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

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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 A_2 (PLA_2) 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, PLA_2 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.

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

Abbreviations: RT-qPCR, reverse transcriptase quantitative PCR; TGase, transglutaminase; PLA, phospholipase; ROS, reactive oxygen species; UGP, ungerminated pollen grains; GP, germinated pollen grains; SP, soluble proteins; CWW, proteins from cell wall; NBT, nitroblue tetrazolium; HEPC, 2-hexadecanoylthio-1-ethylphosphorylcholine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic Acid); NAD(P)H, nicotinamide adenine dinucleotide phosphate; DPI, diphenyleneiodonium; TMB, 3, 3′,5, 5-tetramethylbenzidine; MTT, 2,5-diphenyl tetrazoliumbromide; LG, linkage group

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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 1–3 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 ($sPLA_2$) through the formation of a transient dimer of active $sPLA_2$ [21]. The PLA_2 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 PLA_2 families have been reported in plants: the low molecular weight protein sPLA2 and the patatin-like PLAs [22]. PLA_2 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 $sPLA_2$ or co-inhibiting tTGase and PLA_2 displayed strong *in vivo* anti-inflammatory activity in allergic conjunctivitis [19,23]. Despite the proven involvement of plant $sPLA_2$ in many biological functions [22,24], the $sPLA_2$ 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 $sPLA_2$, 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 PLA_2 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 min and allowed to germinate (1 mg/mL in germination medium: 10% sucrose, 324 μM H_3BO_3, 1.27 μM $CaNO_3$) for 2 h 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