Amino Acids (2012) 42:1007–1024
DOI 10.1007/s00726-011-1043-7

ORIGINAL ARTICLE

Simulated environmental criticalities affect transglutaminase of *Malus* and *Corylus* pollens having different allergenic potential

Rosa Anna Iorio · Alessia Di Sandro · Roberta Paris · Giulia Pagliarani · Stefano Tartarini · Giampaolo Ricci · Donatella Serafini-Fracassini · Elisabetta Verderio · Stefano Del Duca

Received: 16 March 2011 / Accepted: 26 May 2011 / Published online: 17 August 2011

Abstract Increases in temperature and air pollution influence pollen allergenicity, which is responsible for the dramatic raise in respiratory allergies. To clarify possible underlying mechanisms, an anemophilous pollen (hazel, *Corylus avellana*), known to be allergenic, and an entomophilous one (apple, *Malus domestica*), the allergenicity of which was not known, were analysed. The presence also in apple pollen of known fruit allergens and their immunorecognition by serum of an allergic patient were preliminary ascertained, resulting also apple pollen potentially allergenic. Pollens were subjected to simulated stressful conditions, provided by changes in temperature, humidity, and copper and acid rain pollution. In the two pollens exposed to environmental criticalities, viability and germination were negatively affected and different transglutaminase (TGase) gel bands were differently immunodetected with the polyclonal antibody AtPng1p. The enzyme activity increased under stressful treatments and, along with its products, was found to be released outside the pollen with externalisation of TGase being predominant in *C. avellana*, whose grain presents a different cell wall composition with respect to that of *M. domestica*. A recombinant plant TGase (AtPng1p) stimulated the secreted phospholipase A_2 ($sPLA_2$) activity, that in vivo is present in human mucosa and is involved in inflammation. Similarly, stressed pollen, hazel pollen being the most efficient, stimulated to very different extent $sPLA_2$ activity and putrescine conjugation to $sPLA_2$. We propose that externalised pollen TGase could be one of the mediators of pollen allergenicity, especially under environmental stress induced by climate changes.

Keywords Climate changes · Environmental pollution · Phospholipase A_2 · Pollen allergenicity · Respiratory allergies · Transglutaminase

Abbreviations

AtPng1p	*Arabidopsis thaliana* peptide *N*-glycanase
BSA	Bovine serum albumin
DMC	*N*′,*N*′-dimethyl casein
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FDA	Fluorescein diacetate
FITC	Fluorescein isothiocyanate
GP	Germinated pollen
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
nsLTP	Nonspecific lipid transfer protein
o/n	Overnight
PAs	polyamines
PBS	Phosphate-buffered saline

R. A. Iorio · A. Di Sandro · D. Serafini-Fracassini · S. Del Duca (✉)
Department of Biology E.S., University of Bologna, Via Irnerio 42, 40126 Bologna, Italy
e-mail: stefano.delduca@unibo.it

R. Paris · G. Pagliarani · S. Tartarini
Department of Fruit Tree and Woody Plant Sciences, University of Bologna, Viale Fanin 46, 40127 Bologna, Italy

G. Ricci
Department of Pediatrics, University of Bologna, Via Massarenti 11, 40138 Bologna, Italy

E. Verderio
School of Science and Technology, Nottingham Trent University, Nottingham NG11 8NS, UK

PMSF	Phenyl methyl sulfonyl fluoride
PR	Pathogen related
PU	Putrescine
$sPLA_2$	Secreted phospholipase A_2
rHu	Relative humidity
T	Temperature
TGase	Transglutaminase
tTGase	Tissue transglutaminase
UGP	Ungerminated pollen

Introduction

Changes in global climate have a negative impact on human health especially on respiratory diseases. When inhaled, biological aerosol carrying antigenic proteins such as pollens penetrate the airway mucosa and access the cells of the immune system leading to an immunoglobulin E (IgE)-mediated response (respiratory allergy). Raises in atmospheric greenhouse gasses, such as CO_2, temperature and rainfalls, in combination with outdoor air pollution, have been shown to influence the allergenicity of pollen and increase the severity and frequency of respiratory allergic diseases (hay fever, bronchial asthma, seasonal allergic conjunctivitis), which have dramatically increased in recent years (D'Amato and Cecchi 2008; Shea et al. 2008).

The mechanism underlying the influence of outdoor air pollution on pollen allergy remains unclear. It has been proposed that changes in climate may affect respiratory diseases such as asthma, either directly by damaging the airway mucosa, thus facilitating the access of allergens to the cells of the immune system (Adhikari et al. 2006; Cecchi et al. 2010; D'Amato and Cecchi 2008; D'Amato et al. 2007; Peden 2002), or indirectly by enhancing the allergenic potential of airborne allergens (Weber 2002; D'Amato et al. 2007). The release of allergens from pollen may also be favoured by environmental factors such as thunderstorms, which could cause the osmotic break of pollen (Cecchi et al. 2010). Increased CO_2 concentrations (Ziska and Caulfield 2000), temperature (Rogers et al. 2006) and ultraviolet radiations have been all shown to lead to stronger pollen allergenicity (Motta et al. 2006), which is further exacerbated by air pollution (particulate matter, ozone, nitric oxides) (Adhikari et al. 2006). It is known that high CO_2 concentration increases plant biomass, propagation and pollen production, and that increased temperature stimulates earlier flowering and longer pollen seasons for some plants. Thus, the aeroallergen characteristics of our environment may change considerably in the future as a result of climate changes (Beggs 2004; D'Amato and Cecchi 2008; Cecchi et al. 2010).

Anemophilous plants have a greater allergenic potency than the entomophilous plants. Because anemophilous pollen is produced in greater amount and it is dispersed in the environment by the wind, it most likely comes in contact with human mucosa. Differences in potency may also be due to allergens produced in greater amount and released early, different allergen composition and presence and/or easier release of factors activating the allergenic cascade, as well as the organisation of the pollen cell wall which, in the case of anemophilous pollen, may facilitate the release of allergens.

Pollinosis causes a type I hypersensitivity response and the manifestation occurs only 30 s after pollen allergen mucosa contact. Thus, allergens should be located on the surface of pollen or rapidly released. Alternatively, it can be hypothesised that their release can occur during tube emergency that however takes longer to begin. Accorsi et al. (1982) reported that germinated (GP) pollens with relatively large tubes have been detected in the mucus of allergic patients but not in healthy controls. This aspect has not been further investigated. In addition to the reported effects, it is possible that the climate changes directly affect some pro-inflammatory agent located in the higher airways of humans.

Transglutaminases (TGases) are a widely distributed family of enzymes present in animals, plants and unicellular organisms, which are responsible for the post-translational modification of proteins by acting as calcium-dependent protein cross-linkers (Metha et al. 2006; Griffin et al. 2002). The presence and activity of TGases in plants is well documented (Serafini-Fracassini et al. 2009). We have previously detected and characterised TGase from *Malus domestica* pollen where it was found to be expressed both inside the cell, with a possible role in the modification of cytoskeleton proteins during apical growth, and extracellularly, co-localised with possible cross-linking substrates (Iorio et al. 2008; Di Sandro et al. 2010). During in vitro germination, pollen TGase is also released into the medium where it catalyses the conjugation of secreted polyamines (PAs) to secreted proteins. A similar distribution of the enzyme activity was observed *in planta* in pollen germinating inside the style (Di Sandro et al. 2010).

