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Comparative analysis of allergen genes and pro-inflammatory factors in pollen and fruit of apple varieties

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HIGHLIGHTS

- Apples are source of nutraceutical molecules, but also of allergenic factors
- The allergenic properties of apple varieties differ greatly
- Some allergens expression is lower in hypo-allergenic variety compared to the others
- The activities of some sensitizing enzymes are lower in the hypo-allergenic variety
- Next step: specific allergens and enzymes could be used as markers for allergenicity

Abstract

Allergy to freshly consumed apple fruits is often associated to pollinosis and manifested as oral allergy syndrome (OAS). The allergenic properties of apple varieties differ greatly, spanning from low allergenic to high allergenic varieties. The knowledge of the genetic determinants for allergenicity has been of great interest in scientific community for several years, but the molecular mechanisms involved are still little understood. Here, factors putatively involved in allergenicity were investigated at biochemical and molecular level in pollen and in fruits of apple varieties differing in their allergenic potential. Among putative sensitizing factors, transglutaminase (TGase) and phospholipase A₂ (PLA₂) were considered together with reactive oxygen species (ROS) and known apple allergen genes, with particular attention devoted to the *Mal d 1* gene family, the most important one in sensitization. We found that the expression of

some allergen genes and the activities of TGase, PLA₂ and ROS producing enzyme are lower in the hypo-allergenic variety ‘Durello di Forlì’ in comparison with the high-allergenic genotypes ‘Gala’ and ‘Florina’. These results highlight correlations among allergen expressions, enzymatic activities and apple cultivars; these data underline the possibility that some of them could be used in the future as markers for allergenicity.

Abbreviations: Reverse Transcriptase quantitative PCR (RT-qPCR), Transglutaminase (TGase), phospholipase (PLA), Reactive Oxygen Species (ROS), Ungerminated pollen grains (UGP), Germinated pollen grains (GP), soluble proteins (SP), proteins from cell wall (CWW), Nitroblue tetrazolium (NBT), 2-Hexadecanoylthio-1-Ethylphosphorylcholine (HEPC), 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB), Nicotinamide adenine dinucleotide phosphate (NAD(P)H), diphenyleneiodonium (DPI), 3, 3', 5, 5'-Tetramethylbenzidine (TMB), 2,5-diphenyl tetrazoliumbromide (MTT), Linkage Group (LG).

Keywords

Malus domestica, apple allergy, plant breeding, hypoallergenicity, Phospholipase, Transglutaminase, Mal d 1 genes

1. Introduction

A regular consumption of fruit and vegetables is the basis of a healthy daily diet able to prevent diseases and reduce the incidence of cardio-vascular ones as well as asthma, diabetes and cancer [1]. The benefits derive both from their nutritional components (vitamins, minerals and fibers) and from secondary metabolites (phenols, flavonoids and carotenoids) that play an important antioxidant role. Unfortunately, fruit allergy is increasing and it has been estimated to affect 2.2–11.5% of children aged 0–6 years and 0.4–6.6% of adults worldwide [2]. Up to now the only treatment is still the avoidance affecting in a significant negative way health-related quality of life, family economics, social interactions, school and work attendance. Fruit allergy is frequently associated with pollinosis as 70% of patients allergic to pollens develop an hypersensitivity reaction against pollen-related food allergens, termed as birch pollen-related food allergy and clinically manifested as oral allergy syndrome (OAS) [3]. As responsible of one of the most prevalent food allergies, apples rank fourth out of 24 foods examined in an extensive Pan-European survey and first among Rosaceae fruits [4]. The reactions to apple are the best described so far and it is known that the allergenic properties of apple varieties differ greatly, showing broad range of allergenic potential spanning from low allergenic varieties till high allergenic ones for the majority of patients. This is due both to ‘external’ factors, like growing and storage conditions [5,6] and ‘internal’ factors like genotype [5,7]. The knowledge of the genetic basis for low and high allergenicity has been of great interest in scientific community for several years [8,9].

There are four classes of allergens currently identified in apple (Mal d 1, Mal d 2, Mal d 3 and Mal d 4), all organized in multigene families with genes differentially expressed in varieties and fruit tissues [10-12]. Mal d 1, 2 and 3 are pathogenesis-related (PR) proteins, belonging to the class PR-10 (ribonuclease-like proteins), PR-5 (thaumatin-like proteins, TLPs) and PR-14 (lipid-transfer proteins, LTPs), respectively; Mal d 4 is a profilin with an actin-binding role. Mal d 1 is thought to be the major allergen in Central and Northern Europe [2] mainly due to the IgE mediated cross-reaction with the major birch-pollen allergen Bet v 1 to which Mal d 1 is highly homologue. Actually, systemic allergic reactions to apple fruits are not rare in areas where birches are widespread and may occasionally be severe. These allergic reactions are often associated with sensitization to Mal d 1 rather than other apple allergens. A proof of the importance of Mal d 1 in the allergic reactions came from a RNAi experiment in which it has been showed that by silencing Mal d 1 genes the resulting fruits are less allergenic [13]. Many allergens from other fruits belong to PR-10 family (Pru p 1 from peach, Pru ar 1 from apricot and Pru av 1 from cherry) and the cross-allergenicity between fruits of the *Rosaceae* species is well known [2]. Mal d 1 proteins are encoded by multiple genes (31) that are organized in five subfamilies according to the DNA sequence similarity [14]. Up to now no reliable method is available for quantification of total Mal d 1 protein content or single isoforms but it is possible to study their specific gene expression and it is known that 20 out of 31 *Mal d 1* genes are expressed in apple fruits [12].

Molecular mechanisms involved in allergic sensitization and increased pollen/food allergenicity are still little understood but it has been recently reasonably proposed that factors related to cell stresses can play key roles in these mechanisms. These factors may act directly on allergenic proteins modifying their binding-capacity to IgE and/or they can activate the inflammation responses in humans after contact with allergenic food or pollen. Among these factors, the plant transglutaminase (TGase), described also in apple pollen [15,16], can be involved in allergies. TGases are a widely distributed family of calcium-dependent acyl-transferase enzymes able to post-translationally modify proteins [17] and promote cross-linking reactions that result in stable networks with structural rigidity, proteolytic and temperature resistance [16], thus stabilizing proteins against proteases [17,18].

In mammals, the role of TGase in inflammatory diseases, such as celiac disease, asthma and allergic conjunctivitis [19,20] has been already demonstrated. It has been associated with the activation of pro-inflammatory secretory phospholipase A2 (sPLA₂) through the formation of a transient dimer of active sPLA₂ [21]. The PLA₂ is a superfamily of lipolytic enzymes that is involved in a number of essential biological processes, such as development, host defence, signal transduction and inflammation. This superfamily is generally categorized into five principal families of lipolytic enzymes, but only two of these PLA₂ families have been reported in plants: the low molecular weight protein sPLA₂ and the patatin-like PLAs [22]. PLA₂ hydrolyses glycerophospholipids, the first rate limiting step in the eicosanoid cascade, and mammalian TGase is reported to be a regulator of eicosanoid production in asthma [20]. Chimeric peptides able to inhibit tissue TGase (tTGase)-mediated modification of sPLA₂ or co-inhibiting tTGase and PLA₂

displayed strong *in vivo* anti-inflammatory activity in allergic conjunctivitis [19,23]. Despite the proven involvement of plant sPLA₂ in many biological functions [22,24], the sPLA₂ genes are essentially still uncharacterized.

In plants, pollen TGase activity is significantly increased under stress conditions and it has been shown that an intense release of TGase in the medium due to pollen damage occurred after stress induced by factors as temperature, relative humidity and pollutants, three factors that simulated climate changes [25]. Its extracellular localization facilitates the contact with the mucosa of the respiratory tract reached by the pollen [26]. Moreover, it has been demonstrated that also TGase from pollen affects the activity of the mammalian sPLA₂, increasing its catalytic activity after post-translational modifications [25].

Recent studies conducted in murine models have shown that also the NAD(P)H oxidase of pollen plays an important role in the pathogenesis of allergies [27,28]. In fact, the release of reactive oxygen species (ROS), produced by NAD(P)H oxidase, can damage the mucosa, thus participating in the process that leads to sensitization reaction. Moreover, pollen grains with different allergenic potentials, show differences in NAD(P)H oxidase activity, both in intensity and localization [27,28].

Here, factors putatively involved in allergenicity were investigated not only in pollen but also in fruits of different apple varieties at biochemical and molecular level. Among putative sensitizing factors, TGase and PLA₂ were considered together with apple allergens. In fact, while the role of antigenic pollen proteins in the induction of allergic airway inflammation is well characterized [29], the contribution of other constituents of pollen grains to this process is partially unknown.

We worked on both pollen and fruits of three genotypes, chosen according to their ranking in allergenicity after prick-to-prick skin prick test conducted with fruits collected in the same orchard [7]: ‘Gala’ and ‘Florina’, two commercial varieties, are high allergenic for peel, but less allergenic when pulp was tested, while ‘Durello di Forlì’, an old Italian apple variety, is the lowest allergenic one. We also analysed an additional genotype (‘Golden Delicious’ for pollen and ‘Fiesta’ for fruit) chosen because the high allergenicity considering both pulp and peel. For this aspect, ‘Golden delicious’ and ‘Fiesta’ can be considered comparable. Finally we verified the gene expression of interesting genes in ‘Elise’, reported to be hypo-allergenic [5, 30].

