Abstract: The cultivated almond displays a gametophytic self-incompatibility system, which avoids self-fertilization, and it is controlled by a multi-allelic locus (S-locus) containing two genes specifically expressed in pistil (S-RNase) and pollen (SFB). Studies on almonds with the same S-haplotype but different phenotype pointed to the existence of unknown components in this system to explain its functioning. The increase of knowledge on this reproductive barrier would allow better management of fruit production and germplasm selection. This work proposes candidates to components of the almond gametophytic self-incompatibility system, by identifying differentially expressed proteins (DEPs) after compatible and incompatible pollen–pistil interactions in almonds with the same S-haplotype but a different incompatibility phenotype using iTRAQ and 2D-nano-LC ESI/MSMS analyses. The protein quantitation analysis revealed 895 DEPs, which were grouped into different functional categories. The largest functional group was “metabolic proteins”, followed by “stress resistance and defense proteins”, with higher up-regulation after pollination. The identity of certain DEPs, such as Thaumatin, LRR receptors, such as kinase and pathogenesis related protein PR-4, resistance and defense proteins”, with higher up-regulation after pollination. The identity of certain DEPs, such as Thaumatin, LRR receptors, such as kinase and pathogenesis related protein PR-4, indicated that some pollen–pistil interactions in almond could have the same bases as host–parasite interactions. Furthermore, additional RT-qPCR analysis revealed the differentially expressed transcription regulator GLABROUS1 enhancer-binding protein-like (GEBPL) could be involved in the gametophytic self-incompatibility system in almond.

Keywords: gametophytic self-incompatibility; iTRAQ; Prunus dulcis; RT-qPCR; 2D-nano-LC ESI/MSMS

1. Introduction

The cultivated almond (Prunus dulcis (Miller) D.A. Webb) is nowadays widely grown (FAOSTAT data 2019), and it is one of the most economically important fruit tree species in the world due to the multiple nutritional benefits of its seed. Almond fruit production is often limited by gametophytic self-incompatibility, a genetic barrier that avoids self-fertilization and fertilization among individuals with identical incompatibility genotypes. Like in other Prunus species, self-incompatibility is controlled by the S-locus, which contains at least two linked genes; one is expressed in the pistil as ribonucleases (S-RNases), and the other encodes for S haplotype-specific F-box proteins (SFBs) that are specifically expressed in pollen [1]. Although the specificity of the incompatibility response is determined by the S-locus, other loci seem to be required for the functioning of this system in almonds. In this sense, it was proposed that two quantitative trait loci (QTL) located in linkage groups 6 and 8 are associated with self-incompatibility [2].

The majority of almond cultivars have been found to be self-incompatible; however, self-compatible cultivars have also been identified mainly in Apulia (Italy) [3,4]. Since the Sf-haplotype (Sf-RNase and SFBf) is present in nearly all self-compatible almonds from Apulia, the self-compatible phenotype had unequivocally been associated with the Sf-haplotype a. However, some almond cultivars with the Sf-haplotype have been reported to
be self-incompatible [5,6]. To unravel this inconsistency, in a previous study we compared Sf-RNase expression levels in self-compatible and self-incompatible almonds and found that the self-compatible phenotype in almonds seems to be due to the very low levels of Sf-RNase transcripts [7]. In this study, we also hypothesized that the regulation of the S-RNase expression in almonds may be a case of combinatorial regulation, in which several transcription factors acting as activators would be involved [7]. Therefore, the low transcription of Sf-RNase in self-compatible almonds may be due to inactivation or absence of one of the activators, which in turn would result in levels of this S-RNase in the style below the threshold required for pollen rejection [7]. This clearly indicates the involvement of other components apart from S-RNase and SFB in the almond self-incompatibility system.

Transcriptomic, proteomic, and metabolic fingerprinting studies have been developed in Prunus species to find additional components of the gametophytic self-incompatibility system [7–13].

Apart from self-incompatibility, multiple pollen–pistil interactions, including guiding of pollen by the female tissues, take place during growth of pollen tubes from the stigma to the ovary. Some of these interactions occur at the style transmitting track tissue during the proagamic phase (from pollination to fertilization). As indicated in [14,15], several pollen tube and transmitting track components have been identified to either influence pollen growth rates and/or guide pollen directional growth to the ovule. Some of these components are Transmitting Tissue-Specific protein (TTS), small CRPs secreted by the stylar transmitting tract, Stigma/style Cysteine-rich Adhesin (SCA), LTP5 (a SCA-like LTP), chemocyanin, plantacyanin, gamma-aminobutyric acid (GABA), pollen receptor kinases LePRK1 and LePRK2, Style Interactor for LePRKs (STIL), cytosolic free calcium, and reactive oxygen species (ROS).

