANOVA ($p < 0.05$; SameSpots Stats) and a cutoff of two-fold change was established for further analyses. Gels were stained using a fresh solution of Colloidal Coomassie. In brief, gels were shaken for 1 h in an aqueous solution containing 16% ammonium sulfate, 3% (v/v) phosphoric acid and 32% (v/v) methanol. Gels were transferred to a 6.6% Coomassie Blue G-250 methanolic solution for 3 hours. De-staining was achieved by rinsing gels in water.

2.6. In-gel Tryptic Digestion

Spot coordinates were picked with an ExQuest Spot cutter (Bio-Rad Life Science, Hercules, CA). Excised gel spots were in-gel tryptic digested followed with shotgun analyses by nanoLC-MS/MS (LTQ-Orbitrap, Thermo Fisher, Waltham, MA, USA).

Gel spots were immersed in 100 μ L wash solution (50% acetonitrile/ 50% 50 mM NH_4HCO_3) for approximately 10 minutes, before agitation, and centrifugation, repeated twice.

Reduction and alkylation, was performed to unfold the tertiary structure of proteins by breaking disulfide bonds (Cysteine). Approximately 100 μ L 10 mM dithiothreitol (DTT) were added to each gel sample. Gels were incubated for 1 hour, incubated, centrifuged and then the filtrate solution was discarded. Approximately 100 μ L 55 mM Iodoacetamide were added and gels were incubated for 1 hour in obscurity. After 1 hour, gels were centrifuged and the filtrate solution discarded. Gels were washed twice with 100 μ L 25 mM ammonium carbonate then centrifuged. Additionally, gels were washed and dehydrated (three times) with 100 μ L acetonitrile, discarding the wash solution after each wash step. Gels were dried overnight at 57° C. Two mL 25 mM NH_4HCO_3 was added to 20 μ g (100 ng) sequencing grade modified trypsin

porcine (Promega, Promega corporation, Madison, WI, USA). Gel plates were incubated for 6 hours at 37°C. After incubation, plates were centrifuged and the trypsin solution was collected. Tryptic peptides were extracted by adding 20 µL 25 mM ammonium bicarbonate to each sample, then centrifuging to collect the solution. Twenty µL 50% (v/v) ACN, 0.5% (v/v) trifluoroacetic acid (TFA) was added. Samples were incubated for 20 minutes then centrifuged to collect the supernatant, repeated twice. Approximately 10 µL acetonitrile was added and the supernatant collected. Peptide extracts were dried in a speed vacuum concentrator for approximately 2 hours. The dried filtrate was suspended in 25 µl of 2% (v/v) ACN, 0.05% (v/v) TFA, 0.08% (v/v) formic acid before HPLC injection.

2.7. Mass spectrometry analyses

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analyses were performed with an UltiMate 3000 LC system Thermo Scientific Dionex (Voisins le Bretonneux, France) connected by a nanoelectrospray interface to a linear ion trap mass spectrometer coupled to an Orbitrap detector (LTQ-Orbitrap, Thermo Fisher, Waltham, MA, USA). Tryptic peptide mixtures (4 µl) were loaded at flow rate 20 µl min⁻¹ onto precolumn Pepmap C18 (0.3 by 5 mm, 100 Å, 5 µm; Dionex). After 4 min, the precolumn was connected with the separating nanocolumn Pepmap C18 (0.075 by 15 cm, 100Å, 3 µm), and the linear gradient was started from 2 to 36% of buffer B (0.1% formic acid, 80% acetonitrile (ACN)) in buffer A (0.1% formic acid, 2% ACN) at 300 nl min⁻¹ over 50 min. Ionization was performed on liquid junction with a spray voltage of 1.3 kV applied to an uncoated capillary probe (PicoTip EMITER 10-µm tip inner diameter; New Objective, Inc, Woburn, MA). Peptides ions were automatically analyzed by the data-dependent method as follows: full MS scan (m/z 300 to 1,600) on Orbitrap analyzer and MS/MS on the four most abundant

precursors on the LTQ linear ion trap. In the present study only +2 and +3 charged peptides were subjected to MS/MS experiments with an exclusion window of 1.5 min, with classical peptide fragmentation parameters as follows: Qz = 0.22, activation time = 50 ms, and collision energy = 35%.

The raw mass data were converted to mzXML format with the ReAdW software (<http://tools.proteomecenter.org/software.php>). Protein identification was performed querying MS/MS data against a *Malus domestica* database (uniprot.org), together with an in-house contaminant database, using the X!Tandem software (X!Tandem Cyclone (2011.12.01), <http://www.thegpm.org>) with the following parameters : one trypsin missed cleavage allowed, alkylation of cysteine and conditional oxidation of methionine, precursor and fragment ion set at 2 ppm and 0.005 Da, respectively. A refined search was added with similar parameters except that semi-tryptic peptides and possible N-ter acetylation of proteins were searched. All peptides matched with an E-value lower than 0.01 were parsed with X!Tandem pipeline software. Proteins identified with at least two unique peptides and a log (E-value) lower than 4 (10^{-4}) were validated. In the case of identification with only two or three MS/MS spectra, similarity between the experimental and the theoretical MS/MS spectra were visually checked.

3. Results and Discussions

Separation of proteins differentially expressed in bitter pit and healthy tissues were carried out in a 2D-DIGE system. In the first dimension, proteins were separated according to isoelectric point. In the second dimension, proteins were separated by molecular mass (Figure 1). SameSpots computer software was used to compare spot intensities by comparing a pool sample (Cy2 - Internal calibration) against healthy or bitter pit cyanine dyed samples to reduce potential gel variability issues. Scanned Gel images were automatically compared and analyzed using SameSpots tools which reported fold values and p-values (one-way anova) of all spots. For a protein to be considered significant (differentially expressed), the p value was less than 0.05 and the fold change was at least two.