2. Materials and methods

2.1. Chemicals

All chemicals (unless otherwise indicated) were obtained from Sigma-Aldrich (Milan, Italy)

2.2. Plant material

All material was collected from apple plants grown in Cadriano experimental orchard of the University of Bologna (Italy). Mature pollen of varieties ‘Golden Delicious’, ‘Florina’, ‘Gala’ and ‘Durello di Forlì’, sampled from flowers at balloon stage, was handled and stored as described in literature [31]. Fruits of the varieties ‘Fiesta’, ‘Florina’, ‘Gala’, ‘Durello di Forlì’ and ‘Elise’ were collected at the physiological ripening stage. For each genotype, peel and flesh from three different fruits were separately pooled as previously described [12], frozen in liquid nitrogen and stored at -80°C until RNA extraction.

2.3. Pollen viability assay

Pollen viability was tested by MTT (Methylthiazolyldiphenyl-tetrazolium bromide). MTT produces a yellowish solution that is converted to dark blue, water-insoluble MTT formazan by mitochondrial dehydrogenases of living cells. The test solution contained 1% concentration of the MTT substrate in 5% sucrose. After 15 min incubation at 30°C pollen samples were visualized under a light microscope. Pollen was considered viable if it turned deep purple. At least 60 pollen grains were observed per cultivar, and each cultivar was analyzed three times in order to ensure the consistency of results.

2.4. In vitro pollen germination

Pollen was hydrated at 30°C-100% rHu for 30 minutes and allowed to germinate (1 mg/mL in germination medium: 10% sucrose, 324 μ M H_3BO_3 , 1.27 μ M $CaNO_3$) for 2 hours into glass Petri dishes at 30°C-100% rHu. The ratio of germinated pollen grains on total (germinated plus ungerminated) was evaluated under inverted microscopy, in two different experiments. Pollen employed in enzyme assay was collected after germination and separated from the culture medium by filtration with vacuum pump on Millipore disks (5 μ m pore size). For total RNA extraction, 2 hours germinated pollen was collected and separated from the culture medium by centrifugation at 500 rpm for 1 min, room temperature.

2.5. Total RNA isolation and cDNA synthesis

Total pollen RNA from four apple varieties (‘Golden Delicious’, ‘Gala’, ‘Florina’ and ‘Durello di Forlì’) was extracted, starting from 10-20 mg of both frozen ungerminated (UGP) and germinated (GP) pollen, following the method described by Zamboni and collaborators [32]. RNA purity and concentration were evaluated by measuring the absorbance (Abs) at 260 and 280 nm wavelengths using a NanoDrop Spectrophotometer ND-1000 (EuroClone S.p.A. Italy). RNA was considered pure if the Abs 260/280 ratio was greater than 1.8 and Abs 260/230 ratio almost 2. First-strand cDNA was synthesized using the cDNA synthesis kit with Oligo(dT) (Applied Biological Materials Inc, Richmond, Canada), starting from 1 μ g DNA-free RNA in a final volume of 30 μ l. The cDNA from pollen was employed at a 1:10 dilution.

Total fruit RNA extraction and cDNA synthesis were performed as described previously [12]. For each genotype, two different RNA extractions were performed from two different pools (biological replications).

2.6. *In silico* search for PLA, TGase and profilin Mal d 4.04 genes

A preliminary *in silico* approach was needed to retrieve the *Malus x domestica* sequences coding for TGase, PLA and profilins from the public databases.

The TGase sequence of *Arabidopsis thaliana* AtPng1p (Genebank ID: AM745095) was used as query for a Blast search versus the predicted coding sequences of the apple genome v 1.0 [33].

Diversely, to identify the apple Phospholipase gene sequences, the term *Phospholipase A* was used as query against the NCBI (www.ncbi.nlm.nih.gov) apple EST database. The retrieved sequences were aligned with the software MegAlign (DNASTAR, Madison, WI, USA) and consensus sequences were searched by Blastx at NCBI for homology with other species. These sequences were also searched in the apple genome v 1.0 to identify their position in the apple genome and in the Plaza 3.0 dicots platform (http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/) to retrieve *Arabidopsis thaliana* orthologs.

Finally, putative profilin genes were searched by BlastN in the apple genome website (www.rosaceae.org), using the existing profilin sequences found in the official allergen website as a query (www.allergen.org). The aminoacidic sequence of the putative fourth profilin was compared to the sequences retrieved from the genome and from public databases (www.ncbi.org; www.allergen.org), by Clustalw software aligning (www.embl.org): an aminoacidic identity between 67 and 95% was used to discriminate the new putative allergenic profilin isoform [34].

2.7. Gene expression analysis in pollen and fruit tissues by RT-qPCR

Gene expression levels were analyzed by quantitative RT-qPCR, using SYBR green based assays and specific primer pairs.

The most important pan-allergens (*Mal d 1*, *Mal d 3* and *Mal d 4*) known to be involved in pollen development and germination were analyzed in the pollen four genotypes: ‘Gala’, ‘Florina’, ‘Durello di Forlì’ and ‘Golden Delicious’. In particular, nine genes were studied: *Mal d 1.01*, *1.02*, *1.06A*, *1.07*, *Mal d 3.01*, *Mal d 4.01*, *4.02*, *4.03* and *4.04* genes. *Mal d 1*, *Mal d 3.01*, and *Mal d 4.01*, *4.02* and *4.03* primer sets were retrieved from previously published works (see Table 2). Specific primers for *Mal d 4.04*, *PLA21*, *PLA23* and *TGase* were developed in the present work, using the software Primer3 version 0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) and primerBLAST (www.ncbi.org) for a first specificity evaluation *in silico*. RT-qPCR reaction mixes and amplification protocols were performed as previously

described [12], in three technical replicates, using a StepOne™ Plus Real Time PCR System (Life Technologies). The primer pair specificity and the single amplification products were also verified by the analysis of the melting curve, performed after the amplification step, in each qRT-PCR experiment. *Actin* and *18S* were used as internal housekeeping genes (Table 2). Gene expression was measured using the standard curve method [35] with a custom standard curve made of a serial dilution of genomic DNA from ‘Golden Delicious’ variety. The final results are expressed as the relative expression of target genes obtained by dividing the non-normalized values by a normalization factor calculated as the geometric mean of two reference genes, *Actin* and *18S* [35, 36]. The relative expression level was reported in arbitrary units (A.U.).

In the fruits, the expression level of 8 selected *Mal d 1* genes was characterized as previously described [12]. Attention was focused on *Mal d 1* gene family as it is reported as the most important fruit allergen. Among the 31 genes of which the *Mal d 1* family is composed, the eight genes enrolled in this study were selected among the once known to be expressed in fruits and representing four subfamilies: *Mal d 1.01* and *Mal d 1.02* from subfamily I; *Mal d 1.06A* and *B* from subfamily III; *Mal d 1.07* and *Mal d 1.08* from subfamily IV and *Mal d 1.11A* and *Mal d 1.12* from subfamily V. Subfamily II was not represented as no gene belonging to this group have been found to be expressed in fruits [12]. Variations in gene expression were tested among peel and pulp of fruits of ‘Fiesta’, ‘Gala’, ‘Florina’ and ‘Durello di Forlì’ apple varieties. For these analyses, ‘Fiesta’ was used instead of ‘Golden Delicious’ as hyper-allergenic variety, and results compared with those recently obtained in ‘Golden Delicious’ [37]. The transcripts levels of target genes were normalized to the transcript levels of *Actin* in fruits (Table 2). The mean normalized expression levels and the standard error of the mean were calculated using the two biological replicates. Each reaction was performed in triplicate (technical replicates).

2.8. Pollen protein extraction

For total protein extraction, pollen was lysed on ice using a grinder in cold lysis buffer (50 mM Tris-HCl, pH 8.5, 0.5 mM EGTA, 0.5 mM EDTA, 2 mM MgCl₂, 2 mM dithiothreitol and protease inhibitor cocktail) and then centrifuged for 2 min at 10,000g. All steps were performed at 4°C. Total soluble proteins (SP from UGP and GP pollens) were extracted according to Del Duca et al. [18], whereas proteins from cell wall (CWW) in UGP and GP pollen were extracted with different buffers (10 mg pollen/0.5 ml buffer) following the method reported by Cai et al. [38]. Proteins quantification was performed following the bicinchoninic acid method with bovine serum albumin as the standard protein. The samples were further used for the enzymatic activity assays.

2.9. Microtitre plate-based assay for TGase activity

SP and CWW proteins extracted from UGP and GP pollen grains were tested for TGase activity measured by

the conjugation of biotinylated cadaverine to *N,N'* dimethylcasein (DMC)-coated 96-well microtitre plates (NUNC, Life Technologies) as described by Lilley et al. [39] with minor modifications. The level of enzyme activity was expressed as Ca^{2+} -dependent increase in Abs 450, after subtraction of the value of the 20 mM EGTA treated control. Specific activity was determined as a change in Abs 450 of 0.1 per hour per mg of non-hydrated pollen.

2.10. *sPLA*₂ enzyme assay

SP and CWW proteins extracted from UGP and GP pollen grains were tested for *PLA*₂ activity measured in a 96-well microtitre plates (NUNC, Life Technologies). Briefly, pollen protein extracts (50 μl) were incubated in total 50 μl reaction buffer containing 100 mM Tris-HCl buffer pH 8, 5 mM 2-Hexadecanoylthio-1-Ethylphosphorylcholine (HEPC) as *PLA* substrate, 10 mM 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) as Ellman's reagent, 5 mM CaCl_2 as positive control or 20 mM EGTA as negative control. The reaction was carried out for 2 hours at 37°C and then the absorbance was immediately read at 405 nm using a Wallac Victor Multiscan ELISA (Perkin Elmer).