The objectives of the present work were to obtain proteomic profiles of pistils with the Sf-haplotype and a different incompatibility phenotype and to identify the proteins that are differentially expressed after compatible and incompatible pollinations. To reach these aims, we performed isobaric tags for relative and absolute quantitation (iTRAQ) and two dimensional-nano-liquid chromatography–electrospray ionization tandem mass spectrometry (2D-nano-LC ESI/MSMS) analyses. The iTRAQ technique is a labelling method that allows for the quantitation of multiplexed peptides and protein changes by comparing four samples using synthetic isobaric peptide standards [16]. It has several advantages when compared to other proteomic methods, including ease of implementation, replication and speed [17]. Two D-nano-HPLC-ESI-MS/MS spectrometry has been defined as a highly sensitive and selective analytical technique that has become a powerful method for the identification of proteins present in complex mixtures [18]. Additional reverse transcription quantitative PCR (RT-qPCR) analysis was used to validate the expression of a differentially expressed protein in pollinated and unpollinated pistils.

2. Materials and Methods
2.1. Plant Material and Controlled Pollinations

The plant material used consisted of around 100 flower buds at ‘D-E’ developmental stage [19] from the almond selections A2-198 and ITAP-1, both with the Sf haplotype but a different incompatibility phenotype. A2-198 is self-compatible and carries the SfSf genotype [20], whereas ITAP-1 has been genotyped as SfSf and it is self-incompatible [6]. Controlled pollination of A2-198 with ITAP-1 resulted to be compatible, and in contrast, pollination on the opposite direction (ITAP-1 × A2-198) resulted to be incompatible [21]. Two samples of 0.5 g of pistils from each selection were independently frozen at −80 °C on the same day the flower buds were collected and were kept frozen until iTRAQ analysis or RNA extractions were performed.

The rest of the flower buds were prepared for controlled pollinations by removing their stamens and petals, leaving only the pistils attached to the calyx. The pistils were then set with the calyx, inserted in wet floral foam blocks on plastic trays, and placed inside an incubation chamber with controlled conditions (22 °C and 60% relative humidity) to be
pollinated (Figure 1a). At the same time, A2-198 anthers were kept at room temperature for 12 h to allow dehiscence. After this time, pollen viability was determined as detailed in [22].

The next day, A-2198 pollen was used to hand-pollinate A2-198 and ITAP-1 pistils, which after pollination were kept for either 48 h (for RNA extraction) or 72 h (for protein extraction) under the same controlled conditions to ensure that gametophytic self-incompatibility interaction took place [23] (Figure 1b). After these times, samples of 0.5 g of pollinated pistils from each selection were devoid of the calyx and frozen separately at −80 °C (Figure 1c).

![Figure 1](image-url)

**Figure 1.** Controlled pollination. (a) Pollinated pistils on floral foam, (b) detail of pollinated pistil during the incubation period, (c) pistil devoid of the calyx before being frozen for subsequent analyses.

In summary, the samples to be analyzed both by iTRAQ and RT-qPCR consisted of four different pools of pistils: unpollinated A2-198 pistils, unpollinated ITAP-1 pistils, self-pollinated A2-198 pistils (compatible pollination), and ITAP-1 pistils pollinated with A2-198 (incompatible pollination).

2.2. iTRAQ and 2D-nano-HPLC-ESI-MS/MS Analyses

2.2.1. Protein Extraction, Digestion, and Tagging with iTRAQ-4-plex® Reagent

The different pistil samples were independently ground in liquid nitrogen to obtain a fine powder, from which proteins were extracted as detailed in [11]. After extraction, the samples were quantified using the Bradford method [24] and delivered on dry ice to the ProteoRed service at CNB-CSIC (Madrid, Spain) for further analysis. Sample digestion was performed as indicated in [11], and iTRAQ labelling was performed separately using a 4-plex design for each condition studied (Tag-114: ITAP-1 pistils; Tag-115: A2-198 pistils; Tag-116: ITAP-1 × A2-198; Tag-117: A2-198 × A2-198). Finally, samples of the same protein content were combined, and the labelling reaction was stopped by the addition of 100 µL of 50% ACN and evaporation in a vacuum concentrator. The digested, labelled, and pooled peptide mixture was desalted using a Sep-Pak C18 Cartridge (Waters) following the manufacturer’s indications. The cleaned tryptic peptides were then evaporated to dryness and stored at −20 °C for further analysis.