This group of enzymes is drawing increasing attention in mammal pathology for the role played on the onset of specific pathologies and associated autoimmune inflammatory conditions (Lorand and Graham 2003). During cellular stress and associated increase in intracellular calcium, the activity of tissue transglutaminase (tTGase) is typically increased inside the cell (Verderio et al. 2004). tTGase is also rapidly secreted into the extracellular environment, following cell insult, where it plays an important role in extracellular matrix stabilisation and cell survival (Verderio et al. 2004). In mammals, the role of

tTGase in inflammatory diseases has been associated with activation of pro-inflammatory secretory phospholipase A_2 ($sPLA_2$) through the formation of a transient dimer of active $sPLA_2$ (Cordella-Miele et al. 1993; Verderio et al. 2004). PLA_2 hydrolyses glycerophospholipids, the first rate limiting step in the eicosanoid cascade, and tTGase is reported to be a regulator of eicosanoid production in asthma (Hallstrand et al. 2010). Chimeric peptides able to inhibit tTGase-mediated modification of $sPLA_2$ or co-inhibiting tTGase and PLA_2 displayed strong in vivo anti-inflammatory activity in allergic conjunctivitis (Sohn et al. 2003; Miele 2003).

There is no demonstration of the possible involvement of pollen TGase in respiratory allergies. Here, we hypothesise that extracellular pollen TGase may be activated by environmental stress conditions such as those caused by climate changes or air pollution and contribute to increase the activity of pro-inflammatory $sPLA_2$ present in the human mucosa of subjects predisposed/affected by allergic respiratory pathologies.

Here, we evaluate comparatively the allergenicity of an anemophilous pollen (hazel, *Corylus avellana*) known to be allergenic, and of an entomophilous one, not reported as being allergenic (apple, *Malus domestica*). Three classes of allergens (Cor a 1, 2 and 8) have been reported in hazel, both in the nut and in pollen. Allergens have not been similarly described in apple pollen; however, sensitisation and allergy to the fruit have been frequently reported in the European Community (Burney et al. 2010) and four main allergen genes families (Mal d 1, 2, 3 and 4) have been identified in the skin and pulp of apples to different extents (Pagliarani et al. 2009).

We show the influence of simulated climate conditions on viability, germination and the physiological level of TGase expression, activity and distribution in vivo and in vitro of both pollens. Finally, we show the role played in vitro by pollen exposed to environmental stress on the activity of mammalian $sPLA_2$.

Materials and methods

Chemicals and antibodies

All chemicals (unless otherwise indicated) were obtained from Sigma-Aldrich (Milan, Italy). The tTGase inhibitors 283 (1,3-dimethyl-2[(oxopropyl)thio]imidazolium) (now available also at Zedira, Darmstadt, Germany) and 281 (*N*-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine), two specific site-directed inhibitors of TGase, developed to covalently bind the Cys-based active site of the calcium-activated form of mammalian TGase were synthesised at Nottingham Trent University, UK (Griffin et al. 2008). 5-((5-aminopentyl) thioureidyl) fluorescein, dihydrobromide salt (fluorescein-cadaverine) was purchased from Molecular Probes (Invitrogen, UK). Biotin-cadaverine was obtained from Molecular Probes (Eugene, OR, USA). Polyclonal antibody AtPng1P was produced in chicken at the CSIC of Barcelona, Spain (Della Mea et al. 2004). The list of secondary antibodies includes: HRP-conjugated rabbit anti-mouse IgG (DakoCytomation, Glostrup, Denmark); fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Roche Applied Science, Mannheim, Germany); goat anti-rabbit IgG HRP-conjugated (Bio-Rad, Milan, Italy); anti-chicken IgG alkaline phosphatase conjugate (Sigma-Aldrich, Milan, Italy).

Plant material and growth

Mature pollen of *Malus domestica* Borkh cv. Golden Delicious was collected from plants grown in experimental plots (Dipartimento di Colture Arboree, University of Bologna), whereas mature pollen from *Corylus avellana* cv. Tonda Gentile delle Langhe was kindly donated by Professor R. Botta, University of Torino. Handling and storage were performed as reported by Del Duca et al. (1997) for the former and by Botta et al. (1994) for the latter. Pollen was hydrated in the environmental phytotron chamber and the treatments included simulation of different atmospheric temperatures and relative humidity (rH) (10°C–70% rHu o/n, 20°C–50% rHu o/n, 20°C–70% rHu o/n, 30°C–100% rHu o/n or in some experiments with *M. domestica* pollen 30 min), acid rain pollution (obtained adjusting a water solution to pH 5.6 with H_2SO_4:HNO_3 at a ratio of 2:1) and copper pollution (a solution of 3.10 μg l^{-1} of $CuCl_2$); the two solutions were sprayed on the pollen grains before the hydration as reported in Bellani et al. (1997).

To mimic natural conditions, 10°C–70% rH o/n for *C. avellana* and 20°C–50% rHu o/n for *M. domestica* have been assumed as control according to the seasonal media condition at the moment of their anthesis. Then, pollen grains were allowed to germinate (1 mg ml^{-1} in germination medium) into glass Petri dishes up to 120 min in the case of apple pollen (Calzoni et al. 1979) or 13 h (overnight, o/n) for hazel pollen as reported by Botta et al. (1994). Light microscopy digital images of at least three non-overlapping fields were captured using an Olympus DP10 digital camera and quantified in terms of GP pollen, when the tube was larger than the grain diameter. Pollen viability was assessed by staining with fluorescein diacetate (FDA, 10 μg ml^{-1}), based on the hydrolysis of fluorescein by the cytoplasmatic enzymes, or with 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT, 1 mg ml^{-1}), based on the conversion from yellow to purple-coloured formazan by mitochondrial reductase enzymes. The pollen morphology was analysed with the Alexander staining (Alexander 1969).

Pollen protein extraction

Proteins from pollen were extracted as described by Del Duca et al. (2009) and quantified following the bicinchoninic acid method with bovine serum albumin (BSA) as the standard protein.

SDS-PAGE and Western blot analysis

Fifty microgram of GP proteins obtained after extraction was electrophoresed on 10% (w/v) polyacrylamide SDS-PAGE slab gels (Laemmli 1970), using the usual Page Ruler Prestained Mw standards (Fermentas, Germany). Western blotted protein bands were revealed by AtPng1p polyclonal antibody according to Della Mea et al. (2004) or with serum from patients allergic and non-allergic to apple and developed with anti-human IgE HRP-conjugated secondary antibody.

TGase activity assays

Extracellular pollen TGase activity was measured by the conjugation of biotinylated cadaverine to *N*,*N*′-dimethylcasein (DMC)-coated 96-well microtitre plates (NUNC life Technologies) as described by Jones et al. (1997), with minor modifications. Hydrated pollen grains were allowed to germinate in the microplate for 120 min for *M. domestica* or 13 h (o/n) for *C. avellana* at 37°C in a germination medium buffered as reported in Di Sandro et al. (2010) in the presence or absence of 281 (500 μM), cystamine (250 μM) and the competitive substrates: iodoacetamide (250 μM), putrescine (10 mM) and EGTA (20 mM). The level of enzyme activity was expressed as Ca^{2+}-dependent increase in A_{450}, after subtraction of the value of the 20 mM EGTA-treated control. Specific activity was determined as a change in A_{450} of 0.1 per hour per mg of non-hydrated pollen.

In situ visualisation of TGase activity during pollen germination

Pollen grains hydrated and treated as above reported were allowed to germinate in eight-well glass chamber slides (Nunc Life Technologies, UK) for up to 120 min for *M. domestica* or o/n for *C. avellana*. In situ TGase activity was visualised by incubation of germinating pollen with fluorescein-cadaverine (FITC-cadaverine), in the presence or absence of the inhibitor 281 (500 μM), as reported by Iorio et al. (2008), which was added to the germination medium at 60 min (*M. domestica*) or at 120 min (*C. avellana*) of germination. After fixation in methanol at −20°C for 10 min to remove the unspecifically bound FITC-cadaverine (Verderio et al. 1998), pollens were viewed by a Leica TCSNT confocal laser scanning microscope system (Leica Lasertechnik, Heidelberg, Germany) as reported by Iorio et al. (2008).