2.11. Nitroblue tetrazolium (NBT) assay to determine ROS production through NAD(P)H oxidase activity

Spectrophotometric ROS quantification by absorbance of the reduced nitroblue tetrazolium (NBT) was performed according to Wang et al. [28], with few modifications. Pollen grains (1mg / assay) were hydrated for 30 minutes and then suspended in phosphate buffer (PBS, 500 μl /mg pollen). The reaction to determine NAD(P)H oxidase activity was started with the addition of 2 mM nitroblue tetrazolium (NBT) with or without the addition of 1 mM NAD(P)H (positive control) or 200 μM diphenyleneiodonium (DPI) (negative control). During the reaction, conducted at 37°C, the suspensions were gently rotated. After 30 min, suspensions were centrifuged for 3 min at 10000 rpm. Residual NBT was removed by washing with 1 ml of pure water twice, and the formazan sediment was dissolved in 400 μl of methanol. After extraction with methanol by rotation for 20 min at room temperature and centrifugation, the absorbance of supernatants was measured at 530 nm. In parallel experiments, after 10 min incubation of hydrated pollen grains with 1.2 mM NBT, the samples were immediately visualized under a microscope.

2.12. Determination of IgE patients' sera binding in ELISA

An ELISA assay was carried out in triplicate. Briefly, total, SP and CWW proteins extracted from UGP and GP pollen grains (50 μl /well) were incubated with a diluted pooled human serum from apple allergic patients (1:30, 50 μl /well), followed by incubation for 45 min at 30°C in a 96-well microtitre plate. The serum of the patients utilized were obtained from the same patients declared in an our previous paper, in which the main

clinical characteristic were reported [7]. Detection of IgE antibodies was performed using a goat anti-human IgE peroxidase conjugate (1:500) (100 μ l /well) incubated 1h at 30°C; a substrate solution (100 μ l /well) of 0.3 mM 3, 3', 5, 5-Tetramethylbenzidine (TMB) (10 mg / ml of dimethyl sulfoxide) and 0.03% (v/v) hydrogen peroxide in 100 mM sodium acetate pH 6.0 was added. The staining development was stopped after maximum 30 min with 50 μ l per well of 5 N H₂SO₄. The absorbance was read at 450 nm using a Wallac Victor Multiscan ELISA (Perkin Elmer).

3. Results

3.1. Identification of apple coding sequences homologues to the *A. thaliana* PLA and TGase

The nucleotide sequence of the gene *AtPng1p* (TGase from *A. thaliana*, Genbank ID: AM745095) was used as query for searching the homologous sequence in the apple sequence genome. The BLASTN search resulted in one high homology hit (79% nucleotides identity) on the bottom of linkage group (LG) 12 (MDP0000160665). The apple sequence was used for the design of a specific primer pair. Regarding the apple PLA₂, there was no genomic information and a preliminary search in public databases for apple ESTs described as PLA₂ was performed. Two consensus sequences were obtained homologous to PLA21 (Genbank ID: EEF38745) and PLA23 (EEF48118) of *Ricinus communis*. This sequence showed high homology in citrus pollen [40]. The apple PLA21-like fragment sequence is similar to *A. thaliana* PLA₂- β and it is positioned on apple LG13 where, according to v 1.0 of apple genome, two putative coding sequences for phospholipase (MDP0000154214 and MDP0000249250) were found very close to each other, differing only for a couple of SNPs (Table 1). PLA23 was identical to MDP0000366609, also located on LG13 next to the others and it was more similar to *Arabidopsis* PLA₂- α . Another similar sequence (97%) was found in the LG16 (MDP0000130878), paralog to the one of LG13 (Table 1).

The known profilin sequences retrieved from the allergen website (www.allergen.org) *Mal d 4.0101* (Genbank ID: AF129428), *Mal d 4.0201* (Genbank ID: AF129427) and *Mal d 4.0301* (Genbank ID: AF129426) were employed for searching additional sequences in the apple genome. Two long sequences were found on different LGs: MDP0000290855 on LG15 and MDP0000437009 on LG2, each containing a *Mal d 4.02* gene plus an additional complete profilin, erroneously linked in the same predicted coding sequence. The two putative new profilins had an aminoacidic similarity of 95%. The comparison with profilins from allergen database and apple genome resulted in an aminoacidic similarity ranging from 77 to 86%, which allowed discriminating the two profilins as new *Mal d 4* isoforms (Table 1).

3.2. Expression and activity of pro-inflammatory factors in apple pollen of different genotypes

To determine whether putative sensitizing factors (*TGase* and *PLA₂*) were expressed in apple pollen, we conducted an analysis based on quantitative PCR on ungerminated (UGP) and germinated (GP) pollen grains of different apple genotypes. The results shown in Figure 1 were in agreement with previous results obtained using a semi-quantitative approach (Supplementary Figure 1). Both isoforms of *PLA₂* and the *TGase* genes are clearly expressed in UGP and GP of different varieties. Interestingly, *PLA₂₃* was much more expressed than *PLA₂₁*, peaking in germinated pollen of ‘Golden Delicious’.

A great variability in the expression level of nine selected *Mal d* genes was observed in pollen of different apple genotypes (Figure 1). In details, we analyzed four *Mal d 1* genes and *Mal d 3.01*, based on the knowledge they are expressed in fruits; indeed, the small gene family of profilins, *Mal d 4*, represented only by four genes in the apple genome, was fully evaluated because profilins are known pollen allergens. Surprisingly, *Mal d 4.04*, unknown before this work, was expressed 10-30 fold higher than the other three genes, while *Mal d 4.01* was the one with lowest expression.

Mal d 3.01 was highly expressed only in UGP of ‘Golden Delicious’. The expression level of all the others was comparable to that of *TGase* and *PLA₂₁*. Most of these genes seem to be more expressed in UGP than GP (with the exception of *Mal d 4.04*), and this is particularly evident for *Mal d 1.01* and *1.02* in ‘Durello di Forlì’.

Among varieties, the pollen grains of ‘Golden Delicious’ were the richest of pro-inflammatory transcripts and allergens such as non-specific lipid transfer (*Mal d 3*) and profilins (*Mal d 4*), but not for *Mal d 1* genes. ‘Durello di Forlì’ never ranked at the highest position for expression amount, but showed levels generally lower or comparable to the other genotypes, with the exception of *Mal d 1.01* and *Mal d 1.02* genes.

The activity of *TGase* was checked in two fractions, total soluble proteins (SP) and proteins from cell wall (CWW), in GP and UGP pollen of four apple varieties. *TGase* activity was lower in GP than UGP (Figure 2A); this result is in agreement with several evidences underlying the extrusion of this enzyme upon pollen rehydration and mostly at the beginning of germination [15]. Proteins derived from ‘Durello di Forlì’ pollen showed always the lowest *TGase* activity while proteins derived from ‘Gala’ pollen showed the highest *TGase* activity. *PLA₂* activity, tested on total protein, was also found higher in cultivars ‘Gala’ and ‘Florina’ in comparison to ‘Durello di Forlì’, with intermediate level in ‘Golden Delicious’ (Figure 2B).

3.3. ROS production in pollen grains of different apple varieties

The NBT assay was performed in order to test if apple pollen from different varieties showed differences in the production of ROS. Pollen grains from apple varieties ‘Gala’, ‘Florina’, ‘Golden Delicious’ and ‘Durello di Forlì’ were investigated. All the pollen types reduced the redox-sensitive NBT to formazan (Figure 3). Results indicate that NAD(P)H oxidase activity and ROS production levels are quite low in pollen grains of ‘Durello di Forlì’, especially in comparison to ‘Gala’ (Figure 3A). Moreover, we gave

evidence that this different production of ROS was not dependent on viability among the several pollen grains of a cultivar, as showed by the MTT assay (Figure 3B). To further confirm these observations, an *in situ* NBT assay on entire pollen grains was performed and it was observed that ROS caused the purple formazan deposits due to reduction of NBT in the four varieties of apple hydrated pollen grains (Figure 3C).

3.4. Binding capacity of IgE from allergic patients' sera to protein extracts from different apple varieties

Total proteins and proteins derived from both CWW and SP were tested in ELISA using a pool of sera derived from patients allergic to apple, in order to check the reactivity of the GP and UGP of four varieties of apple with the sera IgE (Figure 4A). In general, GP showed a slight decrease in IgE recognition and CWW and SP showed a similar trend. Proteins derived from 'Durello di Forlì' pollen showed always the lowest IgE reactivity while proteins derived from 'Florina' pollen showed generally the highest IgE binding capacity. This observation was confirmed by ELISA tests performed with the same pool of patients on a total protein extract of UGP pollen derived from the four varieties (Figure 4B). Here it was clear that 'Durello di Forlì' and 'Florina' pollens were the ones with the lowest and the highest IgE binding activity, respectively, suggesting that 'Durello di Forlì' could be included in apple cultivar with low level of allergen whereas 'Florina' in high level one.