Typhoon scanned gel layers (Cy2, Cy3, and Cy5) were aligned and 200 ± 5 protein spots were visually detected in both gel replicates using SameSpots software (Figure 1). According to statistical analysis ($p\text{-value} < 0.05$) run in SameSpots, a total of 41 spots (Table 1, Figure 2) were identified as being differentially expressed in healthy and bitter pit material. Figures 3 and 4, respectively, provide a comparison of spot intensities between healthy (9 spots) and bitter pit (32 spots) affected tissues.

Target gel spots were picked, in-gel digested, injected into a LC system and isolated fractions were recorded by LTQ-Orbitrap. The forty-one spots were identified by their peptide sequence listed in the *Malus domestica* database, using X!Tandem and De novo pipeline software.

Thirteen unique (non-duplicated) proteins were identified as being significant in either healthy (Table 2) or bitter pit (Table 3) samples. Of the 13 differentially expressed proteins for healthy (Figure 5) and bitter pit (Figure 6) samples, isoelectric point values were identified as being between 3.5 and 5.2

For the sake of simplicity, the results and discussion will be structured with respect to proteins differently expressed in healthy or bitter pit tissues, respectively. The identified spots will be named with their spot ID between parentheses. Non-significant protein spots are listed in Table 4.

3.1. Proteins overly expressed in bitter pit

As shown in Table 3, ten bitter pit proteins expressed greater ratiometric normalization values when compared to healthy proteins. Chitinase (spot 3061), UTP-glucose-1-phosphate uridylyltransferase (spot 3047), and several proteins (spots 3042, 2275, 2268, 3065, 3067, 3063, 3055 and 3060) belonging to pathogenesis-related (PR) subgroups were identified. PR proteins are expressed in biological systems as a defense response to abiotic stress, fruit ripening and bacterial/fungal attacks (Atkinson et al., 1996, Pühringer et al., 2000). PR proteins have been shown to exhibit distinct biochemical characteristics, which are necessary when the plant is under pathogenic infection or any unwanted stress. As many as seventeen PR protein families are recognized based on their amino acid sequence similarities, enzymatic activities, biological properties and are numbered in the order in which they were discovered (Sinha et al., 2014)

In the present work, PR proteins from three different families: PR-5 (spots 3055 and 3060 and 3067), PR-8 (spots 3063 and 3065) and PR-10 (spots 3042, 2268 and 2275) were identified as significantly overly expressed in bitter pit tissues.

PR-10 family: PR-10 proteins are consistently studied due to their type I allergic reactions caused by fruit, vegetable and nut food allergies, a result due to white birch (*Betula verrucosa*) pollen more commonly known as Bet v 1-homologous proteins. The most notable Bet v 1 homologue is Mal d 1, a major apple allergen that shares 56% of its protein sequence identity with Bet v 1 (Hoffmann-Sommergruber, 2000,

345 Bohle, 2007, Ebner et al., 2001). In this present study, Mal d 1 (3042) and two Mal d
346 1 isomers (2268 and 2275) were identified. In a recent group study (data unpublished
347 data), Mal d 1 was identified for the first time in bitter pit affected fruit using
348 MALDI-TOF. Results in the present study indicate that Mal d 1 was 6.8 times greater
349 expressed in bitter pit samples when compared to healthy samples, suggesting a
350 possible effect caused by the bitter pit disorder.

351 PR-5 family: Three PR-5 spots (3055, 3060 and 3067,) were identified as thaumatin-
352 like proteins (TLPs). Normalized TLPs spot volumes revealed bitter pit as being 10.3
353 times greater expressed when compared to healthy samples. Previous studies have
354 recognized TLPs as a new class of panallergens (Breiteneder, 2004), plant defense
355 (Venisse et al., 2002), and stress induced (van Loon et al., 2006b).

356 Spot 3067 was identified as Mal d 2, a major apple allergen (Hsieh et al., 1995). In
357 addition to Mal d 1, in a recent group study (data unpublished), Mal d 2 was also
358 identified as a potential protein marker for bitter pit, which is in agreement with data
359 presented in this study. The abundance of both allergens (Mal d 1 and Mal d 2) in
360 apple fruit affected with bitter pit raises an interesting question about at what moment
361 of bitter pit development are allergens expressed in fruit. Bitter pit apples are not
362 considered “market acceptable” and therefore they usually don’t arrive to the
363 consumer. However, it can be hypothesized that Mal d 1 and Mal d 2 are expressed
364 even before visual symptoms appear in the skin (when the fruit is still marketable).
365 Under this assumption, apples would exhibit a higher allergenicity potential than their
366 sound equivalents at the moment of consumption. Since allergenicity in apples is
367 highly dependent on cultivar (Bolhaar et al.), apple varieties classified as highly
368 allergenic (like the worldwide consumed Golden Delicious studied in this work)
369 would be especially susceptible to an increase in Mal d 1 and Mal d 2 levels; a result

that should be taken into account when apple breeding.

PR-8 family: PR-8 (3063, 3065 and 3061) spots were found to be 35.5-times more abundant in bitter pit samples when compared to healthy samples. PR-8 proteins are known to be type III chitinases and are involved in plant defense mechanisms (Park et al., 2004). The role of chitinases as part of an inducible plant defense response is well documented (Boller, 1987, Collinge et al., 1993). Chitinase activity is found at low levels in plants but is induced in response to various stimuli, e.g., the phytohormone ethylene, salicylic acid (Jung et al., 1993, Margis-Pinheiro et al., 1994), virus infections (Ohme-Takagi et al., 1998, Payne et al., 1990), and various pathogenic microorganisms (Kästner et al., 1998, Mohr et al., 1998, Münch-Garthoff et al., 1997) resulting in the activation of defense signaling pathways.