In situ immunofluorescence of TGase during pollen germination

Immunostaining with polyclonal anti-TGase AtPng1p was performed on fixed pollen as reported in the previous paragraph. First, the pollen was incubated at 37°C for 1 h with 3% BSA in PBS 1X to decrease background and then treated with AtPng1p (1:200) in PBS 3% BSA overnight at 4°C incubation. The samples were washed with PBS and incubated with anti-chicken secondary antibody (1:40 in PBS 3% BSA) FITC conjugated for 2 h at 37°C. Vectashield and coverslip were mounted after washing the samples with PBS, and then stored at 4°C (only overnight) or −20°C.

$sPLA_2$ enzyme assay

$sPLA_2$ enzyme assay was performed following the R&D Systems instructions (Abingdon, UK), incubating at 37°C for 30 min in the 96-well microplate 50 μl of standard (supplied by the manufacturer), 250 μg of pollen samples or 50 μg of AtPng1p recombinant protein with 100 μl of 1× reaction buffer and 50 μl of $sPLA_2$ substrate. As negative control, 10 mM putrescine, 500 μM 281 or 283 were used. The use of both dithiothreitol (DTT) and ethylene diamine/ethylene glycol tetraacetic acid (EDTA/EGTA) was avoided following validation experiments to prevent any possible interference with the seven disulphide bridges of $sPLA_2$ and its strict dependence upon the Ca^{2+} concentration.

Radioactive assay of TGase activity

The samples (whole pollen grains from *M. domestica* and *C. avellana*) were incubated for 2 h at 37°C with 0.2 μM (370 KBq) of [1.4(n)^3 H]-PU] (specific activity 1.5 TBq $mmol^{-1}$, NEN, Milan) and 5 μg PLA_2 as substrate in 20 mM Tris–HCl, pH 8.5, buffer containing 10 mM DTT and 5 mM $CaCl_2$ as positive control or 20 mM EGTA as negative control. After the reaction, the incorporation of radioactivity of covalently conjugated PAs was detected as reported in Del Duca et al. (1997).

Pollen protein extraction from cell wall

Hydrated and germinated pollen grains were treated with different buffers (10 mg pollen/0.5 ml buffer) following the method reported by Cai et al. (2011) to obtain proteins located in the cell wall. The samples were further used for

Table 1 Allergens, TGase and actin housekeeping gene primer sequences for semi-quantitative analysis in apple

Gene	Primer name	5′–3′ sequence	PCR product (bp)	Ta
Mal d 1	Mal d 1.02 F	TCCACCACCAAGATTGTTCA	420	60
Subfamily I	Mal d 1.02 R	TGGCCCTTGAGGTAGCTCTC		
Mal d 1	Mal d 1.04 F	CGAAGGCGATGGAGGTGT	315	60
Subfamily II	Mal d 1.04 R	GATCCTCCATCAGGAGATGC		
Mal d 1	Mal d 1.06A R	TTCCAACCTTAACATGTTCTTCT	170	60
Subfamily III	Mal d 1.06A R	CTATAGCTATAGCTTGATTGAAGGG		
Mal d 1	Mal d 1.03 F	TCAACTTTGGTGAAGGTAGCACA	198	60
Subfamily IV	Mal d 1.03 R	TGGTGTGGTAGTGGCTGGTA		
Mal d 2.01	M2nFor	AAAGCTAGCCGATCAGTGGA	478	60
	M2nRev	GAGGGCACTGCTTCTCAAAG		
Mal d 3.01	M301For2	GTGGCCAAGTGACCAGCAG	249	60
	M3Rev2	TGGTGGAGGTGCTGATCTTG		
Mal d 3.02	M302For2	TGGCCAGGTGAGCTCCAA	247	60
	M3 Rev2	TGGTGGAGGTGCTGATCTTG		
Mal d 4.01	Md401RTF1	GGCCAAGCTTTGGTTTTC	99	58
	Md401RTR1	GCCTTGATCAATCAGGTAGTCT		
Mal d 4.02	qMd4.02F	GTGTTACTGTCAAGAAGAGCACAA	126	58
	qMd4.02R	GCTCAATGAGATAATCCGCA		
Mal d 4.03	Md403RTF1	CAGCCAGGCCCTGTTAATC	104	58
	Md403RTR1	AGAGACCCTGCTCAATAAGGTAA		
TGase	TGaseRTF	CTGGCATCTGTTGGATAAACG	199	60
	TGaseRTR	ACCTAATTGCAGCCGCGA		
Actin	ACT F	GTTGCAATTCAGGCTGTCCT	217	60
	ACT R	GCTCAGGAGTTGTGGTGAAA		

the immunoblot analysis with serum from patients allergic or non-allergic to apple.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from 30 mg of frozen UGP and GP *M. domestica* pollen (collected at 15, 30, 60, 90 and 120 min) using the NucleoSpin RNA Plant (Macherey–Nagel, Germany), according to the manufacturer's instructions. The purity and concentration of the extracted RNAs were evaluated by measuring the absorbance at 260 and 280 nm wavelengths. RNA was considered pure if the A260/280 ratio was >1.8, and 10 µg was treated with 2.5 Units DNase I (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) at 37°C for 20 min. First-strand cDNA was synthesised following the instruction from Stratagene (Germany), starting from 1 µg DNA-free RNA, and 2 µl was used for the semi-quantitative PCR analyses.

Primer design

Specific primer pairs (Table 1) were designed with the software Primer3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/) and further tested with the software PrimerSelect® v8.0-MegAlign for the formation of primer homo and heterodimers. For the complex *Mal d 1* gene family, four different primer pairs were designed, each one specific for a gene belonging to a *Mal d 1* subfamily and chosen according to their expression profile in fruit (Pagliarani et al. 2009): *Mal d 1.02* (subfamily I), *Mal d 1.04* (subfamily II), *Mal d 1.06A* (subfamily III) and *Mal d 1.03* (subfamily IV). To this aim, *Mal d 1* sequences were retrieved from GeneBank database and aligned with the software Lasergene® v8.0-MegAlign (DNASTAR, Inc., Madison, WI, USA). The other listed allergen gene-specific primer pairs were from Pagliarani et al. (2009), except for *Mal d 4.02* (Pagliarani, personal communication). TGase-specific primers were designed on the base of an apple EST sequence published in the Michigan State University database (Gene id: MD4C271570) and compared to AtPng1p (Gene id: AC AM745095). As endogenous control, the *actin* gene of *M. domestica* (Gene id: MD09G010810 http://bioinformatics.psb.ugent.be/plaza/) was used.

Allergen gene expression analysis in apple pollen by semi-quantitative PCR

The PCR reactions were performed in a 17.5 µl reaction containing 2 µl of cDNA, 0.1 µM specific primers (Table 1), 1.5 mM $MgCl_2$, 100 µM dNTPs, 0.5 unit DNA