3.5. *Mal d* genes expression analysis in fruits of different apple varieties

Mal d 1 was investigated in deep in fruit material as it is suggested to be the main apple allergen gene family. The expression at transcriptional level of 8 specific *Mal d 1* genes in fruit peel and flesh was studied in different apple varieties to possibly find correlations among *Mal d 1* genes expression and allergenicity. RT-qPCR results showed great variability in gene expression according to genotype, gene and fruit tissue (Figure 5). First of all, most of the genes are prevalently expressed in the apple peel (except for the ones of subfamily V). Secondly, the genes belonging to subfamily I, *Mal d 1.01* and *Mal d 1.02*, are the most expressed ones while *Mal d 1.12* is the less expressed (about 7000 times). Finally, the allergen genes expression in 'Durello di Forlì' was in general statistically lower than that of the other varieties. In particular, *Mal d 1.01*, *Mal d 1.02*, *Mal d 1.06A* and *Mal d 1.07* were expressed much less in 'Durello di Forlì' fruits than in the other genotypes. The only exception is the gene expression of *Mal d 1.08* and *Mal d 1.11A* that were not statistically different between 'Durello di Forlì' and 'Fiesta'. *Mal d 1.12* is not expressed at all in 'Durello di Forlì', 'Fiesta' and 'Gala'. Furthermore, *Mal d 1.06B* and *Mal d 1.08* were differentially expressed among the fruits but with a less clear correlation with allergenicity of the apple varieties: *Mal d 1.06B* is expressed in 'Durello di Forlì' at a significantly different level compared to 'Fiesta' but not to 'Gala' and *Mal d 1.08* is similarly expressed in 'Durello di Forlì' and 'Fiesta' and at higher levels in 'Florina' and 'Gala'. As an additional endorsement that hypoallergenic varieties toward OAS syndrome

could have a lower transcript amount of some *Mal d 1* genes, in particular *1.01* and *1.02* isoforms, transcript RT-qPCR data were obtained on ‘Elise’ variety, a well-known hypoallergenic variety [5, 30]. *Mal d 1.01* and *1.02* in ‘Elise’ were transcribed at the same level as ‘Durello di Forlì’ (Supplementary Figure 2), thus confirming that a reduction of *Mal d 1* transcripts in both varieties could be correlated to hypo-allergenicity features found using patients sera [5,7,30].

These data confirmed the results reported previously in the pollen of ‘Golden Delicious’ [25] and in ‘Florina’ and ‘Gala’ apples [11].

4. Discussion

Allergic reactions to apple vary greatly according to both apple genotype and allergic individual [7], and this hampers the successful identification of reliable common and specific factors involved in allergenicity. In this research, the expression/production/activation of sensitizing factors (specific allergens expression, ROS production, TGase and PLA₂ activity) were studied in relation to the known allergenic potential of apple varieties, and, based on our results, we hypothesize that some allergens and these pro-inflammatory factors are actually involved in raising allergic reactions.

The presence of PLA₂ transcripts in pollen was recently demonstrated in *A. thaliana*, where three out of four PLA₂ paralogs (PLA₂-β, -γ and -δ) were expressed during pollen development; in this process, as well as during pollen germination and tube growth, endoplasmic reticulum and Golgi-localized PLA₂ plays critical roles [41]. Moreover, the expression of two isoforms of secretory phospholipases (sPLA₂ α and β) was found to increase during germination of *Citrus clementine* pollen [40]. As in *Citrus*, we also identified the putative isoforms -α and -β of PLA₂ genes and located them in tandem on chromosome 13. Another similar sequence was found in the homologue chromosome 16, probably as the result of ancient genome duplication events [33]. From our analyses, both PLA₂ sequences are expressed in pollen grains of different apple varieties, with PLA₂₃ by far more expressed than PLA₂₁. Interestingly, pollen grains were found to secrete significant amounts of pro-inflammatory eicosanoid-like substances, namely leukotriene-like and prostaglandin E-like substances [42,43] and sPLA₂ are known key enzymes in the synthesis of eicosanoids that may have a role in raising sensitization and allergic reaction. Thus, it is reasonable to hypothesize that sPLA₂ could be involved in generation of pro-inflammatory eicosanoids also in pollen.

The finding of a single one sequence similar to *AtPng1p* suggests that TGase is a single gene in *Malus x domestica*, as it was found in *A. thaliana* [44]. Given the presence of TGase in apple pollen grains of all varieties tested in this study, revealed both at molecular and biochemical level, it is now plausible to believe a TGase-mediated modification of pollen proteins, possibly allergens themselves. It is known that the TGase is involved in some allergic diseases because its ability of transamidation of proteins present in the human mucosa following conditions of cell stress and its peculiar extracellular location also in the pollen cell wall [19,20]. Moreover, this enzyme itself can represent an immunogenic protein, as suggested by studies on

celiac disease. Pollen proteins as well as mammalian sPLA₂ are post-translationally modified by TGase [18,45] and TGase seems to drastically stimulated the sPLA₂ activity in guinea pig liver [21] and this is important for allergies because sPLA₂ is a key factor in the regulation of the inflammatory response [25]. In this study we found that the hypo-allergenic variety ‘Durello di Forlì’ is the one with the lower TGase and PLA activity and this result can indirectly be used as a proof of their possible involvement in triggering pollen allergenicity.

For TGase and PLA₂, gene expression and enzyme activity were not always in agreement; this result can depend on a multitude of factors that may account for discrepancies between gene expression and enzyme activity, *i.e.* post-transcriptional and post-translational regulation, affecting not only enzyme abundance but also its activity. Many regulatory mechanisms are known to affect the activity of these proteins. Plant TGase and PLAs regulations are well known to be pH-dependent, with TGase having a basic pH optimum [46] and PLAs a basic or an acidic optimum according to the different enzymes studied [47,48,49]. Furthermore, both enzymes have strong substrate specificity [44,48] and require Ca²⁺ ions [44,47,49]. While some TGase need a proteolytic cleavage for its activation [46], PLA activity undergoes even more control steps, as its activity is regulated also by soluble Gα in the neighboring cytoplasm probably by influencing the architecture of the enzyme inserted in a Gα-containing complex [50,51]. These Gα-containing complexes were also shown to contain cyclophilins, chaperone-like proteins that modulate the conformation-based signal transfer within a Gα-target complex [51]. Finally, gene expression and enzyme activity may show discrepancies as both TGase and PLA present isoforms with different subcellular localizations [41,52] and enzyme activity assays are not selective for a specific one. Taken together, these evidences underscore the complexity of the problem of predicting enzyme activities from mRNA data and for this reason, besides gene expression, also enzyme activity assays were performed for the two investigated enzymes.

Among the allergen genes tested in pollen, we found a great variability of expression levels, probably linked to the roles that the encoded proteins play in pollen viability and germination. Most of them (*Mal d 1* genes and *Mal d 3.01*) were more expressed in UGP than GP. As they encode for putative pathogenesis-related proteins, we can suggest that they have a defensive role in UGP against various biotic and abiotic stresses [53]. Noteworthy, *Mal d 3.01* is expressed at greater level in hyper-allergenic variety as ‘Golden Delicious’ respect to the other ones. Further investigations will be necessary to verify the effect of *Mal d 3.01* on allergic patients, especially in the Mediterranean area where LTPs are considered the main fruit allergens and where allergy to nsLTPs is more diffused [2]. Also the genes encoded for profilins were all found expressed in pollen and this result is compatible with the role of these proteins in pollen growth and germination. Profilins are low molecular weight proteins (10-15 kDa) that bind to ATP-G-actin with 1:1 stoichiometry and form moderate affinity profilin–actin complexes. In pollen, profilin is present at levels equimolar with total actin and it is thought that profilin sequester G-actin monomer and let them free locally,

when it is necessary their polymerization to F-actin; for this reason, profilin is a very important factor to organize microfilament elongation and indeed tube growth.

Interestingly, *Mal d 4.04*, a new profilin gene identified in this work for the first time, was expressed by far more than the others, especially in GP of ‘Golden Delicious’ and ‘Florina’, suggesting an important role in pollen germination. As profilins are, together with LTPs, common pan-allergens, it would need further investigation to understand their role in the pollen-food allergy syndrome, not only limited to apple but also to other fruits and vegetables that can cause cross-reactions.

In general, our results suggest the hypothesis that a combination of factors is involved in the determination of the severity of the allergic reaction: the presence and the amount of both particular allergens and pro-inflammatory factors as TGase and PLA₂. In future, further research on the genetic characterization of the gene families of the inflammatory factors and the identification of new ones (i.e. eicosanoid-like substances) will be necessary to add details in the complex mechanism of sensitization/allergy.

So far allergenic pollen grains of different plant species were shown to be able to produce ROS through NAD(P)H oxidase [28]; here we confirmed that apple pollen has an intrinsic NAD(P)H oxidase activity and is able to produce ROS, with different intensity according to the variety. In particular, our data suggest that the oxidative stress induced by ‘Durello di Forlì’ pollen in human mucosa could be of smaller extent compared to the other apple pollens tested. These data are in agreement with what has been recently reported regarding a strong pro-oxidant activity of pollen grains and their allergenic extracts, which induces profound oxidative stress in the lung or conjunctiva within minutes after exposure [54,55]. Inhibition of this immediate oxidative insult significantly decreases the allergic inflammation in sensitized mice after allergen challenge [56]. It has also been demonstrated that the pro-oxidant effect of pollen grains or extracts is due to the activity of NAD(P)H oxidases [57]. It would be interesting in the future to study these factors also in fruits.