Some of the above mentioned proteins (TLP, Mal d 1) have been reported to be enhanced and overly expressed during ripening in response to ethylene production (Botton et al., 2008). Enhanced ethylene production is an active response to pathogens and is associated with the induction of defense reactions (van Loon et al., 2006a). Pesis (2009) addressed the study of bitter pit development in cold-stored transgenic apples suppressed for ethylene biosynthesis. The authors monitored apple fruit ethylene during and after cold storage concluding that ethylene concentrations remained relatively low during cold storage and apples developed bitter pit. Also in this study it was suggested that apple decay could be related to bitter pit incidence as samples expressing greater bitter pit incidence also had greater visible decay. In the present study, apples were stored for a period of 6 months in cold storage and although fruit respiration/ transpiration levels decreased, fruit may have decayed at a much greater rate due to the storage environment. In several previous studies, which stored fruit in low ethylene, low oxygen environments (Stow et al., 2000, Zanella,

2003, Both et al., 2014), the reduction in oxygen and ethylene was effective in reducing incidence of fruit softening and apple scald. The cause of bitter pit whether calcium or cell death (Fukuda, 1986) is still unknown and additional studies are needed to investigate whether apples stored in low oxygen, low ethylene conditions will reduce fruit decay which may result in lower occurrences of bitter pit. Additionally, mycology studies would be beneficial to monitor fruit decay and cellular disruptions over time during storage to identify any known apple fungi such as *Penicillium* spp. or *Botrytis cinerea* (Kim & Xiao, 2008) that are often present during decay.

3.2. Proteins overly expressed in healthy tissues

Three healthy proteins (4 spots) had greater average normalized volumes (1358, 1453 and 1859) when compared to bitter pit proteins (Table 2).

Spot 1358: Identified as 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO), this enzyme has shown to catalyze the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Chervin et al., 2004, Sun et al., 2010). The ACO protein normalized volume was 3.3 fold greater in healthy tissues than bitter pit tissue. In a previous study (Dandekar et al., 2004) transgenic ‘Greensleeves’ apples with reduced ACO activity (antisense) displayed reduced autocatalytic ethylene production, slower ripening and less fruit softening when compared to the control. In our presented study, the healthy tissue samples expressed greater ACO activity suggesting increased ethylene production, faster ripening and more instances of fruit softening. Additionally, it is believed that once healthy fruit begin to decay, due to ripening and endogenous ethylene caused by ACO activity, they are more vulnerable for bitter pit development.

Spot 1453: this spot was unable to be identified using the *Malus domestica* database

(no data entry found) and was ultimately sequence matched with another genera from the general Roseaceae database (also found at uniprot.org). Spot 1453 was identified as UTP-glucose-1-phosphate uridylyltransferase also referred to as UDP-glucose pyrophosphorylase or UGPase. In the current study UTP-glucose-1-phosphate uridylyltransferase was 2.3-fold greater in healthy tissue when compared to bitter pit tissue. Its function is to catalyze the formation of UDP-glucose, which has a central role in the production of glycolipids and glycoproteins, from glucose-1-phosphate and UTP (Thoden & Holden, 2007).

Apple cell wall tissues are known to contain soluble and insoluble glycoproteins (Knee, 1973, Knee, 1975). It has been reported that UTP-glucose-1-phosphate uridylyltransferase is induced by ripening and ethylene treatment (Zheng, 2013). UTP-glucose-1-phosphate uridylyltransferase produces glycolipids, which in human studies, the decay-accelerating factor (Schmelz et al.), a 70-kDa glycoprotein that binds to C3b and C4b, prevented the assembly and accelerated decay of C3 and C5 convertases of both the classical and alternative pathways (Brooimans et al., 1992). While UTP-glucose-1-phosphate uridylyltransferase is involved in several apple functions, it is hypothesized that UDP-glucose concentrations were greater in healthy samples due to healthy cell wall glycolipids that had not yet deteriorated due to fruit decay, as mentioned above. It is thought that as apple tissues begin to decay, cell wall glycoproteins are compromised which would explain why UTP-glucose-1-phosphate uridylyltransferase was upregulated in healthy tissues.

Spot 1859 peptide fragments were identified as being involved in metal binding. Apple residue has been reported to play an important role in metal binding in the presence of carboxyl and phenolic functional groups (Lee et al., 1999). Other hypotheses suggest plants will often “hyper-accumulate” metals to help protect

445 against herbivores and pathogens (Boyd et al., 2007). Healthy protein expressed 11.1-
446 fold more protein abundance when compared to bitter pit. It is postulated that in the
447 presented study healthy apples accumulated metal binding proteins in order to protect
448 themselves from stresses associated with post-harvest. It is also suggested that as fruit
449 mature in storage, metal binding proteins begin to decrease, increasing the chance to
450 develop a disorder.

451 Identified protein functional categories of the thirteen proteins are provided in Figure
452 7. Six bitter pit proteins (originating from the PR-5 and PR-10 families) were
453 classified as toxins and defense, a result, which is in agreement with other articles
454 (Hoffmann-Sommergruber, 2000, van Loon et al., 2006b) as these proteins are known
455 major allergens. Three proteins were characterized as being responsible for
456 carbohydrate metabolism (bitter pit) and nitrogen metabolism (healthy) transport. In
457 regard to bitter pit carbohydrate metabolism and transport, to date no studies have
458 reported any effects of bitter pit carbohydrate metabolism in apples. In the current
459 article, two bitter pit proteins contributing to carbohydrate metabolism were identified
460 suggesting that bitter pit tissue although desiccant in appearance may function to
461 metabolize carbohydrates. Additionally, nitrogen metabolism and transport was
462 identified in healthy samples, validating nitrogen's many roles in chlorophyll, and
463 photosynthesis. Three proteins (2 healthy and 1 bitter pit) were characterized as
464 responsible for transferases and one bitter pit protein was identified as other enzyme.