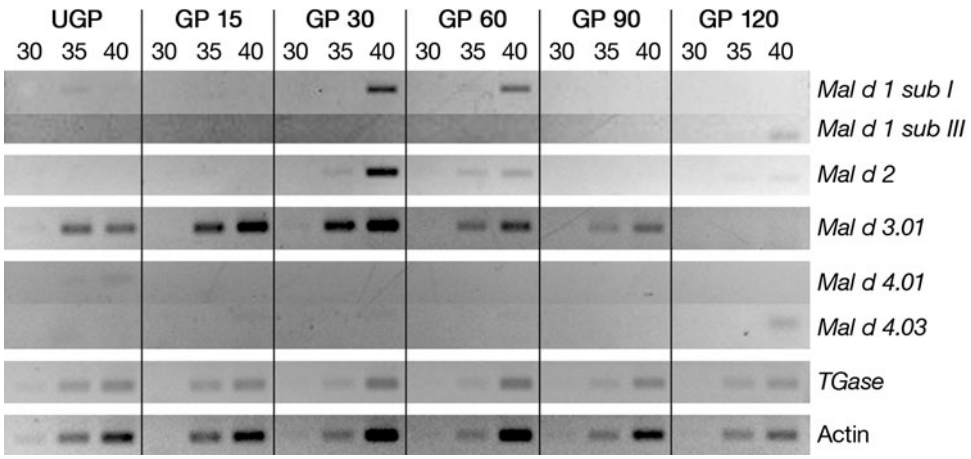

Fig. 1 Expression of apple allergen and TGase genes in ungerminated and germinating *M. domestica* pollen. The cDNAs obtained from mRNA of pollen UGP and GP at different times was tested for different allergen genes belonging to the *Mal d 1*, *Mal d 2*, *Mal d 3* and *Mal d 4* families and for the TGase gene through semi-quantitative RT-PCR, after 30, 35 or 40 cycles of PCR. *Lanes*: pollen total extract after hydration, UGP; 15 min of germination, GP 15; 30 min of germination, GP 30; 60 min of germination, GP 60; 90 min of germination, GP 90; 120 min of germination, GP 120. Actin expression was used as reference gene

polymerase (Fisher Molecular Biology, Hampton, NH, USA) and 1× reaction buffer.

The reaction included an initial 3 min of a denaturation step at 94°C, followed by 30, 35 and 40 PCR cycles at the optimised annealing temperature, 2 min at 72°C and 30 s at 94°C.

The number of amplification cycles was chosen to be in the exponential phase of amplification. The amplicons were visualised with a transilluminator (Vilber Lournet, Genenco) at 302 nm after electrophoresis in 1% (w/v) agarose gels in TAE 1X buffer, containing 0.5 μg ml^{-1} ethidium bromide, and photographed (Nikon E5400 Coolpix). The experiment was repeated twice.

Statistics

The values reported are expressed as mean ± SD and represent one of at least three or four different experiments undertaken in triplicate. Differences between sample sets were determined by the Student's *t* test with 95% confidence limits. When indicated, statistical analysis was performed using GraphPad Prism (version 5.03 Windows GraphPad Software Inc., La Jolla, CA, USA).

Results

Allergen and transglutaminase expression during germination and evaluation of the allergenicity of apple pollen

To determine whether allergens found in apple fruits were also expressed in pollen, transcripts for PR10-like proteins (*Mal d 1*), thaumatin-like protein (TLP, *Mal d 2*), nonspecific lipid transfer proteins (nsLTP, *Mal d 3*) and profilins (*Mal d 4*) were tested during pollen germination and tube growth (Fig. 1). In *M. domestica* pollen, the only allergen gene expressed in the ungerminated grain (UGP) was *Mal d 3.01*, showing a trend of increasing expression during the early phases of pollen tube growth (GP), with a maximum expression at 30 min of germination. Among the four *Mal d 1* genes tested, only *Mal d 1.02* (subfamily I) revealed a clear expression at 30 and 60 min, while *Mal d 1.06A* (subfamily III) showed a faint band at 120 min. *Mal d 2* gene expression occurred around 30 min of germination and then decreased. *Mal d 4* genes were either not expressed or produced faint bands.

TGase was expressed in UGP as well as in GP where it increased up to 60 min of germination and then slightly decreased (Fig. 1).

To further verify the allergenicity of *M. domestica* pollen, proteins extracted from pollen cell wall with buffers having increasing extraction capacity were separated by SDS-PAGE, and blotted and incubated with the serum of patients allergic and non-allergic to apple fruit (Fig. 2). The serum of the allergic patient, characterised by a high antibody titre against *Mal d 3*, immuno-recognised proteins easily released from pollen cell wall with molecular masses in the 36–63 kDa range, which are consistent with sizes of possible aggregates of allergen whose molecular masses are in the range 9–23 kDa.

Transglutaminase localisation and activity in germinating pollen

The localisation of TGase was obtained by indirect immunofluorescence in hydrated and germinated non-permeabilised pollen of *M. domestica* (Fig. 3Aa) and *C. avellana*

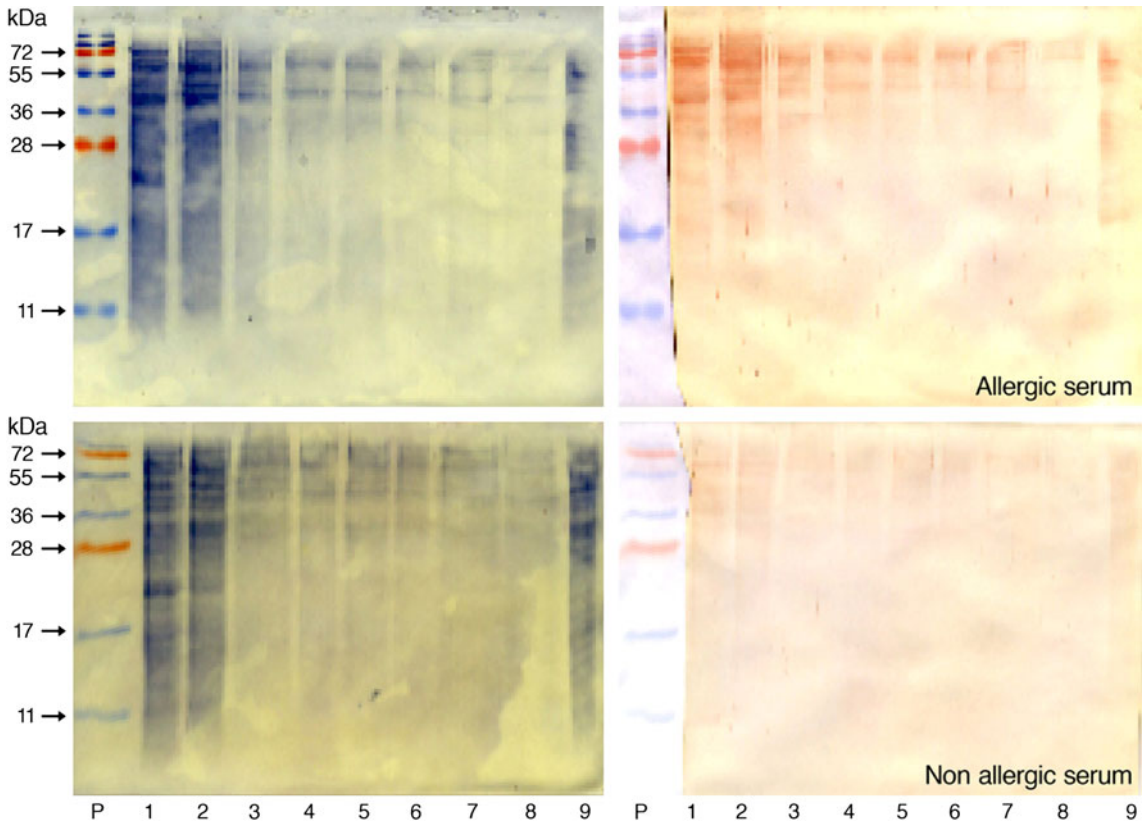

Fig. 2 Immunodetection of different fractions of *M. domestica* pollen proteins from cell walls with sera from patients allergic and non-allergic to apple fruit. Proteins were extracted with buffers having increasing extraction capacity from cell walls of *M. domestica* pollen, hydrated at 30°C–100% rHu o/n plus acid rain and germinated. Proteins (50 µg) were separated on a 10% SDS–PAGE, transferred to a nitrocellulose, probed with serum from patients allergic and non-allergic to apple fruit. The two nitrocellulose membranes on the left were stained with chloramine T; the two membranes on the right were developed with anti-human IgE HRP-conjugated secondary antibody. *Lanes*: P. standard molecular weight; *Lanes 1–9* proteins extracted with: HEEMS buffer (1. SL1; 2. SL2); homogenisation buffer (3. SN3; 4. SN4); Triton X-100 buffer (5. SNT1; 6. SNT2); NaCl buffer (7. SNA1; 8. SNA2); SDS buffer (9. SNB)

(Fig. 3Bb) using the anti-plant TGase polyclonal antibody AtPng1p. At the beginning of germination, TGase was localised mainly into the cell wall of both pollen grains and in the cytoplasm. *M. domestica* was more intensely labelled. TGase was also located in a finely punctuate pattern (apparently in the intine) in the emerging tube as shown in Fig. 3Bb. As germination progressed, with the increase in tube length of both pollens, the TGase punctuate pattern became widespread along the tube with some more intense spots of TGase in the shanks of the proximal region of the tube, especially in apple pollen (Fig. 3Aa) and at the apical tip (Fig. 3Bb and insert).