The reactivity of the IgE in the allergic patients sera with pollen proteins showed a slight decrease in IgE recognition for GP and this is in agreement with several evidences underlying the extrusion of cytoplasmic material, containing huge amounts of allergenic proteins, upon pollen rehydration and germination [42]. Furthermore, it is known that IgE binding proteins can be localized both in the cell wall (e.g. *Mal d 2* and *3* are apoplastic proteins) and in the cytoplasm of the pollen (e.g. *Mal d 1* and *4* are cytosolic proteins). This is the situation of the apple pollen that was tested in this study because in our results CWW and SP showed a similar trend in IgE recognition. The binding activity of the IgE to proteins from different varieties seems to be correlated to the intensity of the allergic reactions caused in patients, as it was low in ‘Durello di Forlì’ and high in ‘Florina’. The existence of a good correlation between the proteins-antibody recognition level and allergenicity is promising for future studies of prediction of the allergenicity, in particular for the subjects with severe and dangerous reactions. Even if apple pollen is entomophilous and only rare cases of respiratory allergy to *M. domestica* pollen were reported in literature [58], here we proved the presence of pro-inflammatory factors and fruit allergens in apple pollen and their immune-recognition by

serum of allergic patients. Indeed we can confirm that apple pollen is potentially allergenic as well as fruits [25].

After the study in apple pollen, the analysis turned on the apple fruits, the edible part of *M. domestica* and thus, interesting for what concerns food allergies. *Mal d 1* is the major apple allergen due to its cross reactivity with *Bet v 1*, the major allergen of birch pollen, and this is the reason of our in deep analysis of this allergen gene families in the peel and pulp of the apple varieties. The differential gene expression among the *Mal d 1* genes belonging to the family, depending on the variety or the fruit tissue has been already reported [6,12] but here the correlation of specific isoallergen gene expression in several apple varieties with known level of allergenicity was investigated, giving additional information to this important topic [37]. *Mal d 1.01* and *Mal d 1.02* were the highest expressed *Mal d 1* genes, as expected [6,12,37]. Interestingly, they were also significantly less expressed in the peel of ‘Durello di Forlì’ respect to the other three genotypes. Vegro et al. [37] also reported that *Mal d 1.01* and *Mal d 1.02* were expressed at high level in hyperallergenic genotype ‘Golden Delicious’ and at low level in hypoallergenic ‘Calvilla Bianca d’Inverno’. We tested the expression of these two genes also in Elise, another genotype with low level of allergenicity, and confirmed that they are expressed at a level similar to those of ‘Durello di Forlì’. We can therefore suppose that the low expression of these two genes could be a kind of marker for low allergenicity. Previous data suggested also that the presence and amount of specific *Mal d 1* isoallergens can determine allergenicity more than total *Mal d 1* content [12]. This is also in agreement with our data where *Mal d 1.06A* and *Mal d 1.07* showed a lower expression compared with *Mal d 1.01* and *Mal d 1.02* but a higher expression levels in the two hyper-allergenic varieties than in low-allergenic ones suggesting also for them a key role in determining fruit allergenicity. Again, Vegro et al [37] reported similar results on a different panel of apple genotypes, reinforcing our results. The suggestion that *Mal d 1.06A* is particularly important for the allergic reaction was also given based on a correlation between low allergenicity of a small set of apple varieties and allelic composition at this locus [59] and on previous gene expression studies [12]. However, we could not find a positive feedback in ‘Elise’ and further analyses are required to understand if *Mald 1.06* could be used as marker of hypoallergenicity. It is advisable for the future to underpin the protein structure of this allergen and to evaluate its allergenicity potential also *in vivo*. The knowledge and identification of possible *Mal d* genes responsible of allergenicity make the choice of these genes as a priority for a specific targeted mutagenesis through the so called new plant breeding techniques [60]. This approach was successfully used for *Mal d 1* genes [13].

The general low level of expression of allergen genes and of pro-inflammatory genes in fruit and pollen of the old variety ‘Durello di Forlì’ is supporting the possible involvement of these genes in the determination of allergenicity and, for some of them, a role as marker for allergenicity can be hypothesized. It would be interesting to verify this hypothesis in a panel of hypoallergenic varieties that have been recently identified by other authors [37,61], possibly cultivated in different environments and with appropriate biological replicates. Interestingly, three different and independent studies show that old varieties are less

allergenic than recently bred ones. This highlights the importance of studying a broad set of apple genetic resources and germplasm collections as genetic sources towards low allergenicity.

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FIGURES LEGENDS

Figure 1. Expression profiles by quantitative RT-PCR of twelve genes in ungerminated and germinated pollen of four apple varieties. Three pro-inflammatory (*PLA21*, *PLA23* and *TGase*) and nine Mal d allergen genes (*Mal d 1.01*, *1.02*, *1.06A*, *1.07*, *Mal d 3.01*, *Mal d 4.01*, *4.02*, *4.03*, *4.04*) expression profile in pollen grains of four apple varieties: ‘Gala’, ‘Florina’, ‘Golden Delicious’ and ‘Durello di Forlì’, as ungerminated (UGP) and germinated pollen (GP). Expression values were calculated as Arbitrary Units using both *Actin* and *18S* as internal reference genes, and then normalized on germination data. Black bars: ungerminated pollen; grey bars: germinated pollen.

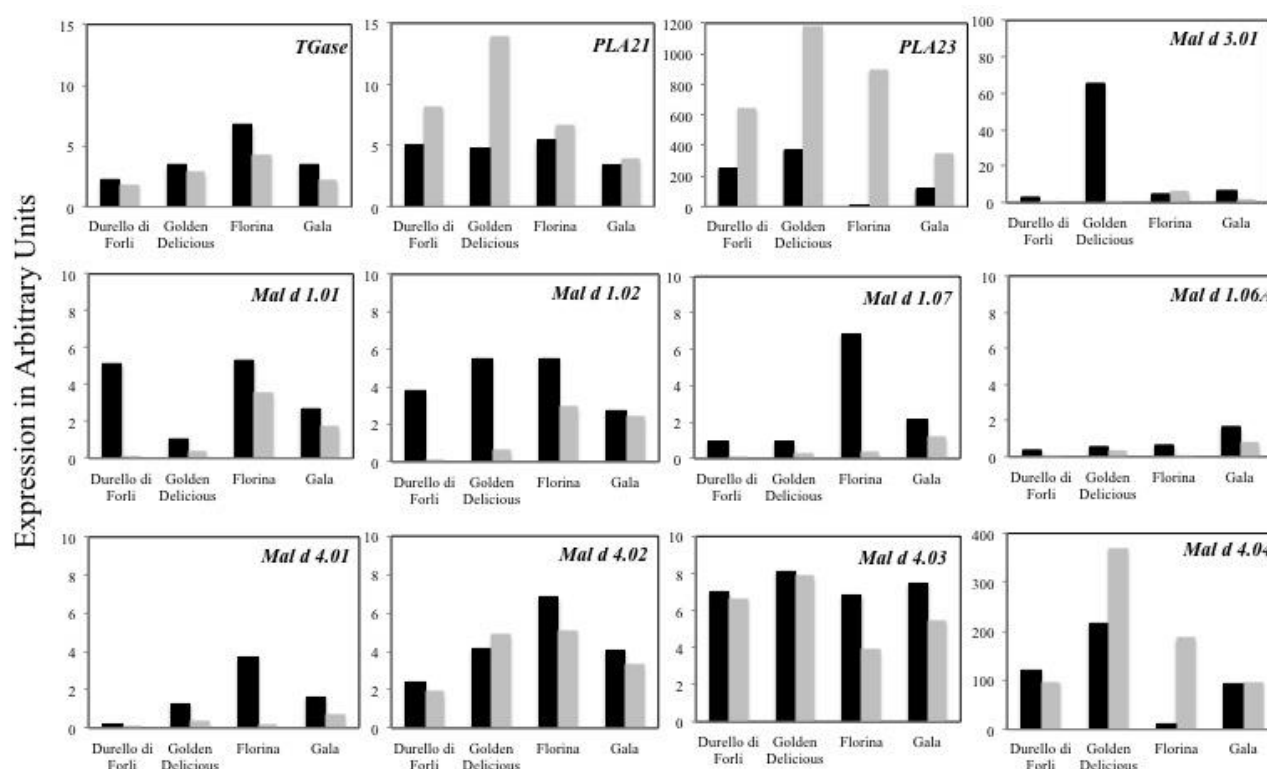


Figure 2. TGase and PLA activities. A) TGase activity has been tested for CWW and for SP of UGP and GP pollen of the four cv of *M. domestica*. TGase activity is expressed as units (U) of specific activity (means $\pm$ S.D.) per mg of pollen. Samples indicated with different letters were significantly different each other. The data were analyzed by two-way ANOVA using Bonferroni's *post-test*. B) PLA activities has been tested in the highly CWW and SP from hydrated UGP pollen of the four varieties of *M. domestica* measured after incubation at 37°C for 30 min with 250 μ g of protein extracts of *M. domestica*. PLA activity is expressed as absorbance units (Abs 450nm) (means $\pm$ S.D.). Each data point represents the mean of three independent experiments undertaken in triplicate. The data were analyzed by one-way ANOVA and Tukey's *post-test*. A *p* value <0.05 was regarded as statistically significant. ***p* < 0.01 . ****p* < 0.001 .