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Conclusion

This work is the first to use a combined 2D-DIGE and LC/MS methodology to study and compare the proteomic profile of sound apple tissues and tissues affected by bitter pit disorder. In the present study, ten proteins expressing greater abundance when compared to healthy proteins could be successfully identified by means of a shotgun proteomic approach. Our results highlight that bitter pit disorder induces a deep change in the physiological condition of affected tissues, expressing a variety of pathogenesis-related (PR) proteins from several families (PR-10, PR-5 and PR-8), including Mal d 1 and Mal d 2, two major allergens. These proteins are probably generated as a response to several stress factors during bitter pit development, and may be expressed even before external symptoms are visible in the skin tissues, thus increasing the apples' allergenicity potential. On the other hand, some proteins were much more abundant in healthy tissues than in the affected ones. These proteins carry out different biological functions such as ethylene production catalysis, synthesis of glycoproteins and glycolipids, as well as metal binding.

Studying alterations in the expression levels of protein indicators of both bitter pit and sound tissues at the time of harvest and its evolution during the post-harvest storage may help to better understand the inner mechanisms of bitter pit development. better knowledge of the factors that trigger this disorder will allow the development of more effective control strategies during breeding.

488 **Acknowledgments**

489 This study was supported by the projects AGL2009-59 08501 (Programa Nacional de
490 Proyectos de Investigación Fundamental), co-financed by the European Social Fund.
491 Fellowship funding JAE-Predoc/CSIC was supported by the Ministerio de Economía
492 y Competitividad (MINECO). ProteoRed is part of the PRB2-ISCI, supported by
493 grant PT13/0001.

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Fig. 2. Composite 2D-DIGE image of separated *Malus domestica* 'Golden Delicious Smoothie' healthy and bitter pit protein spots. Circled spots with numbers represent differentially expressed proteins with $p < 0.05$. pH 3–7 NL IPG strips were used for the second dimension. kDa = kilodaltons (molecular weight). A total of 41 protein spots were identified as differentially expressed.

A.)

B.)

C.)

Figure 3. Selection of proteins identified between bitter pit and healthy samples. Amplification of spot characteristics with image analysis and statistics are for the following proteins: (A.) Differentially expressed healthy - Predicted: Germin-like protein (Spot 1859) (B.) Differentially expressed bitter pit - Major allergen Mal d 1 (Spot 3042) (C.) Non-differentially expressed - Predicted: UTP--glucose-1-phosphate uridylyltransferase (Spot 3194).

Figure 4. Distribution of total bitter pit and healthy *Malus domestica* proteins identified using SameSpots software. Significant proteins ($p < 0.05$) are displayed as circles and the circle overlap represents non significant proteins.

Figure 5. Composite 2D-DIGE image of separated *Malus domestica* 'Golden Delicious Smoothie' healthy spots. Circled spots with numbers represent differentially expressed proteins with $p < 0.05$ and minimum 2-fold change. pH 3-7 NL IPG strips were used for the second dimension. kDa = Kilodaltons (molecular weight).

Figure 6. Composite 2D-DIGE image of separated *Malus domestica* 'Golden Delicious Smoothee' bitter pit. Circled spots with numbers represent differentially expressed proteins with $p < 0.05$ and minimum 2-fold change. pH 3-7 NL IPG strips were used for the second dimension. kDa = Kilodaltons (molecular weight).

IDENTIFIED PROTEIN GENE ONTOLOGY (GO) FUNCTIONAL CATEGORIES

■ Toxin/ Defense ■ Metabolism/ Transport ■ Transferases ■ Other Enzyme

Figure 7. Protein Functional Categories for identified differentially expressed proteins with $p < 0.05$ and minimum 2-fold change.

Table 1. SameSpot software Identified *Malus domestica* protein spots categorized as significant ($p < 0.05$) after DIGE analysis. Spots were spotpicked and proteins analyzed using a LTQ-orbitrap coupled with an online database

#	Anova (p)	Fold	pI	Avg. Normalised Vol.		
				Healthy	Bitter Pit	
1788	0,006	55,9	3,64	0,054	3,042	
3063	0,002	46,7	3,51	0,071	3,292	*
3065	0,004	31,9	3,72	0,123	3,915	*
3061	0,002	27,9	3,54	0,103	2,881	*
3034	0,004	19,9	3,91	0,193	3,834	
3055	0,012	19,1	3,72	0,146	2,783	*
3069	0,003	15,4	6,4	0,164	2,529	
3054	0,01	13	3,6	0,193	2,503	
3070	0,02	12,6	6,43	0,199	2,507	
3047	0,024	10,6	4,91	0,296	3,133	*
3042	5,25E-05	10,3	5,21	0,219	2,253	*
3091	0,002	10,1	3,94	0,271	2,729	
3066	0,013	9,8	4,92	0,299	2,918	
3073	0,032	9,8	3,28	0,27	2,647	
1797	0,006	7,9	3,99	0,381	3,022	
3060	0,043	7,8	3,66	0,305	2,364	*
2261	0,013	7,2	4,9	0,343	2,461	
2366	0,005	6,4	3,96	0,436	2,795	
2268	0,012	5,8	4,64	0,446	2,59	*
2263	8,25E-04	5,5	4,19	0,445	2,471	
3080	0,011	4,8	6,01	0,368	1,772	
2275	0,005	4,3	4,41	0,614	2,635	*
2253	0,002	4,1	4,04	0,479	1,987	
3067	0,013	4,1	3,89	0,541	2,212	*
3077	0,013	4	4,63	0,616	2,484	
3058	0,007	3,6	3,76	0,485	1,76	
3057	0,013	3,4	3,76	0,579	1,978	
3062	0,011	3	4,52	0,723	2,196	
3032	0,003	2,9	4,9	0,737	2,122	
3085	0,044	2,8	4,14	0,724	2,041	
955	0,006	2,1	5,65	0,617	1,314	
1928	0,004	2	6,33	0,778	1,594	
1859	0,004	11,1	4,13	2,061	0,186	*
3039	0,006	6	6,23	1,263	0,212	
1843	0,039	4	5,14	1,04	0,26	
1072	0,033	3,4	6,13	1,108	0,327	
1358	0,027	3,3	4,53	1,38	0,417	*
1439	0,018	2,7	5,07	1,288	0,477	
1453	0,014	2,3	5,12	1,345	0,576	*
1308	0,003	2,3	6,25	1,085	0,469	
3084	0,017	2,1	4,47	1,396	0,671	