The fluorescent-tagged amine substrate (FITC-cadaverine), routinely used to visualise tTGase activity in animal cells (Verderio et al. 1998), was added to the germination medium after the pollen tubes started to grow (60 min for *M. domestica* and 120 min for *C. avellana*, whose growth rate is slower). FITC-cadaverine, a competitor of endogenous amine-donor substrates (ε-amino group of lysine residues or PAs), limited or blocked tube elongation of both pollens (Fig. 3C, D). This primary amine was found to be cross-linked around the pollen grain of *M. domestica*, mainly in the cell wall and in the apical part of the tube (Fig. 3Ca and insert). A similar incorporation of FITC-cadaverine was detected in the pollen tube of *C. avellana* (Fig. 3Da). However, during germination, the pollen grain wall of *M. domestica* was more intensively stained with the anti-TGase antibody than that of *C. avellana*.

To further verify whether a Ca^{2+}-dependent TGase activity in pollen was responsible for this fluorescent signal, a specific inhibitor of TGase activity was used, a synthetic CBZ-glutaminyl glycine analogue which targets the calcium-activated form of tTGase (inhibitor 281) (Baumgartner et al. 2004). Data in Fig. 3Cb, Db show that the in situ TGase activity detected in the tube of both pollens was inhibited by 281, as the fluorescence in the tubes disappeared almost completely, as previously reported in apple (Iorio et al. 2008). By comparing the pollen grains, only in *C. avellana* the inhibitor was effective, whereas in *M. domestica* pollen grain the fluorescence due to FITC-cadaverine persisted clearly evidently around the grain also in the presence of the TGase inhibitor and this

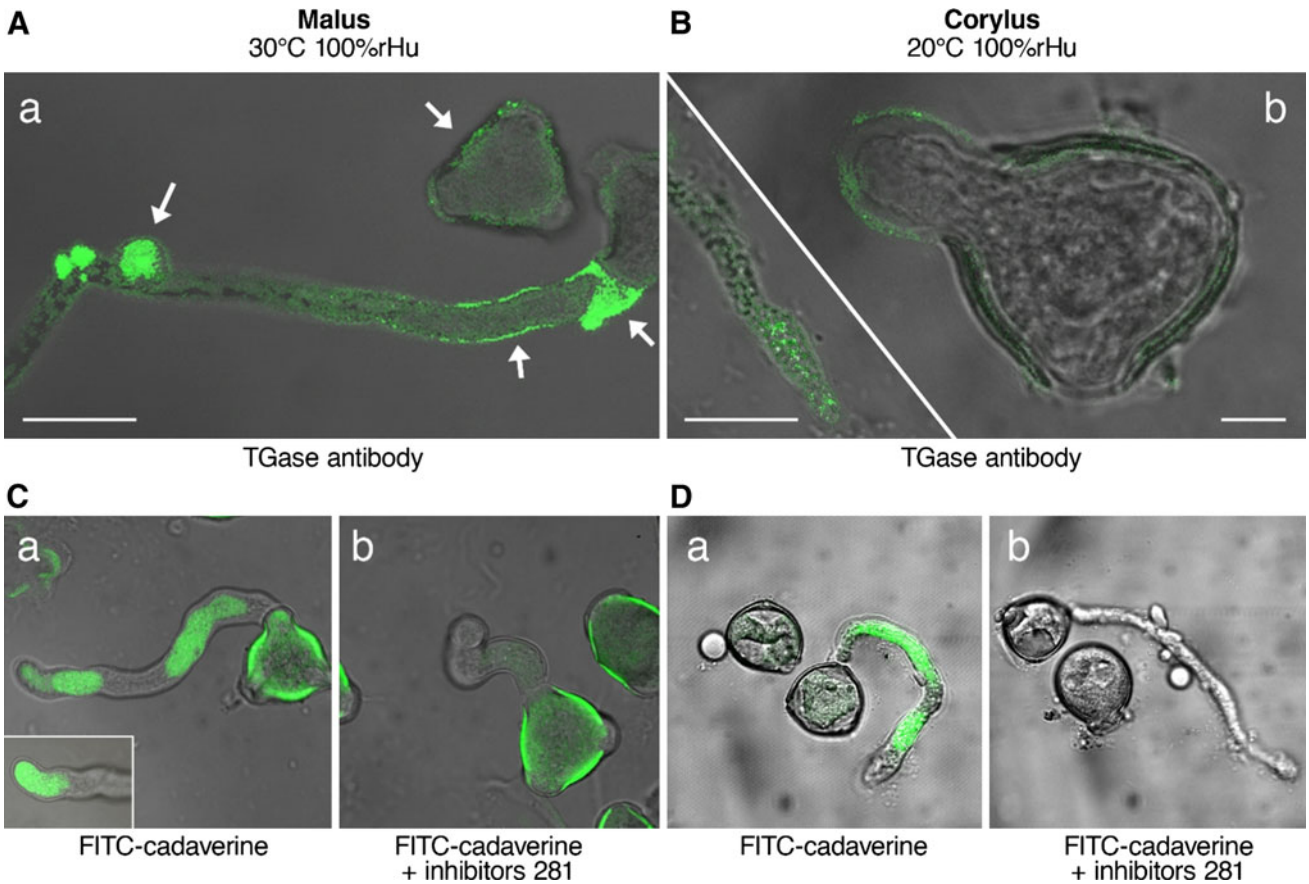

Fig. 3 TGase characterisation in *M. domestica* and *C. avellana* pollen **A** and **B**. In situ TGase immunodetection during pollen germination. **A** *M. domestica* pollen was allowed to germinate for 120 min. Following fixation, pollen was incubated with the polyclonal antibody directed to plant TGase, AtPng1p, which was subsequently detected with a chicken FITC-conjugated antibody and fluorescence was visualised by confocal laser microscopy. *Arrowheads* indicate TGase staining. **B** *C. avellana* pollen was allowed to germinate for 13 h, fixed and incubated with the polyclonal antibody AtPng1p, as described above. **C** and **D**. In situ detection of TGase products during pollen germination. **C** *M. domestica* pollen and **D** *C. avellana* pollen were allowed to germinate for 60 min or 120 min, respectively, fluorescein-cadaverine (FITC-cadaverine) (500 μM) was added to the germination medium and pollen was allowed to germinate for a further 60 min (**C***a* and insert) for the former, or 11 h for (**D***a*) the latter. Following fixation, specimens were viewed by confocal laser microscopy. TGase-specific inhibitor 281 (500 μM) was added together with FITC-cadaverine to germinating pollen of *M. domestica* (**C***b*) or *C. avellana* (**D***b*). Series of optical images at different focal depth were produced to acquire 3D information on TGase activity. *Bars*, 20 μM

may be due to the particular intense level of staining observed at this location. When EGTA was utilised to inhibit the activity of TGase in an alternative way, this treatment was found to diminish pollen grain signal, but to severely affect the morphology of the tube (data not shown).