2.2.2. Liquid Chromatography and Mass Spectrometer Analysis

A triple analysis was performed for each sample. An aliquot of the tryptic peptides was analyzed by LC ESI-MSMS using a nano-liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, AB SCIEX, Foster City, CA, USA) coupled to a high-speed Triple TOF 5600 mass spectrometer (AB SCIEX, Foster City, CA, USA) with a dual
spray ionization source. The following silica-based reversed phase column with a 3 µm particle size and a 120 Å pore size was used: C18 ChromXP 75 µm × 15 cm (Eksigent Technologies, AB SCIEX, Foster City, CA, USA). The nano-pump was set at a flow rate of 300 nL/min under gradient elution conditions using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Data was acquired with a TripleTOF 5600 System (AB SCIEX, Concord, ON, USA), using the information-dependent acquisition mode with Analyst TF 1.5 software (AB SCIEX, Framingham, MA, USA). The switching criteria were set to include ions greater than the mass to charge ratio (m/z) 350 and smaller than m/z 1250 with a charge state of 2–5 and an abundance threshold of more than 90 counts. The former target ions were excluded for 20 s.

2.2.3. Data Analysis of Mass Spectrometry

We processed the mass spectrometry and MS/MS data obtained for pooled samples using Analyst® TF 1.5.1 Software (AB SCIEX). The mgf files generated by raw data conversion tools were also searched against the UniProtKB/SwissProt database for Prunus persica (taxon identifier: 3760), which was found to contain 28,639 protein-coding genes and their corresponding reversed entries using the Mascot Server v. 2.3.02 (Matrix Science, London, UK). The search parameters were set as follows: enzyme, trypsin; allowed missed cleavages, 1; fixed modifications, iTRAQ4plex (N-term and K) and beta-methylthiolation of cysteine; and variable modifications, oxidation of methionine. Peptide mass tolerance was set to ±20 ppm for precursors and 0.05 Da for fragment masses.

As in [25], the confidence interval for protein identification was set to ≥95% (p < 0.05) and only peptides with an individual ion score above the 1% false discovery rate (FDR) threshold were considered correctly identified. For quantitative analysis, only the correctly identified proteins having at least two quantitated peptides were considered in the quantitation. To obtain iTRAQ protein ratios, the median was calculated over all distinct peptides assigned to a protein subgroup in each replicate. Then, each iTRAQ channel was normalized by dividing each protein ratio by the median of the ratio in each channel. This normalized median in each replicate was used to obtain the final geometric media of the corresponding protein. After calculating log2 of geometric media, frequency distribution histograms were obtained from Excel 2010. Log2 protein ratios were fitted a normal distribution using least squares regression. Mean and standard deviation values derived from the Gaussian fit were used to calculate p values and FDR (at quantitation level). The FDR for quantitation was then calculated as the FDR = (E value/protein rank), with E value = (p value * total number of quantified proteins) and the protein rank the individual position of the specific protein after ordered it by its p value. A 5% quantitation FDR threshold was estimated to consider the significant differentially expressed proteins.

The identity of each protein was assessed after a BLAST search on 30 September 2021 in the UniProtKB/SwissProt database (https://www.uniprot.org (accessed on 30 September 2021)). In those particular cases in which the proteins appeared as un-characterized in UniProtKB/SwissProt database, the associated accession numbers were BLAST searched in the NCBI database (https://www.ncbi.nlm.nih.gov (accessed on 30 September 2021)). Finally, the genes associated with the identified proteins were located in the Prunus persica Whole Genome v1.0 [26] using Phytozome database v9.1 (http://www.phytozome.net (accessed on 30 September 2021)). For the selection of candidate genes, priority was given to those genes located in linkage groups 3, 6, and 8, according to previous studies in Prunus proposing the location of genes putatively involved in gametophytic self-incompatibility in those linkage groups [2,27,28].

2.3. RNA Isolation and cDNA Synthesis

Total RNA was extracted according to [29] from 0.5 g of frozen samples of unpollinated pistils of A2-198 and ITAP-1, and of A2-198 and ITAP-1 pistils after 48 h of pollination with A2-198. The quality and quantity of the RNA samples were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and after checking them
in an RNAse-free agarose gel, cDNA was synthetized using SSIII Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) for subsequent amplification by standard and quantitative PCR.