Greater Bitter Pit
Avg. Normalised Vol.

Greater Healthy
Avg. Normalised Vol.

* = Spots Identified using LTQ-orbitrap coupled with an online database

Table 2. Identified healthy *Malus domestica* protein spots after DIGE analysis. To be considered differentially expressed, DIGE spots had a p<0.05 and a minimum 2-fold difference, database (uniprot.org), after LTQ-Orbitrap protein identification. Spot ID = Spot Identified using SameSots Software; Proteins were identified using the reviewed *Malus domestica* database. Fold = Fold Change (quantity change); pI = Isoelectric Point; Acc. # = UniProtKB Accession number; GO (Gene Ontology); Scan = The spectrum's scan number within this MS/MS run; Rt = Retention time (in minutes); m/z = mass-to-charge ratio (The charge state of the best peptide assignment for this spectrum); MH+Obs. = Observed MH+ (Monoisotopic observed mass for the peptide + one proton) of the best peptide assignment for this spectrum MH+ theo Theoretical MH+of the best peptide assignment for this spectrum. DeltaMH+ Error in the precursor mass between observed and theoretical data (Da). Delta-ppm = Error in the precursor mass between observed and theoretical data (ppm).

Protein Name	Spot ID	Anova (p)	Fold	Avg.Norm. Vol. Healthy Bitter Pit	pI	Acc. #	Function	Gene Ontology (GO)	Scan	Rt	Sequence	m/z	MH+Obs	MH+theo	DeltaMH+	Delta-ppm	Identification
Predicted: Germin-like protein	1859	4,00E-03	11,1	2,061	0,186	4,13	Manganese Ion Binding/ Nutrient reservoir activity	Molecular Function	423	Unknown	FVTYLQK	2	Unknown	Unknown	Unknown	Unknown	DENOVO
							Unknown Manganese Ion Binding/ Nutrient reservoir activity	Molecular Function	523	Unknown	AFQLDDK	2	Unknown	Unknown	Unknown	Unknown	DENOVO
Predicted: UTP--glucose-1- phosphate uridylyltransferase	1453	0,014	2,3	1,345	0,576	5,12	Cytoplasm/ Nucleotidyltr ansferase/ Transferase	Molecular Function/ Cellular Component	525	Unknown	FLPVK	2	1506,729	1506,729	1,00E-04	0,066369	X!Tandem
							Unknown Cytoplasm/ Nucleotidyltr ansferase/ Transferase	Molecular Function/ Cellular Component	664	7,18	SDVASLSQI SENEK	2	Unknown	Unknown	Unknown	Unknown	DENOVO
1-aminocyclopropane-1- carboxylate oxidase 1	1358	0,027	3,3	1,38	0,417	4,53	Ethylene biosynthesis/ Fruit Ripening/ Oxidoreduct ase	Biological Process/ Molecular Function	316	5,08	FKEMVAAK	2	939,4964	939,4972	-8,00E-04	-0,851519	X!Tandem
							sp/Q00985 Ethylene biosynthesis/ Fruit Ripening/ Oxidoreduct ase	Biological Process/ Molecular Function	347	5,3	GPNFGTK	2	720,3672	720,3679	-8,00E-04	-1,110544	X!Tandem
							sp/Q00985 Ethylene biosynthesis/ Fruit Ripening/ Oxidoreduct ase	Biological Process/ Molecular Function	1266	11,17	MSIASFYNP GNDSFISAP AVLEK	3	2571,246	2571,244	0,0021	0,816725	X!Tandem
							sp/Q00985 Ethylene biosynthesis/ Fruit Ripening/ Oxidoreduct ase	Biological Process/ Molecular Function	1404	11,91	MSIASFYNP GNDSFISAP AVLEK	3	2555,248	2555,249	-9,00E-04	-0,352216	X!Tandem

Table 3. Identified bitter pit *Malus domestica* protein spots after DIGE analysis. To be considered differentially expressed, DIGE spots had a p<0.05 and a minimum 2-fold difference. database (uniprot.org), after LTQ-Orbitrap protein identification. Spot ID = Spot Identified using SameSots Software; Proteins were identified using the reviewed *Malus domestica* database. Fold = Fold Change (quantity change); pI = Isoelectric Point; Acc. # = UniProtKB Accession number; GO (Gene Ontology); Scan = The spectrum's scan number within this MS/MS run; Rt = Retention time (in minutes); m/z = mass-to-charge ratio (The charge state of the best peptide assignment for this spectrum); MH+Obs. = Observed MH+ (Monoisotopic observed mass for the peptide + one proton) of the best peptide assignment for this spectrum MH+ theo Theoretical MH+ of the best peptide assignment for this spectrum. DeltaMH+ Error in the precursor mass between observed and theoretical data (Da). Delta-ppm = Error in the precursor mass between observed and theoretical data (ppm).