The presence of extracellular TGase was confirmed by measuring the enzyme activity by incorporation of biotin-cadaverine into DMC (Fig. 4A for *M. domestica* pollen and Fig. 4B for *C. avellana* pollen), using the in vivo assay previously developed in apple pollen (Di Sandro et al. 2010). TGase activity was not different in the two GPs, and, as expected, was specifically inhibited to 30–20% residual activity by a range of TGase inhibitors, namely 281 and cystamine, and the competitive inhibitors iodoacetamide, putrescine and EGTA (Fig. 4A, B). TGase activity was also assayed in the crude supernatant of either UGP or GP (highly soluble UGP and GP proteins) in both pollens (inserts in Fig. 4). The specific activity in these fractions was found to be higher in UGP than GP and overall higher in *C. avellana* with respect to *M. domestica* pollen.

Influence of simulated atmospheric conditions and pollution on pollen

To evaluate the effects of atmospheric pollution on pollen morphology, viability and germinability, environmental conditions, such as atmospheric variation in relative humidity (rHu) and temperature (T), heavy metal pollution

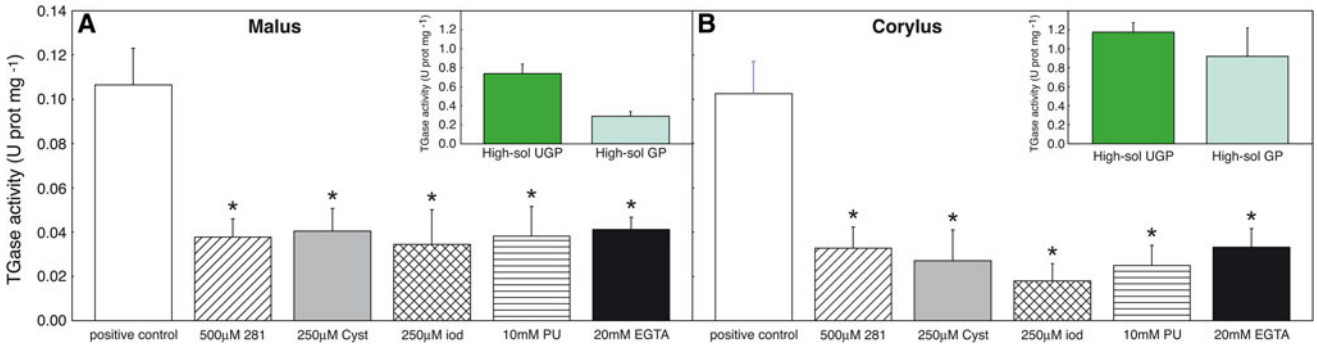

Fig. 4 Extracellular TGase activity of germinated *M. domestica* and *C. avellana* pollens in the presence of irreversible and competitive inhibitors. Pollen was allowed to germinate for 120 min (**A**) (*M. domestica*) or 13 h (**B**) (*C. avellana*) into microwells pre-coated with DMC in appropriate germination medium containing biotinylated cadaverine. At time zero of germination, 5 mM of Ca^{2+} as positive control, or irreversible (500 µM 281 and 250 µM cystamine (cyst)) and competitive (250 µM iodoacetamide (iod), 10 mM putrescine (PU) and 20 mM EGTA) TGase inhibitors were added. Following time germination, pollen was removed and the level of biotinylated cadaverine incorporated into immobilised DMC was revealed by extravidin peroxidase. *Inserts*: the TGase activity has been tested for the highly soluble proteins from UGP and GP *M. domestica* and *C. avellana* pollens. TGase activity is expressed as units (U) of specific activity (means ± S.D.) per mg of pollen. The significance of each sample was calculated with respect to the positive control [* $p \leq 0.05$ with the Student's t test (two-tailed distribution, two-sample equal variance)]

(copper) and acid rain (at pH 5.6) were simulated and applied to pollen in a phytotron chamber. Conditions of 20°C–50% rHu o/n and 10°C–70% rHu o/n were used as control conditions of normal viability for, respectively, *M. domestica* and *C. avellana* pollen. The viability of the two pollens, investigated after exposure to the stressors was assessed by FDA and MTT, the former showing a more remarkable staining. *M. domestica* pollen was not significantly affected by temperature and humidity changes, whereas a prolonged treatment at 30°C–100% rHu caused the death of pollen. When it was exposed to copper and acid rain pollution, even at optimal temperature, the viability was significantly reduced at low level of relative humidity (Fig. 5A). *C. avellana* pollen was more resistant to high temperature and humidity changes, whereas it was more sensitive to copper and acid rain treatment (Fig. 5B). After exposing both pollens to copper and acid rain pollution at high temperature and humidity, the presence of the pollen grain cytoplasm was clearly reduced as shown by the Alexander dye, which stains in fuchsia the cytoplasm and in green the cell walls (grain pictures in Fig. 5A, B); apple pollens assumed a typical triangular form indicative of failure of hydration. The treatment with acid rain sometimes led in apple pollen to a precocious emission of abnormal pollen tubes (data not shown). When germination was assessed after the same treatments, a decrease of approximately 50% of pollen germination was observed in non-extreme conditions compared to control germinations [81.2 ± 8.5% (*M. domestica*) and 53.4 ± 4,1% (*C. avellana*)]. When the same treatments were applied in the presence of copper and acid rain, these resulted in a very severe inhibition of germination (Table 2).

Influence of simulated atmospheric conditions and pollution on pollen TGase

The enzyme

To verify the effects of environmental stress factors on pollen TGase, protein lysates from *M. domestica* and *C. avellana* pollens exposed to the simulated atmospheric conditions were anlaysed by Western blotting with the polyclonal antibody AtPng1p.

Immunoprobing of *M. domestica* extracts revealed immunoreactive bands at 94, 87, 72, 55 and 38 kDa in the control (20°C–50% rHu o/n) (Fig. 6A), which were generally decreased by copper and acid rain treatment and were accompanied by aggregates of high molecular weight proteins visible after acid rain treatment. Immunoprobing of *C. avellana* extracts showed that the main bands of 94 and 55 kDa in the control (10°C–70% rHu o/n), and their intensity increased after copper treatment. Further bands of 72 and 38 kDa were immunorecognised following copper treatment, and bands with molecular masses 55–94 and 38 kDa after acid rain (Fig. 6B).

The intensity of the band at 55 kDa, which was constantly revealed by AtPng1p and previously reported in *M. domestica* pollen cell wall (Di Sandro et al. 2010), was quantified in both *M. domestica* (Fig. 6D) and *C. avellana* (Fig. 6E) to describe the different behaviour of the two pollens under different treatments. In apple pollen, 55 kDa was increased by prolonged high temperature and humidity exposure (30°C–100% rH o/n). In hazel pollen, the band at 55 kDa was at its lowest expression level after pollen exposure to high temperature and humidity (30°C–100%rH o/n). Copper and acid rain affected both pollens in a