2.4. Full-Length cDNA Amplification and Sequence Analysis

A full-length cDNA was amplified by standard PCR from cDNA of ITAP-1 and A2-198 pistils using specific primers from peach coding sequence ppa006690m, available in the GDR Rosaceae database (https://www.rosaceae.org (accessed on 30 September 2021)) (Forward primer: 5′-ATG GCA CCC AAG CGC C-3′; Reverse primer: 5′-CTA ATG CTC AGA TGA CTT TAA CGC C-3′). High-fidelity PCR was performed using KOD Hot Start DNA polymerase (Novagen), in a 25 uL mix with 150 ng of cDNA and 7.5 µM of primers. The PCR reaction was incubated at 94 °C for 2 min. for the initial denaturalisation step, followed by 35 cycles of 94 °C for 30 s, 62 °C for 1 min. and 68 °C for 1 min. A final extension step at 68 °C was set for 10 min. The PCR product obtained was cloned into One Shot™ TOP10 Chemically Competent E. coli cells (Invitrogen, Carlsbad, CA, USA), using a Zero Blunt Topo PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). Three positive plasmids from each sample were sent for sequencing to SECUGEN (Madrid, Spain). The plasmid sequences obtained were aligned and translated using SeqManII and EditSeq tools from DNASTAR software (Madison, WI, USA). In order to study the molecular function of the protein, the BLAST search in the NCBI database was performed using the predicted protein sequence.

2.5. RT-qPCR Analysis

Reverse transcription qPCR experiments were carried out to investigate the expression pattern of a differentially expressed gene in unpollinated pistils of ITAP-1 and A2-198, and in compatible (A2-198 × A2-198) and incompatible (ITAP-1 × A2-198) pollinations, with Step One Plus real-time PCR system (Applied Biosystems). Specific primers (Primer forward: 5′-ATG GCA CCC AAG CGC C-3′; Primer reverse: 5′-TCC GGA TTC TTC CTC TTC TTC-3′), based on the almond sequence obtained here were designed using Primer 3 software (http://primer3.ut.ee/ (accessed on 30 September 2021)) [30].

For all real-time qPCR reactions, a 10 µL mix was made with 5 µL Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 10ng of cDNA, and 2.5 µM of each primer. The qPCR conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The melting temperature in these experiments was set to 60 °C~95 °C, increasing by 0.3 °C/s. Each biological sample was implemented in triplicate. RNA Polymerase II (RPII) was used as a reference gene for data normalization [31], and the levels of relative expression were calculated by the comparative method [32]. Expression of A2-198 and ITAP-1 pollinated pistils was relative to the expression level of the controlled unpollinated pistils, respectively.

3. Results

3.1. iTRAQ Analysis

A total of 1132 proteins were obtained in the iTRAQ analysis of unpollinated and pollinated pistils. However, after mass spectrometry, only 895 proteins were considered in the quantitation analysis, since at least two peptides for these proteins could be quantified. This criterion was used to ensure the validity of the results. To analyze the quantified proteins, the following comparisons were performed: unpollinated A2-198 pistils vs. unpollinated ITAP-1 pistils, self-pollinated A2-198 pistils (compatible pollination) vs. unpollinated A2-198 pistils, ITAP-1 pistils pollinated with A2-198 (incompatible pollination) vs. unpollinated ITAP-1 pistils, and self-pollinated A2-198 pistils vs. ITAP-1 pistils pollinated with A2-198.

Variable numbers of differentially expressed proteins (DEPs) were obtained for the comparisons analyzed (Table 1), and some of these DEPs were identical among the different comparisons. The number of common DEPs between compatible and incompatible pollinations is indicated in Figure 2. Nine proteins were common in all comparisons, and 13 were
common between self-compatible and self-incompatible pollinations vs. corresponding unpollinated pistils. Furthermore, 9 and 10 DEPs were common in the comparison of pollinated pistils and in the comparison of self-compatible and self-incompatible pollinations, respectively. On the other hand, as observed in the Venn diagram (Figure 2), the largest group of DEPs was obtained when comparing the compatible interaction with the incompatible interaction.