Protein Name	Spot ID	Anova (p)	Fold	Avg-Norm. Vol.	pI	Acc. #	Function	Gene Ontology (GO)	Scan	Rt	Sequence	m/z	MH+Obs	MH+theo	DeltaMH+	Delta-ppm	Identification	
				Healthy	Bitter Pit													
PR-5 Family																		
Thaumatococcal-like Protein 1b	3055	0.012	19.1	0.146	2.783	3.72	spP83336	Plant Defense/ Stress Response	Biological Process/ Cellular Component	379	5.38	AADGSVISCK	2	1007,483	1007,483	0.0001	0.099257	X!Tandem
							spP83336	Plant Defense/ Stress Response	Biological Process/ Cellular Component	591	6.65	SACLAFGDSK	2	1055,483	1055,483	-0.0003	-0.28423	X!Tandem
Thaumatococcal-like Protein	3060	0.043	7.8	0.305	2.364	3.66	Unknown	Plant Defense/ Stress Response	Plant Defense/ Stress Response/ Secreted	395	Unknown	AADGSVLSCK	2	Unknown	Unknown	Unknown	Unknown	DENOVO
Thaumatococcal-like protein 1a (Mal d 2)	3067	0.013	4.1	0.541	2.212	3.89	spQ9FSG7	Plant Defense/ Stress Response	Plant Defense/ Stress Response/ Secreted	523	6.38	FTCETADCGSGQVAC	2	1662,618	1662,62	-0.0013	-0.781899	X!Tandem
							spQ9FSG7	Plant Defense/ Stress Response	Plant Defense/ Stress Response/ Secreted	543	6.5	CPAPLQVK	2	912,497	912,497	-6.00E-04	-0.657536	X!Tandem
							trQ00MX4	Glycosidase/ Hydrolase	Molecular Function	629	7.01	ASQSVDPAPSWSGR	2	1444,682	1444,682	3.00E-04	0.207658	X!Tandem
							trQ00MX4	Glycosidase/ Hydrolase	Molecular Function	1260	10.97	TFTCSGGPDYVHTCP	3	3612,505	3612,503	0.0014	0.387543	X!Tandem
PR-8 Family																		
Pathogenesis-related protein 8	3065	0.004	31.9	0.123	3.915	3.72	trQ00MX4	Glycosidase/ Hydrolase	Biological Process/ Molecular Function	619	7.01	FYDNGYSASIK	2	1264,584	1264,585	-0.001	-0.790773	X!Tandem
Pathogenesis-related protein 8	3063	0.002	46.7	0.071	3.292	3.51	trQ00MX4	Glycosidase/ Hydrolase	Molecular Function	900	8.7	FYDNGYSASIKDSI	2	1579,727	1579,728	-1.00E-03	-0.63302	X!Tandem
							trQ00MX4	Glycosidase/ Hydrolase	Molecular Function	1116	10.08	MGLPAAPEAAPSGGFP	3	2097,069	2097,069	1.00E-04	0.047686	X!Tandem
Predicted: Chitinase	3061	0.002	27.9	0.103	2.881	3.54	Unknown	Chitin-Binding/ Glycosidase/ Hydrolase	Biological Process/ Molecular Function	1193	10.71	VNYTYDYCSQFGVDP	2	2460,019	2460,012	0.0066	2.682913	X!Tandem
PR-10 Family																		
Major allergen Mal d 1	3042	5.25E-05	10.3	0.219	2.253	5.21	spQ40280	Plant Defense/ Disease/ Pathogenesis-related Protein	Biological Process	312	4.92	SISHYHTK	2	972,4866	972,4902	-0.0036	-3.701837	X!Tandem
							spQ40280	Plant Defense/ Disease/ Pathogenesis-related Protein	Biological Process	560	6.42	HAIELEGDGGPGTIK	3	1493,759	1493,76	-6.00E-04	-0.401671	X!Tandem
							spQ40280	Plant Defense/ Disease/ Pathogenesis-related Protein	Biological Process	527	6.4	HAIELEGDGGPGTIK	2	1493,759	1493,76	-0.0007	-0.468616	X!Tandem
							spQ40280	Plant Defense/ Disease/ Pathogenesis-related Protein	Biological Process	367	Unknown	LDSEIK	2	Unknown	Unknown	Unknown	Unknown	DENOVO
							spQ40280	Plant Defense/ Disease/ Pathogenesis-related Protein	Biological Process	374	Unknown	AHGLF	2	Unknown	Unknown	Unknown	Unknown	DENOVO
							spQ40280	Plant Defense/ Disease/ Pathogenesis-related Protein	Biological Process	529	Unknown	XLEDGGPASLK	3	Unknown	Unknown	Unknown	Unknown	DENOVO
Major allergen Mal d 1.03E	2268	0.012	5.8	0.446	2.59	4.64	trQ4VPI3	Plant Defense/ Pathogenesis-related Protein	Biological Process	336	Unknown	ASHLTK	2	Unknown	Unknown	Unknown	Unknown	DENOVO
							trQ4VPI3	Plant Defense/ Pathogenesis-related Protein	Biological Process	1200	10.7	LIENYLLENQDAYN	2	1711,82	1711,818	0.0023	1.343601	X!Tandem
Major allergen Mal d 1.03E	2275	0.005	4.3	0.614	2.635	4.41	trQ4VPI3	Plant Defense/ Pathogenesis-related Protein	Biological Process	314	5.16	GDVEIKEEHVK	3	1282,665	1282,664	3.00E-04	0.233888	X!Tandem
Major Allergen Mal d 1.06A							trA0AUE3	Plant Defense/ Pathogenesis-related Protein	Biological Process	342	5.32	ASHLTK	2	702,3935	702,3938	-3.00E-04	-0.427111	X!Tandem
							trA0AUE3	Plant Defense/ Pathogenesis-related Protein	Biological Process	894	8.67	TVEILEGDDGGVGTIK	2	1487,796	1487,796	2.00E-04	0.134427	X!Tandem
							trQ4VPI3	Plant Defense/ Pathogenesis-related Protein	Biological Process	1177	10.47	LIENYLLENQDAYN	2	1608,783	1608,791	-8.00E-03	-4.972679	X!Tandem
							trQ4VPI3	Plant Defense/ Pathogenesis-related Protein	Biological Process	1220	10.75	LIENYLLENQDAYN	2	1711,82	1711,818	3.00E-04	0.175252	X!Tandem
Predicted: UTP-glucose-1-phosphate uridylyltransferase	3047	0.024	10.6	0.296	3.133	4.91	Unknown	Metabolic enzymes	Biological Process/ Molecular Function	519	6.37	AAVATGNVDK	2	987,5112	987,511	-4.00E-04	-0.405059	X!Tandem
							Unknown	Metabolic enzymes	Biological Process/ Molecular Function	658	7.21	SDVASLSQISENEK	2	1506,729	1506,729	1.00E-04	0.066369	X!Tandem
							Unknown	Metabolic enzymes	Biological Process/ Molecular Function	862	8.49	VLQLETAAGAAR	2	1312,758	1312,759	9.00E-04	0.685579	X!Tandem