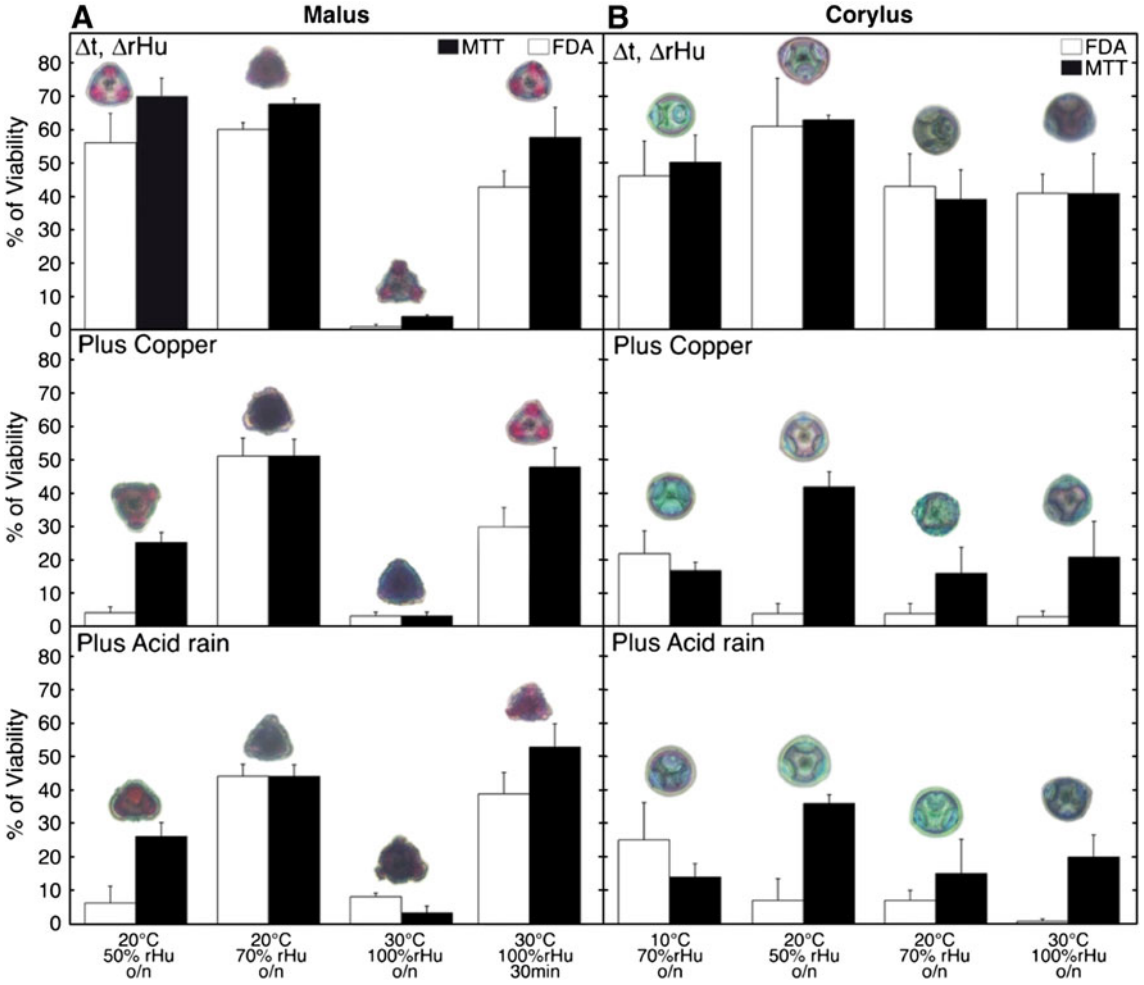

Fig. 5 Effects of environmental conditions on *M. domestica* and *C. avellana* pollen viability **A, B**. Pollens of *M. domestica* (**A**) and *C. avellana* (**B**) exposed to different environmental conditions as reported in "Methods" including various atmospheric temperatures (T) and relative humidity (rHu), copper pollution (3.10 μg l^{-1}) and acid rain at pH 5.6 were left to react with FDA or MTT to test the viability and with Alexander staining to evaluate their morphology; pollens were analysed by fluorescent microscopy in the case of FDA and by light microscopy for MTT and Alexander. The viability was evaluated as percentage of fluorescent grains (FDA) or purple grains (MTT) (means ± SD) of three independent pollen culture wells and by evaluating the data of ten independent fields on the slide. The respective pollen grains stained with Alexander are reported above each column

Table 2 Effects of different environmental conditions on *M. domestica* and *C. avellana* pollen germination

Germination (%)								
Treatments	Malus				Corylus			
	20°C, 50% rHU,o/n	20°C, 70% rHU,o/n	30°C, 100% rHU,o/n	30°C, 100% rHU,30 min	10°C, 70% rHU,o/n	20°C, 50% rHU,o/n	20°C, 70% rHU,o/n	30°C, 100% rHU,o/n
ΔT, ΔrHU	42.2	53.4	3	81.2	53.4	20.3	28.3	2.7
Plus copper	7.3	5.2	1	8.3	2.9	2.7	3.9	0.9
Plus acid rain	2.9	3.8	1	5.7	1.7	1	1.9	1.3

Pollen grains were exposed to different environmental conditions as reported in "Methods" including various atmospheric temperatures (T) and relative humidity (rHu), copper pollution (3.10 μg l^{-1}) and acid rain at pH 5.6. Pollens were allowed to germinate (1 mg ml^{-1} in germination medium) in glass Petri dishes left up to 120 min in the case of *M. domestica* or 13 h for *C. avellana* pollen. Light microscopy digital images of at least three non-overlapping fields covering the central part of each chamber were captured using a video digital camera (Olympus DP10) and quantified in terms of % GP versus UGP, when the pollen tube was either absent or shorter than the pollen grain diameter