Table 1. Number of differentially expressed proteins (DEPs) obtained in each comparison.

| Comparison a | DEPs | Up-Regulated b | Down-Regulated c | Unidentified |
|--------------|------|----------------|------------------|-------------|
| A vs. I      | 13   | 6              | 7                | -           |
| A × A vs. A | 44   | 37             | 7                | 9           |
| I × A vs. I | 42   | 36             | 6                | 9           |
| A × A vs. I × A | 69 | 26             | 43               | 11          |

a A: A2-198 (SfSf, self-compatible); I: ITAP-1 (S11Sf, self-incompatible). b Proteins up-regulated in the first condition. c Proteins down-regulated in the first condition.

Figure 2. Venn diagram of the number of differentially expressed proteins retrieved in the different comparisons of pollinated pistils performed by DE analyses. The comparisons correspond to: A2-198 × A2-198 vs. A2-198: Compatible pollination vs. unpollinated pistils of self-compatible almond; ITAP-1 × A2-198 vs. ITAP-1: Incompatible pollination vs. unpollinated pistils of self-incompatible almond; A2-198 × A2-198 vs. ITAP-1 × A2-198: Compatible pollination vs. incompatible pollination.

The differentially expressed proteins were in different scaffolds of the Prunus persica genome (Tables S1–S4), most of them being in scaffold 1. In addition, BLAST characterization of the DEPs made it possible to determine their function. Taking this knowledge into consideration, together with whether the DEPs were up- or down-regulated in each condition after the compatible or incompatible pollination, certain proteins were considered as good candidates to components of the gametophytic self-incompatibility system in almonds (Table 2). Most of these proteins have a function related to the pathogenesis process or to hydrolase activity, while only two have GTP binding and transcription regulation functions.
Table 2. Differentially expressed proteins for the four comparisons performed. In each comparison, the grey color indicates up-regulation of the protein for the first condition, whereas the orange color indicates down-regulation for the first condition. For each protein accession number description, the function, corresponding accession number in the “Genome Database for Rosaceae” (GDR gen), and scaffold location in the peach genome are indicated.

| A vs. I | A × A vs. A | I × A vs. I | A × A vs. I × A | Description | Function | GDR Gen | Scaffold |
|---------|-------------|-------------|-----------------|-------------|----------|---------|----------|
| Stress resistance and defense | | | | | | |
| M5W1Q5 | M5W1Q5 | M5W1Q5 | M5W1Q5 | Putative LRR receptor-like serine/threonine-protein kinase | Pathogenesis process | ppa007248m | 8 |
| M5W1Q5 | M5W1Q5 | M5W1Q5 | M5W1Q5 | Pathogenesis-related protein PR-4 | Pathogenesis process | ppa012991m | 6 |
| M5WR86 | M5WR86 | M5WR86 | M5WR86 | Pathogenesis-related thaumatin-like protein | Pathogenesis process | ppa019480m | 5 |
| M5WT96 | M5WT96 | M5WT96 | M5WT96 | Pathogenesis-related thaumatin-like protein | Pathogenesis process | ppa024543m | 5 |
| Cellular structure, organization and biogenesis | | | | | | |
| M5WH02 | M5WH02 | M5WH02 | M5WH02 | Xyloglucan endotransglucosylase | Hydrolase activity | ppa009685m | 4 |
| M5WJ79 | M5WJ79 | M5WJ79 | M5WJ79 | Xyloglucan endotransglucosylase | Hydrolase activity | ppa009472m | 6 |
| M5WT30 | M5WT30 | M5WT30 | M5WT30 | Xyloglucan endotransglucosylase | Hydrolase activity | ppa009608m | 3 |
| Signal transduction | | | | | | |
| M5XF62 | M5XF62 | M5XF62 | M5XF62 | GTP-binding nuclear protein | GTP binding | ppa007452m | 1 |
| Transcription regulation | | | | | | |
| M5WQU9 | M5WQU9 | M5WQU9 | M5WQU9 | GLABROUS1 enhancer-binding protein-like | Transcription regulation | ppa006690m | 3 |

Superscript notes:

* A vs. I: Unpollinated A2-198 (Sf/Sf, self-compatible) pistils vs. unpollinated ITAP-1 (S11/Sf, self-incompatible) pistils. b A × A vs. A: Self-pollination of A2-198 vs. unpollinated A2-198 pistils. c I × A vs. I: Pollination of ITAP-1 with A2-198 vs. unpollinated ITAP-1 pistils. d A × A vs. I × A: Self-pollination of A2-198 vs. pollination of ITAP-1 with A2-198. e According to UniProtKB/SwissProt and NCBI databases. Those proteins with an asterisk appeared as un-characterized in UniProtKB/SwissProt database. f Identification code according to the Genome Database for Rosaceae linked to the accession number of each protein. g Location in the Prunus persica Whole Genome v1.0.
3.2. Differentially Expressed Proteins in Unpollinated Pistils