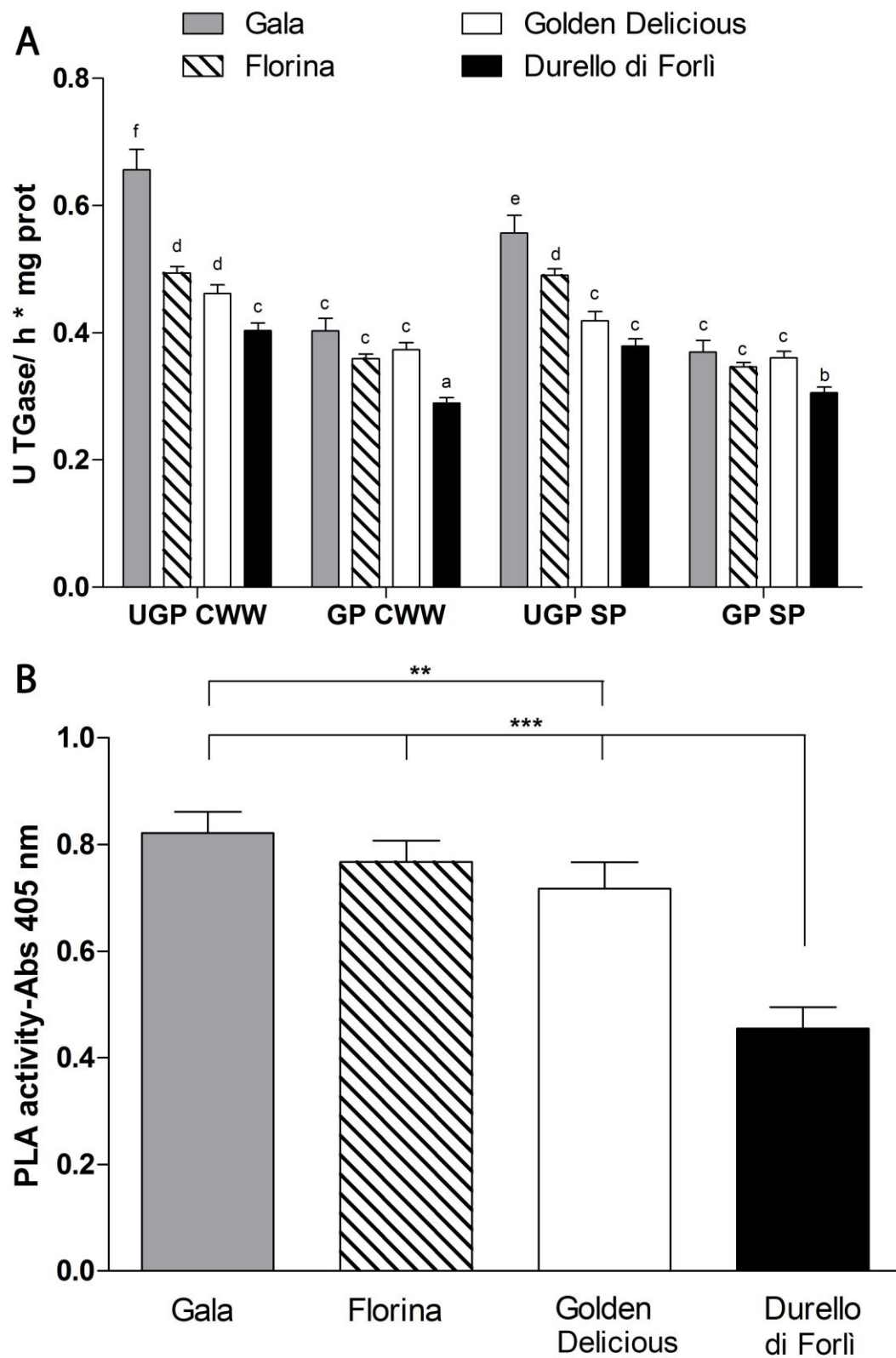


Figure 3. Generation of ROS in four apple varieties hydrated pollen grains. A) ROS quantification has been performed by spectrophotometric assay after reduction of NBT. Data were analyzed by one-way ANOVA and Tukey's *post-test*. A *p* value <0.05 was regarded as statistically significant. ***p* < 0.01. ****p* < 0.001. Data are representative of three independent experiments with similar results. B) Insert shows pollen viability measured by the MTT test. No significant differences among the samples were measured. C) Lower panel shows the purple formazan deposits due to reduction of NBT in the four varieties of apple hydrated pollen grains.

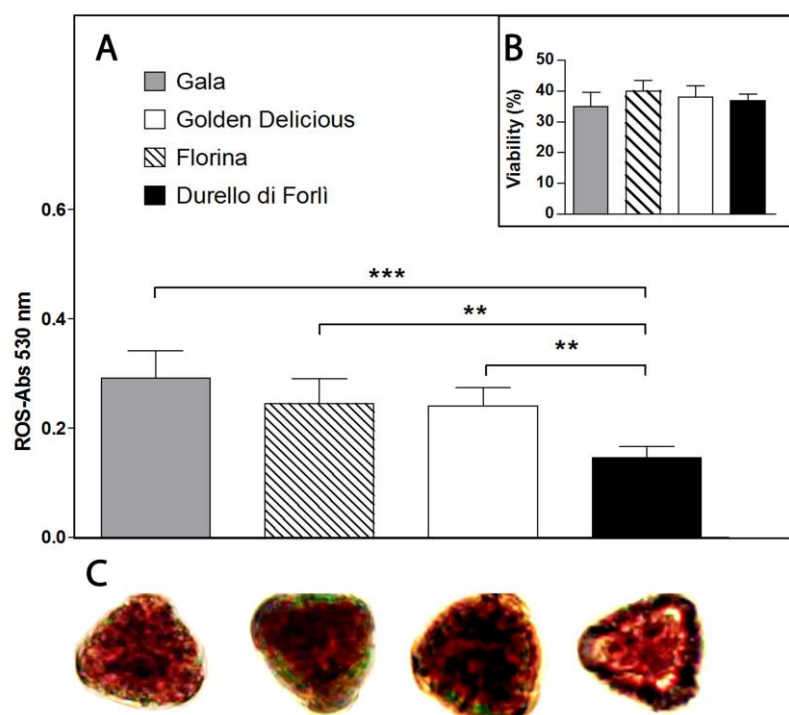


Figure 4. ELISA tests with allergic serum analysed against pollen proteins of four varieties of *M. domestica*. A) IgE reactivity towards UGP and GP pollen proteins present in CWW and SP, is expressed as absorbance units (Abs 450nm) (means $\pm$ S.D.). Samples indicated with different letters were significantly different each other. The data were analyzed by two-way ANOVA using Bonferroni's *post-test*. B) Insert shows the IgE recognition in total pollen extracts derived from the same apple UGP pollen samples. The data were analyzed by one-way ANOVA and Tukey's *post-test*. A *p* value <0.05 was regarded as statistically significant. * $p < 0.05$. *** $p < 0.001$. Data are representative of three independent experiments with similar results.

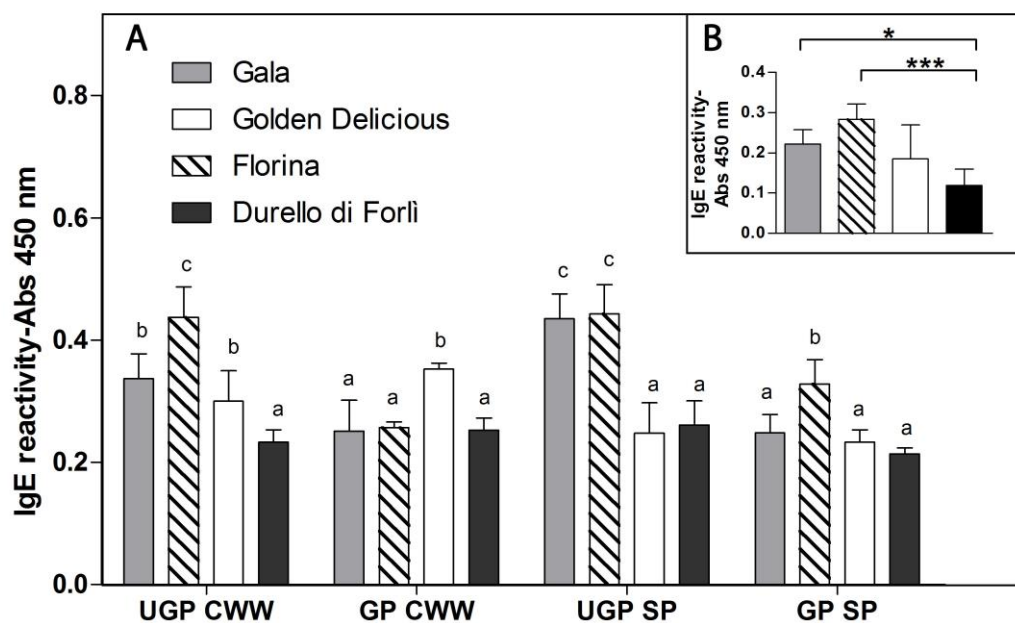


Figure 5. Results of RT-qPCR expression analyses of eight *Mal d 1* genes in samples of peel (black bars) and flesh (grey bars) of four apple varieties ('Durello di Forlì', 'Fiesta', 'Florina' and 'Gala'). The mean normalized expression level of each gene is reported in the y axis and expressed in arbitrary units; bars represent the standard errors. Letters indicate statistically different values ($P \leq 0.05$) as resulted from HSD test (R software). *Actin* was used as reference gene