Table 4. SumoSpot software identified *Makar domestica* protein spots categorized as non-significant (p>0.05) after DMC analysis.

Pick ID	Area (p)	Fold	pI	Avg Norm. Vol.	
				Healthy	Bitter Pe
1300	0.051	1.6	5.97	1.194	0.754
2109	0.051	2.2	3.64	0.074	1.454
1422	0.051	4.1	3.01	0.538	2.201
1790	0.051	2.7	4.74	1.665	0.612
3020	0.053	2.3	5.99	0.496	1.166
1222	0.053	5.8	6.99	3.238	0.554
981	0.054	2.1	6.42	1.195	0.576
5391	0.142	36.8	5.79	0.211	7.773
3970	0.148	3.5	6.57	1.195	0.541
5433	0.154	2.6	3.05	4.119	1.594
3202	0.167	3.5	4.54	1.297	0.375
5447	0.178	2	4.32	1.186	0.591
3647	0.18	3.6	5.42	1.071	0.299
4779	0.18	2.4	3.74	1.533	0.657
4260	0.187	2.9	3.85	2.805	0.972
5465	0.191	1.3	3.9	1.138	0.854
3182	0.202	1.8	6	0.088	1.264
4774	0.204	1.5	5.73	0.661	0.996
5363	0.207	2.2	4.85	1.418	0.655
5415	0.209	3.6	6.56	1.737	0.485
4013	0.211	4.7	5.16	0.682	3.208
5424	0.211	3	3.81	0.939	0.113
5444	0.212	2.1	5.24	1.707	0.809
5173	0.213	3.1	3.05	0.974	0.31
5452	0.226	1.8	4.56	2.017	1.128
4236	0.227	2.3	4.46	1.608	0.708
5395	0.228	9.5	6.32	1.077	0.113
3174	0.231	1.8	6.01	1.518	0.837
4417	0.243	1.5	3.92	0.702	0.499
5422	0.244	3	6.4	1.132	0.375
5448	0.252	2	5.09	0.71	1.422
5453	0.259	4.7	5.13	1.52	0.92
5451	0.278	1.9	6.63	1.025	0.551
5457	0.28	1.5	4.47	1.63	6.7
5407	0.291	5.1	6.43	3.542	0.697
3827	0.296	1.2	5.46	1.942	1.582
3201	0.297	1.6	5.32	1.292	0.823
3731	0.297	2.9	6.58	1.68	0.572
3170	0.3	1.4	5.58	1.36	0.36
5400	0.304	6.3	4.85	0.406	2.573
3028	0.306	1.4	4.26	0.931	0.673
3543	0.31	2.6	5.64	1.628	0.629
5423	0.314	3	5.3	2.22	0.735
3303	0.315	1.5	4.78	1.433	0.975
5393	0.316	12	3.92	0.206	2.48
5426	0.317	2.9	3.91	1.27	3.675
5463	0.327	1.3	3.86	1.025	0.76
3195	0.329	2.2	5.32	0.695	1.542
3888	0.33	1.5	5.6	2.725	1.849
5432	0.333	2.6	4.29	0.744	1.93
5411	0.334	4.3	3.01	1.999	0.464
3526	0.343	1.8	5.22	1.548	0.855
4895	0.346	1.4	4.26	1.095	0.764
5419	0.346	3.1	3.68	0.709	2.214
5442	0.348	2.1	5.63	0.991	0.466
5429	0.35	2.7	5.28	1.426	0.534
5428	0.361	2.7	5.41	0.431	1.157
5439	0.367	2.3	5.3	2.503	1.101
5470	0.367	1.2	6.76	1.218	1.026
5398	0.369	6.9	5.91	3.071	0.442
4700	0.372	1.6	5.47	1.046	0.642
3238	0.379	2.3	6.27	1.66	0.728
2909	0.381	1.2	5.11	0.834	0.67
5394	0.382	9.5	4.28	0.276	2.64
5406	0.383	5.5	6.76	2.613	0.473
5413	0.385	3.7	3.28	0.914	0.246
5450	0.393	2.7	5.58	1.275	3.444
4053	0.396	1.9	5.48	0.706	1.329
3347	0.397	4.6	7	0.528	2.446
4468	0.397	2.6	3.72	1.074	2.765
4051	0.402	1.3	5.37	0.986	0.761
4724	0.402	2.1	5.37	4.16	1.949
4312	0.405	1.3	6.57	1.317	1.017
5438	0.413	2.3	5.8	1.203	2.811
4484	0.422	2.1	5.01	1.375	2.852
5396	0.427	8.6	5.62	0.271	2.328
3282	0.431	1.4	5.79	0.672	0.957
3561	0.436	2.3	4.88	1.201	0.524