The quantitative analysis showed 13 DEPs in unpollinated pistils. Of these DEPs, seven were down-regulated and six were up-regulated in the self-compatible almond A2-198 (Table S1). All DEPs in the comparison of unpollinated pistils were characterized after a BLAST search in the UniProtKB/SwissProt database. The DEPs were then classified into different functional groups according to the role of the proteins in cellular pathways (Figure 3). This distribution showed that the main functional group was “metabolic proteins” (69%), followed by “stress resistance and defense proteins” (15%). In addition, the categories “signal transduction” and “cellular structure, organization and biogenesis” included up-regulated DEPs in the self-compatible selection (Figure 4a).

![Figure 3. The functional distribution of the differentially expressed proteins in each comparison.](image)

![Figure 4. The functional distribution of the differentially expressed proteins after the compatible pollination.](image)
Figure 4. The functional distribution of the differentially expressed proteins according to their regulation in unpollinated pistils (a), after self-compatible pollination (b), after self-incompatible pollination (c), and among self-compatible and self-incompatible pollinations (d) of the self-compatible almond A2-198 (represented by A) and the self-incompatible almond ITAP-1 (represented by I).

3.3. Differentially Expressed Proteins after the Compatible Pollination

Quantitation revealed 44 DEPs in the comparison of self-pollinated pistils of A2-198 with unpollinated pistils of A2-198 (Table S2). Two of these proteins could not be identified in UniProtKB/SwissProt and NCBI databases.

Functional classification indicated that both “metabolic proteins” and “stress resistance and defense proteins” were the main groups with a similar proportion (27% and 30%, respectively) (Figure 3). On the other hand, the number of up-regulated proteins was higher after self-pollination in all functional groups (Figure 4b).

3.4. Differentially Expressed Proteins after the Incompatible Pollination

In the comparison of pistils from ITAP-1 pollinated with A2-198 vs. unpollinated pistils from ITAP-1, a total of 42 proteins were identified as differentially expressed (Table S3). Six of these DEPs were down-regulated and 36 were up-regulated in the incompatible pollination. Two of the 42 proteins could not be characterized in UniProtKB/SwissProt and NCBI databases.

The main functional group in this comparison was “metabolic proteins” (33%) (Figure 3). Furthermore, there was a trend of increasing up-regulated proteins from pollinated pistils after the incompatible pollination (Figure 4c), as also happened after the compatible pollination.

3.5. Differentially Expressed Proteins in Compatible and Incompatible Pollinations

Quantitation revealed 69 proteins that were differentially expressed between the incompatible pollination (ITAP-1 × A2-198) and the compatible pollination (A2-198 × A2-198) (Table S4). Forty-three of these proteins appeared as down-regulated in the compatible pollination, and 26 proteins were up-regulated in the same condition. From the 69 DEPs, 4 could not be characterized in UniProtKB/SwissProt and NCBI databases.

The functional classification of pollinated pistils showed the same trend found in the other comparisons, with the main groups being “metabolic proteins” and “stress resistance and defense proteins”. Nevertheless, the functional groups “transcription regulation” and “translation initiation” appeared solely in the comparison A2-198 × A2-198.
vs. ITAP-1 × A2-198 (Figure 3; Table S4). Each of these groups contained only one protein (M5WQU9 and M5XKU7), which were up-regulated after the incompatible pollination. On the other hand, protein regulation in each functional group was similar in both compatible and incompatible pollinations (Figure 4d).

3.6. M5WQU9 Protein and Analysis of Its Expression by RT-qPCR

The protein M5WQU9 of the minority functional group “transcription regulation”, which was identified in the NCBI database as GLABROUS1 enhancer-binding protein-like (GEBPL) from Prunus persica and corresponds to the peach sequence ppa006690m in Genome Database for Rosaceae (Table 2), was selected to validate the proteomic analysis. The choice of this protein for additional RT-qPCR analyses was strongly determined by its role as a transcription factor, and therefore for its potential involvement in the regulation of the expression of components of the self-incompatibility system. In particular, the proposal of this hypothesis is based on our previous knowledge of different transcription levels of the almond Sf-RNase after compatible and incompatible pollinations [7].