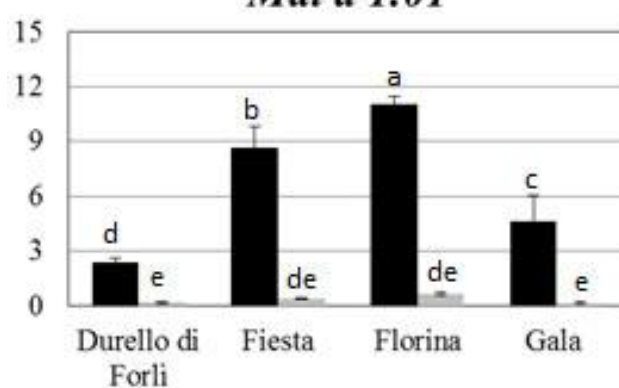
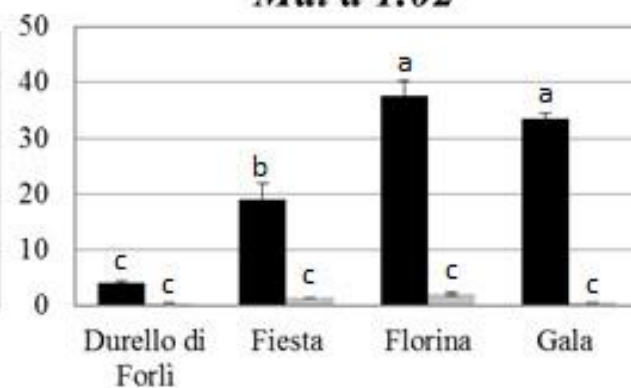
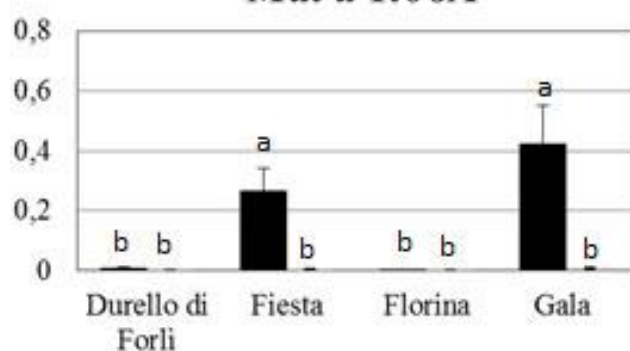
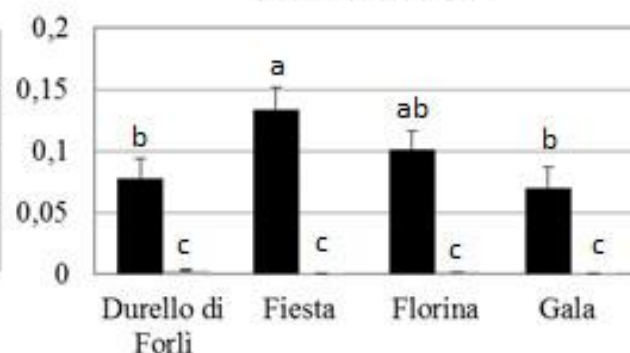
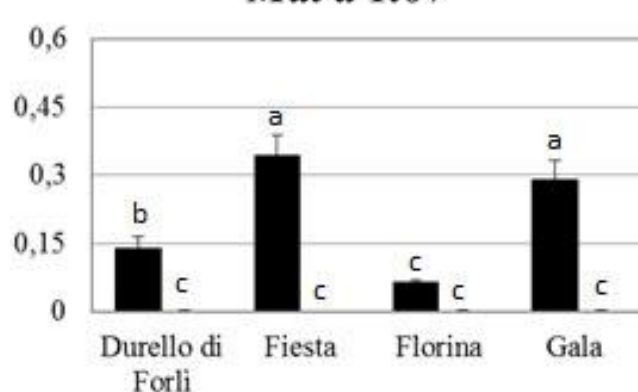
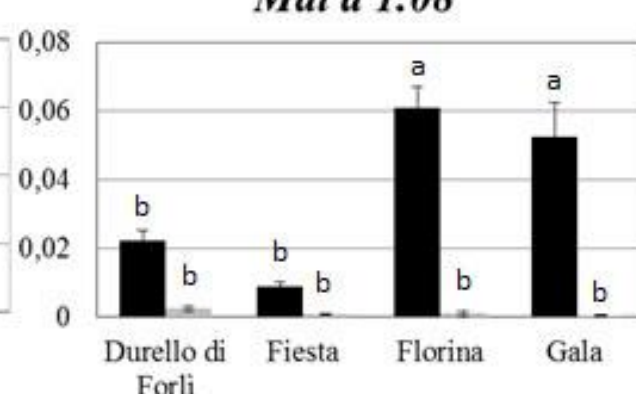
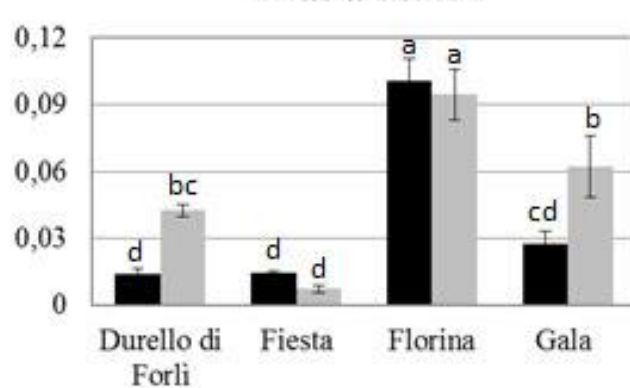
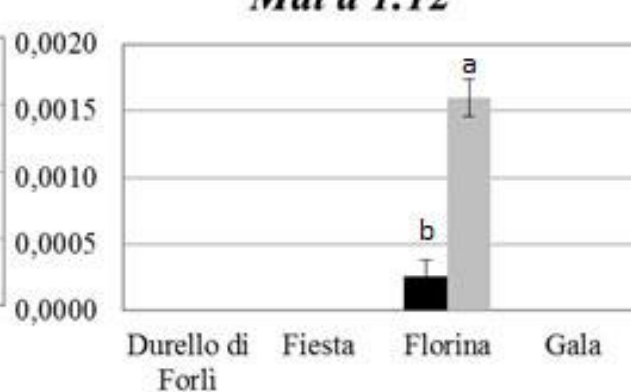
Mal d 1.01*Mal d 1.02**Mal d 1.06A**Mal d 1.06B**Mal d 1.07**Mal d 1.08**Mal d 1.11A**Mal d 1.12*

Table 1.

N a m e	PLA ZA #gene _id	MDC¹	S c o r e	E- v a l u e	position	MDP²	S c o r e	E- v a l u e	position	<i>A.th alia na</i> best hort olog s	Descriptio n
<i>P L A2 1</i>	MD13 G027 410	MDC0 20870. 281	5 7 3	E- 16 2	chr13:3206 4231..3207 3307	MDP0 00015 4214	1 6 4	8. 00 E- 40	chr13:3206 6089..3206 8106	AT2 G19 690	Phospholi pase A2- beta. Encodes one of the four Arabidopsi s phospholip ase PLA2 parologs: AT2G069 25 (PLA2- ALPHA), AT2G196 90 (PLA2- BETA), AT4G294 60 (PLA2- GAMMA) and AT4G294 70 (PLA2- DELTA). Involved in pollen developme nt and germinatio n and tube growth. Also involved in stomatal opening in response to light.
	MD00 G130 490	MDC0 05564. 266	5 7 3	E- 16 2	chr13:3206 4377..3207 2001	MDP0 00024 9250	1 6 4	8. 00 E- 40	chr13:3206 9608..3207 1665		
<i>P L A2 3</i>	MD00 G419 000	MDC0 19114. 235	3 0 1	3. 00 E- 80	chr13:3076 7184..3081 0046	MDP0 00036 6609	5 1 3	E- 14 5	chr13:3078 9189..3079 0902	AT2 G06 925	Phospholi pase A2 family protein

	MD16 G017 200	MDC0 19114. 147	2 8 5	2. 00 E- 75	chr16:1946 2206..1946 9556	MDP0 00013 0878	4 6 6	E- 13 1	chr16:1946 7387..1946 9262		Encodes a secretory phospholipase A2 enzyme, which specifically hydrolyzes the sn-2 position of phospholipids. The enzyme has a preference towards linoleoyl acyl chain over palmitoyl acyl chain. It also has a slight preference for phosphatidylcholine over phosphatidylethanolamine.
Mal d 4.04	MD00 G261 550	MDC0 11209. 236	1 1 1	9. 00 E- 23	chr2:54838 11..550208 6	MDP0 00043 7009	1 2 3	2. 00 E- 27	chr2:54916 68..549555 9	AT2 G19 770	profilin 5
	MD15 G014 150	MDC0 11209. 234	1 0 3	2. 00 E- 20	chr15:1403 0061..1406 3650	MDP0 00029 0855	1 0 3	2. 00 E- 21	chr15:1403 3253..1404 3324		Encodes profilin 5, originally named profilin 4 (PRO4/PFN4). Low-molecular weight, actin monomer-binding protein that regulates the