5204	0.444	3.3	4.54	3.688	1.104
4988	0.451	1.5	5.69	0.824	0.538
5401	0.451	6.1	4.33	0.566	2.237
5436	0.46	2.4	4.15	0.557	1.331
5420	0.464	3	6.8	0.575	1.746
5405	0.48	5.7	3.91	4.142	0.728
5409	0.487	1.2	4.73	0.856	0.71
3512	0.491	1.6	5.01	2.187	1.33
5454	0.501	1.6	3.04	1.21	1.98
3783	0.502	2.6	6.1	2.233	0.849
3722	0.504	1.5	6.3	1.424	0.933
3432	0.507	1.8	5.32	1.149	0.627
3461	0.513	1.4	3.05	2.047	1.473
5414	0.518	3.6	5.56	0.522	1.884
5459	0.523	1.4	6.52	0.797	0.35
3517	0.524	1.5	4.55	1.187	0.809
5045	0.53	4.3	6.08	1.422	0.33
2908	0.533	2.6	4.62	0.491	1.202
5208	0.533	1.4	4.43	0.788	0.564
4760	0.534	1.6	3.47	0.857	0.545
3120	0.559	6.3	4.81	0.771	2.338
3775	0.563	1.2	5.27	1.661	1.345
3446	0.573	2.1	4.85	0.797	1.444
3659	0.576	1.4	5.34	1.478	1.039
5425	0.581	2.9	5.23	1.361	4.003
4273	0.584	1.5	6.09	0.799	1.215
5417	0.594	3.4	6.44	0.788	2.648
3344	0.608	1.1	5.46	1.853	1.678
3745	0.614	1.8	3.62	2.41	1.323
5358	0.62	1.2	6.17	3.211	3.795
3653	0.622	1.2	4.34	1.422	1.186
3044	0.638	3	6.67	1.123	0.38
3015	0.638	1.3	6.18	2.028	1.613
4211	0.644	1.1	6.08	1.545	1.763
4123	0.651	1.2	5.86	1.303	1.081
5460	0.667	1.4	5.36	3.611	2.555
5437	0.684	2.3	5.45	2.915	1.242
3449	0.685	2	5.2	0.698	1.79
3460	0.686	1.3	4.43	0.905	0.688
3547	0.692	1.3	5.6	1.399	1.774
3045	0.693	1.4	5.06	1.035	0.711
5473	0.695	1.1	5.06	1.709	1.554
4773	0.704	1.2	5.32	1.613	1.345
4695	0.708	1.1	4.15	2.042	1.826
5467	0.713	1.2	6.36	4.745	3.81
5472	0.72	1.2	4.54	0.736	0.639
3194	0.737	1.1	6.56	0.994	0.898
4302	0.737	1.4	5.2	0.996	0.705
5445	0.743	2.1	6.39	1.047	2.17
3491	0.746	2.9	6.09	0.701	2.044
4756	0.747	1.1	5.34	3.71	3.99
3143	0.751	1.1	4.61	1.657	1.483
5410	0.757	4.3	5.72	0.381	1.646
5474	0.757	1.1	5.01	1.116	1.019
3275	0.759	1.2	6.19	0.649	0.749
3698	0.765	1	6.27	1.067	1.032
5427	0.77	2.8	5.86	1.716	4.782
5477	0.771	1	5.98	4.424	4.208
4762	0.783	1.1	4.3	2.925	2.607
5458	0.794	1.4	6.56	7.027	4.854
5475	0.801	1.1	6.37	1.236	1.128
3513	0.803	1.1	3.79	1.147	1.009
3417	0.804	1.1	4.38	0.912	0.961
5456	0.804	1.5	6.15	0.702	1.04
3070	0.805	1.3	5.03	1.042	1.397
5462	0.827	1.4	6.87	0.701	0.519
5471	0.835	1.2	3.94	0.795	0.937
3348	0.842	1.1	5.35	1.297	1.434
3302	0.854	1.2	6.98	3.564	2.875
5464	0.854	1.3	35	1.931	1.442
5478	0.868	1	6.68	1.189	1.174
3129	0.872	1.5	4.96	0.686	1.056
2911	0.875	1.1	4.22	0.704	0.798
3858	0.884	1.1	6.46	1.009	1.095
5466	0.886	1.3	4.34	0.872	1.161
3906	0.887	1.4	4.12	0.974	1.333
5455	0.924	1.5	4.03	1.011	1.545
5479	0.928	1	6.23	0.929	0.641
3847	0.931	1.6	6.59	5.962	3.756
5476	0.932	1.1	6.22	0.91	0.909
5468	0.945	1.2	5.79	2.784	2.239
3317	0.95	1.3	4.65	0.672	0.861
3334	0.955	1.2	4.11	0.97	1.132