In the present study, a complete and identical coding sequence of 1200 bp was obtained from ITAP-1 and A2-198 pistils. Homology analysis at the NCBI database identified the full-length cDNA clone as Glabrous1 enhancer binding protein-like, and thus it was named as Prunus dulcis GEBPL (PdGEBPL) and uploaded in the NCBI database under the accession number MK557895.

The results of the qPCR analysis indicated that after the compatible pollination (A2-198 × A2-198), the expression of PdGEBPL was lower than in unpollinated pistils of A2-198, whereas its expression increased after the incompatible pollination (ITAP-1 × A2-198) (Figure 5).

![PdGEBPL](image-url)

**Figure 5.** Normalized expression by RT-qPCR of PdGEBPL gene with the reference gene RNA Polymerase II (RP II). For each condition (A: A2-198 unpollinated pistils, A × A: compatible pollination (A2-198 × A2-198), I: ITAP-1 unpollinated pistils, I × A: incompatible pollination (ITAP-1 × A2-198)), the expression is relative to the unpollinated pistils. Relative expression levels are mean of triplicates (of each biological sample) ± standard error.
4. Discussion

4.1. Classification of Differentially Expressed Proteins

In all cases, functional grouping revealed that the largest number of DEPs corresponded to “metabolic proteins”. Analogous results were shown in a previous iTRAQ analysis performed in anthers and unpollinated pistils of A2-198 and ITAP-1 almonds [11], and in other angiosperm species with gametophytic self-incompatibility [33–35]. Metabolic proteins, specifically those with lipid metabolism, seem to be involved in pollen–pistil interactions [36]. This group of proteins has also been identified after pollination of *Solanum melongena* L., and also in transcriptomic and proteomic analyses carried out in *Oryza sativa* [37,38].

In the present study, a large number of proteins with metabolic function were also up-regulated after the compatible pollination. These results were similar to those obtained by [39] after DIGE analysis in *Brassica*, where the authors suggested that the results correlate with the fact that compatible pollination requires an increase in energy to allow pollen hydration, germination, penetration of the pollen tubes in the style, and their growth to the ovary.

The second most numerous functional group of DEPs observed in the present work was “stress resistance and defense”, which appeared in the same ranking position in a previous iTRAQ analysis of anthers and unpollinated pistils of the same plant material [11]. This group was also abundant in the analysis of expressed sequence tags of almond pistils performed by [40]. These authors suggested that cold or drought stresses are important during the development of pistils. Moreover, growth of the pollen tube was meant to be possible by the accumulation of nutrients in the pistil, which turns the pistils into a target for pathogens. However, pistil infection is unusual because the pistils also accumulate numerous antimicrobial proteins, which could explain the importance of this functional group [41].

4.2. Characterization of Differentially Expressed Proteins

In the present study, the expression profile obtained for unpollinated pistils showed 13 DEPs, 7 of which were also found in a previous iTRAQ analysis using the same plant material [11]. The differences between both studies could be because the proteome is not a static entity and may be affected by multiple modifications, such as changes in external conditions (water availability, temperature, etc.) or by particular physiological conditions [42]. This variability of the proteome is also the reason why proteomic studies are an important tool in the analysis of the biochemical pathways in the response to stimuli of plants [42].

As stated above, DEPs with stress resistance and defense function constituted a large group in the actual study. One of these proteins was Thaumatin (M5WR86; M5WT96), which appeared up-regulated in the unpollinated pistils of the self-compatible almond A2-198. This protein also appeared up-regulated in unpollinated pistils of A2-198 almond in [11], and in pistils of the self-incompatible Japanese pear [43]. Thaumatin has been found to be specifically expressed in the transmitting tissue of the stigma and style of tobacco [33,44], and it seems to be implicated in the recognition of pollen signals [43]. It also interacts with β-1,3-glucans [45]. A well-known β-1,3-glucan is callose, which is present in the pollen tube walls and in the plugs that segment growing tubes [46].

Another protein with a defense function found in the present study was putative LRR (leucine-rich repeat) receptor-like serine/threonine-protein kinase (M5VM54), which was up-regulated for the self-compatible pollination. As reviewed in [47], plant receptor-like kinases (RLK) functions can be classified into two major categories: those implicated in cell growth and development and those involved in plant–pathogen interaction and defense responses. Additional RLK proteins not included in the two major classes include proteins implicated in symbiosis, S receptor kinases (SRK) contributing to self-incompatibility, and SRH5, a protein involved in the control of di-nitrogen fixation. According to these authors, structures of the LRR domains are hypothesized to correlate with the functional
categories. Thus, for disease resistance the LRR domain will be required to bind several ligands and to integrate the binding to an appropriate response. In relation to pollen-pistil interactions, [48] correlated the presence of a LRR domain in extensins of the stylar transmitting tissue of apple with their role as recognition-signal molecules. Likewise, LRR extensins expressed in pollen have been reported to play an important role in pollen germination and tube growth [49].