											organizati on of actin cytoskelet on. Pollen- specific plant profilin present predomina ntly in mature pollen and growing pollen tubes. Derlin for MDP0000 290855
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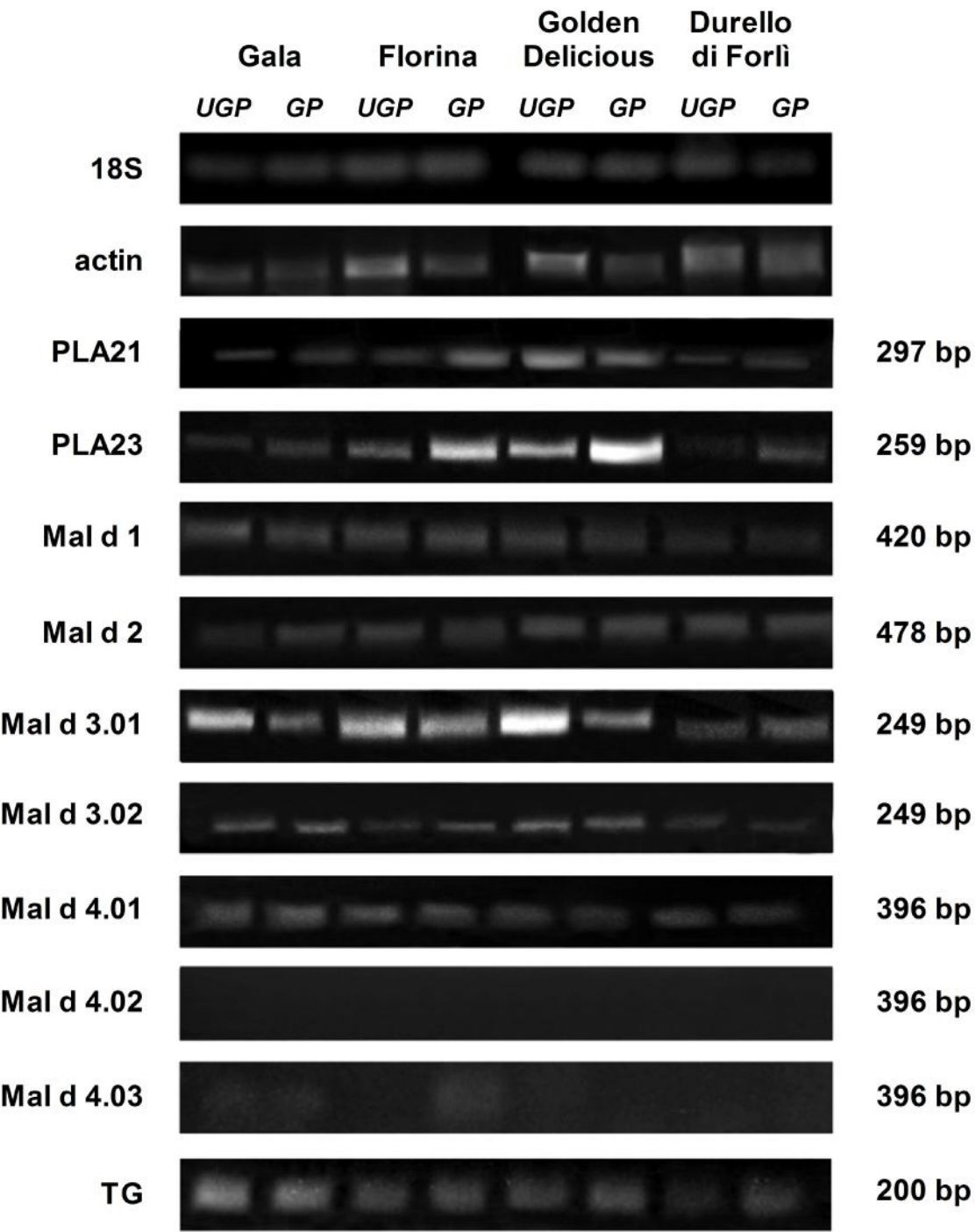
Table 2

Gene	Primer name	Primer sequence (5'-3')	Lenth (bp)	Ta	Reference
Primers for semi-quantitative PCR					
PLA21	PLA 21-For	TCAAGCGCTGCATAGAGAAA	297	52	This work
	PLA 21-Rev	CAACGCAACACAAAATCAGG			
PLA23	PLA 23-For	TGGGAAGTATTGTGGGCTTT	259	52	Iorio et al., 2013
	PLA 23-Rev	AGCAACTAAAGCAGCCTCCA			
Tgase	TGaseRTF	TTCAGTTGGGTAACTCGCC	200	58	This work
	TGaseRTR	ACGCCCTCTTCTTGTTC			
Mal d 1	M1For	TCCACCACCAAGATTGTTCA	420	58	Pagliarani et al., 2009
	M1Rev	TGGCCCTTGAGGTAGCTCTC			
Mal d 2	M2nFor	AAAGCTAGCCGATCAGTGGGA	478	58	Pagliarani et al., 2009
	M2nRev	GAGGGCACTGCTTCTCAAAG			
Mal d 3.01	M301For2	GTGGCAAAGTGACCAGCAG	249	60	Pagliarani et al., 2009
	M3Rev2	TGGTGGAGGTGCTGATCTTG			
Mal d 3.02	M302For2	TGGCCAGGTGAGCTCCAA	247	60	Pagliarani et al., 2009
	M3Rev2	TGGTGGAGGTGCTGATCTTG			
Actin	Act F	TCCTTCGTCTGGACCTTGCT	225	60	Iorio et al., 2012
	Act R	TTGTGATGACTTGGCCATCG			
18S	18S F	TGGGAAGTATTGTGGGCTTT	259	52	Iorio et al., 2012
	18S R	AGCAACTAAAGCAGCCTCCA			
Primers for Real time PCR					
PLA21	PLA 21-For	ATCGCACGACACAGTTCTCC	78	60	This work
	PLA 21-Rev	TATTGTCGGAACAGTGGGCG			
PLA23	PLA 23-For	AGTGCAGCCAAACGTTCCCTA	123	59	This work
	PLA 23-Rev	CAGCCTCCATGACAAGGGTT			
Tgase	TGaseRTF	AGGGATCAAGCTGGCATCTG	108	58	This work
	TGaseRTR	AAGCATTTGCGAGGAAACCTT			
Mal d 1.01	qMd1.01/02F	GATTGAAGGAGATGCTTTGACA	103	63	Pagliarani et al., 2013
	qMd1.01R	GTAATGACTGATGCTCTTGATGG			
Mal d 1.02	qMd1.01/02F	GATTGAAGGAGATGCTTTGACA	111	62	Pagliarani et al., 2013
	qMd1.02R	TTGGTGTGGTAGTGGCTGATA			
Mal d 1.06A	qMd1.06AF	CTATAGCTATAGCTTGATTGAAGGG	167	61	Pagliarani et al., 2013
	qMd1.06AR	TTCCAACCTTAACATGTTCTTCT			
Mal d 1.06B	qMd1.06BF	AAACCGAATACGCATCCATT	105	61	Pagliarani et al., 2013
	qMd1.06BR	ACAGTTTTGACTGCTTGTGGAG			
Mal d 1.07	qMd1.07F	CAACTTTGTGTACCAGTACAGTGTC	126	61	Pagliarani et al., 2013
	qMd1.07R	TAGTGGCTGATGCTCTTGATAAC			
Mal d 1.08	qMd1.08F	TCTTCGGTGAAGGTAGCACAA	200	61	Pagliarani et al., 2013
	qMd1.08R	ACCCTTAGTGTGGTAGTGGCAT			
Mal d 1.11A	qMd1.11AF	GGAGGATGCATCTGTCATTTG	130	62	Pagliarani et al., 2013
	qMd1.11AR	CCATGAGATAGGCTTCCAAAAC			
Mal d 3.01	M301RTF	CCGCTGACCGCCAGAC	85	60	Pagliarani et al., 2009
	M3RevRTF	AGCCCTGCTGCATTGTTAGG			

<i>Mal d 4.01</i>	Md401fw	CAGCCAGGCCCTGTTAATC	104	58	Pagliarani et al., 2009
	Md401rv	AGAGACCCTGCTCAATAAGGTAA			
<i>Mal d 4.02</i>	qMd402F	GTGTTACTGTCAAGAAGAGCACAA	126	58	Pagliarani et al., 2010
	qMd402R	GCTCAATGAGATAATCCGcA			
<i>Mal d 4.03</i>	Md403fw	GGCCAAGCTTTGGTTTTTC	99	58	Pagliarani et al., 2009
	Md403rv	GCCTTGATCAATCAGGTAGTCT			
<i>Mal d 4.04</i>	Md404Afor	GGCAAGCTCTAGTATTTGGAATCTAC	79	58	This work
	Md404Arev	TCGCCCCAACCTCTCAACC			
<i>Actin</i>	qMdActF	CTATGTTCCCTGGTATTGCAGACC	82	60	Paris et al., 2009
	qMdActR	GCCACAACCTTGATTTTCATGC			
<i>18S</i>	RT18S F	GAGAAACGGCTACCACATCCA	65	60	This work
	RT18S R	TCCCCGTGTCAGGATTGG			

Supplementary Material

Supplementary Figure 1. Expression profiles by semi-quantitative RT-PCR of ten target genes and two reference genes in ungerminated and germinated pollen of four apple varieties. On the top, the four cultivar analyzed: Gala, Florina, Golden Delicious; Durello di Forlì as ungerminated (UGP) and germinated pollen (GP). On the right: amplicon dimension expressed in basepairs (bp)



Supplementary Figure 2. RT-qPCR expression analysis of *Mal d 1* genes. Four *Mal d 1* genes (*Mal d 1.01*, *1.02*, *1.06A* and *1.07*) were analyzed in fruit samples of peel (black bars) and flesh (grey bars) from the ‘Elise’ variety. The expression level of each target gene was normalized on *Actin* as housekeeping gene and reported in the y axis as arbitrary units.