The pathogenesis related protein PR-4 (M5W1Q5) appeared to be up-regulated following pollinations, whereas it was down-regulated in unpollinated pistils of the self-compatible selection and also after self-compatible pollination (Table 2). The production and accumulation of pathogenesis-related proteins (PR proteins) in plants in response to biotic or abiotic stresses is well known and is considered as a crucial mechanism for plant defense [50]. Focusing on the PR-4 family, several studies showed the involvement of these proteins in plant defense responses regulated by signal molecules, such as salicylic acid, abscissic acid, jasmonate, and ethylene [50]. PR proteins have been localized in the transmitting tissue of Nicotiana tabacum pistils [51], and expressed in the stigma of Solanum tuberosum [52]. In Solanum tuberosum, [53] found an mRNA transcript with expression in the stigma and the stylar cortex that showed high homology with PR-1 proteins and suggested that it may function to protect the outer tissues of the pistil from pathogen attacks, and also in controlling guidance of pollen tubes.

Xyloglucan endotransglucosylases/hydrolases under the accession numbers M5WH02, M5WJ79, and M5WT30 were up-regulated in pistils after pollination herein. They are a family of enzymes that mediate the construction and restructuring of xyloglucan cross-links, thereby controlling the extensibility or mechanical properties of the cell wall in a wide variety of plant tissues [54]. These proteins are responsible for remodeling and/or breaking down the cell wall of the pollen tube in Arabidopsis thaliana [55].

GTP-binding nuclear protein (M5XF62) appeared up-regulated after the self-compatible pollination. This protein appeared up-regulated in A2-198 anthers in a previous study [11]. In a recent study carried out in Arabidopsis, a similar protein has been proposed as a protein required for optimal pollination and fertilization processes [56]. The authors suggested that the GTP-binding protein related1 (GPR1) is required to maintain optimal pollen germination and tube growth speed and exerts positive effects on ovule and pollen life span. Nevertheless, the molecular mechanisms through which this protein negatively regulates pollen germination and tube growth, and the way it promotes the longevity of both ovules and pollen grains, remain unknown [56].

As indicated, GEBPL protein (M5WQU9) was up-regulated after the incompatible pollination. This protein has been reported to have a central region with unknown motifs and a C-terminal region with a leucine-zipper motif in its structure [57]. Moreover, GEBPL proteins have been reported to be non-canonical transcription factors that are involved in cytokinin response regulation [58]. Interestingly, inhibition of incompatible pollen tube growth has been correlated with a sharp increase in cytokinin content in Petunia style tissues [59]. In the present study, the validation of PdGEBPL (almond homologous transcript) expression by RT-qPCR reinforces its participation in the gametophytic self-incompatibility system.

5. Conclusions

This study provides proteomic profiles of differential expression in mature unpollinated pistils from self-compatible and self-incompatible almonds, and a comparison of proteomic profiles following compatible and incompatible pollinations. This information could serve as a reference for other comparative proteomic studies of pollen–pistil interactions in species with gametophytic self-incompatibility. “Metabolic” and “stress resistance and defense” proteins were over-represented in this study, and thus pollen-pistil interactions seem to have the same functional bases as host-parasite interactions. Moreover, according to RT-qPCR results, the differentially expressed protein involved in regulation of the transcription GLABROUS1 enhancer-binding protein-like could be considered a good
candidate component of the self-incompatibility system in almond, and it could act directly on the \( S \)-genes or on other components. Additional analyses of interactions of this protein with the \( S \)-genes would help to clarify the breakdown of self-incompatibility in almond.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/agronomy2020345/s1: Table S1: Differentially expressed proteins in pistils of A2-198 and ITAP-1 almonds; Table S2: Differentially expressed proteins in self-pollinated A2-198 pistils and unpollinated A2-198 pistils; Table S3: Differentially expressed proteins in ITAP-1 × A2-198 pistils and ITAP-1 unpollinated pistils; Table S4: Differentially expressed proteins in A2-198 × A2-198 and ITAP-1 × A2-198 almonds.

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