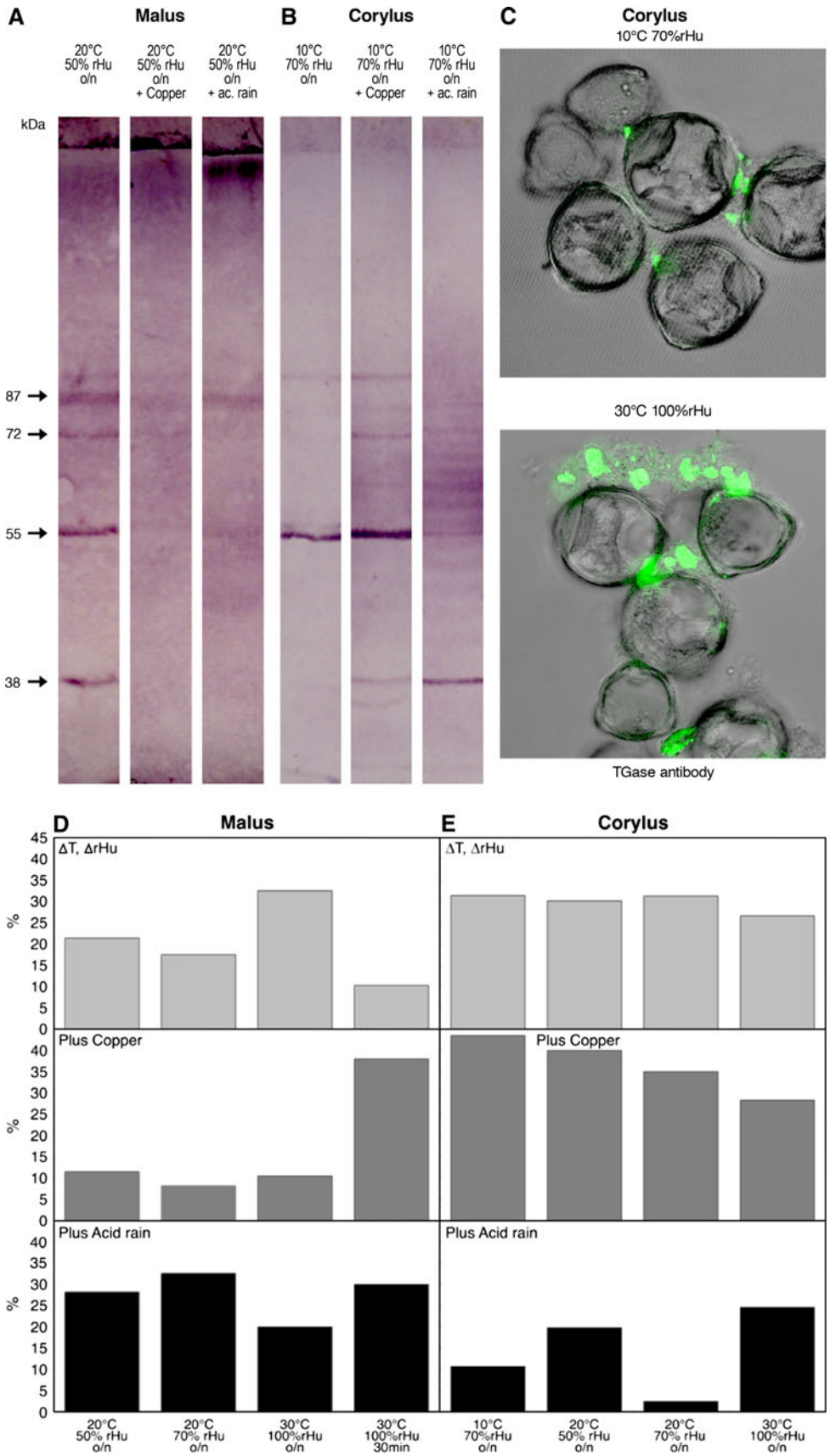

Fig. 6 Immunodetection with polyclonal antibody AtPng1p of proteins extracted from *M. domestica* and *C. avellana* pollen exposed to different environmental conditions. **A** and **B** Proteins extracted from pollens of *M. domestica* (**A**) or *C. avellana* (**B**) exposed to natural conditions (20°C–50% rHu o/n and 10°C–70% rHu o/n, respectively) or with the addition of copper pollution or acid rain at pH 5.6 were separated on SDS–PAGE, blotted and immunodetected with polyclonal antibody AtPng1p. **C** *C. avellana* pollen exposed to natural (10°C–70%rH o/n) or to simulated high temperature and humidity conditions (30°C–100%rH o/n) was incubated with polyclonal antibody AtPng1p, detected with a chicken FITC-conjugated antibody and the fluorescence was visualised by confocal laser microscopy. **D** and **E**. Densitometries of the 55-kDa band (revealed as above) obtained from proteins extracted from pollens of *M. domestica* (**D**) and *C. avellana* (**E**) exposed to different environmental conditions as reported in "Methods" including various atmospheric temperatures (T) and relative humidity (rHu), copper pollution (3.10 μg l^{-1}) and acid rain at pH 5.6. Data are expressed as % of the immunofluorescence of the 55-kDa band on the total fluorescence of the respective lane = 100

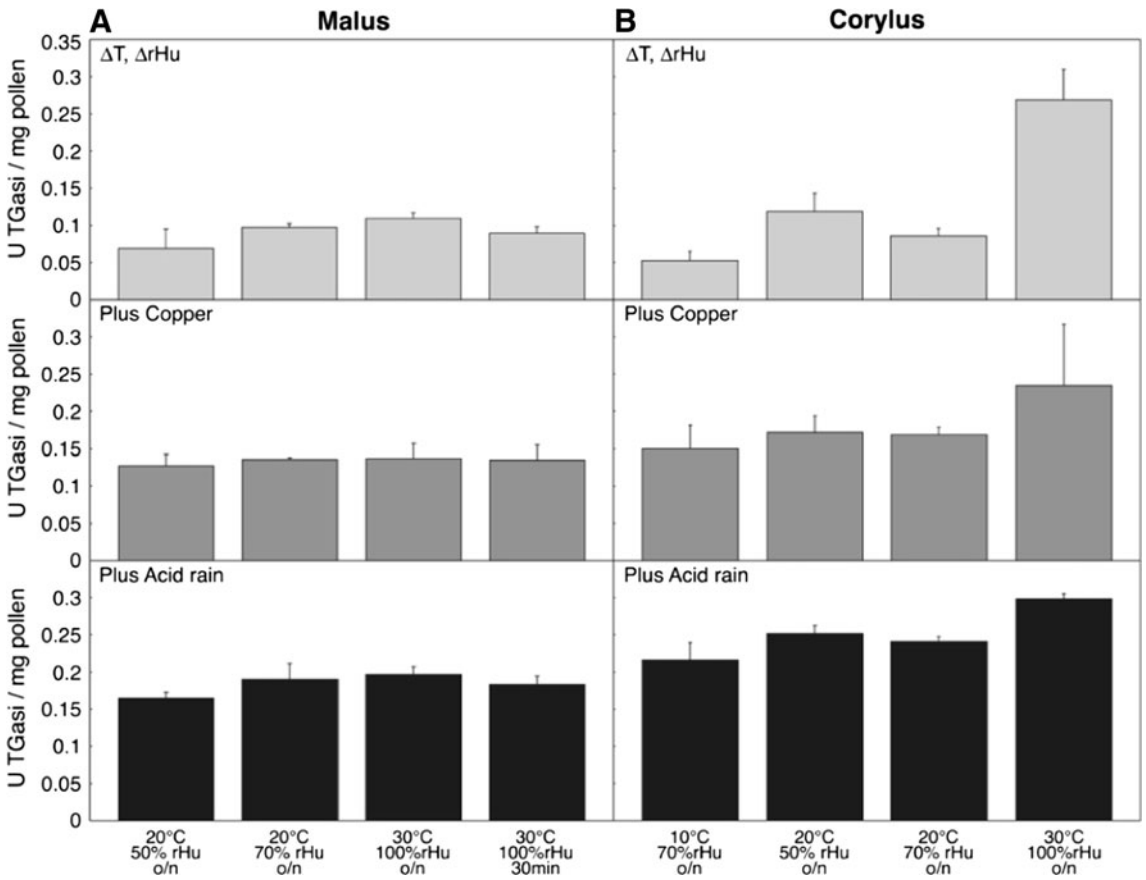

Fig. 7 Effect of environmental conditions on *M. domestica* and *C. avellana* pollen TGase activity. The TGase activity at the surface of *M. domestica* (**A**) and *C. avellana* (**B**) pollens exposed to different environmental conditions, as reported above, was measured by the microplate assay. Pollen was allowed to germinate for 120 min (*M. domestica*) or 13 h (*C. avellana*) in microwells pre-coated with DMC in appropriate germination medium containing biotinylated cadaverine. TGase activity is expressed in units (U) of specific activity (means ± SD of at least three independent experiments performed in triplicate) per mg of pollen

different way: copper was more effective on *Malus*, whereas acid rain on *Corylus.*

To visualise the expression and location of TGase in both pollens either exposed to natural conditions or to climate changes, pollen grains were incubated with the polyclonal antibody anti-AtPng1p followed by FITC-conjugated secondary antibody. Whereas in apple pollen no relevant differences in TGase distribution was observed (data not shown), in *C. avellana* UGP fluorescence confocal microscopy revealed that TGase was much more released in the medium following exposure to high temperature and humidity compared to pollen under natural conditions (Fig. 6C).

The activity

The levels of extracellular TGase activity during germination were measured by a previously described in vivo assay (Di Sandro et al. 2010). This was performed at 37°C in wet conditions to simulate the conditions of the human airway mucosa. In *M. domestica* pollen, TGase activity was not significantly affected by temperature and humidity increases compared to control conditions (20°C–50% rHu o/n), but it did increase after copper and acid rain treatments (Fig. 7A). In *C. avellana* pollen, TGase activity significantly increased after exposure to both changes in temperature and humidity (Fig. 7B), and even more following acid rain and copper treatments compared to control conditions (10°C–70% rHu o/n). The increase in TGase activity was always more marked in *C. avellana* than in *M. domestica* pollen (Fig. 7A, B).

Phospholipase A_2 as a substrate

The mammalian glycoprotein secretory phospholipase A_2 ($sPLA_2$), due to its relevance in the inflammatory cascade, was used as a substrate to assay the capacity of pollen to conjugate [^{3}H]-putrescine (PU) to this enzyme in the presence of calcium. Whole pollen grains of *M. domestica* (Fig. 8A) and especially of *C. avellana* (Fig. 8B) showed an higher conjugating activity when exposed to high temperature and rHu with respect to pollens exposed to natural

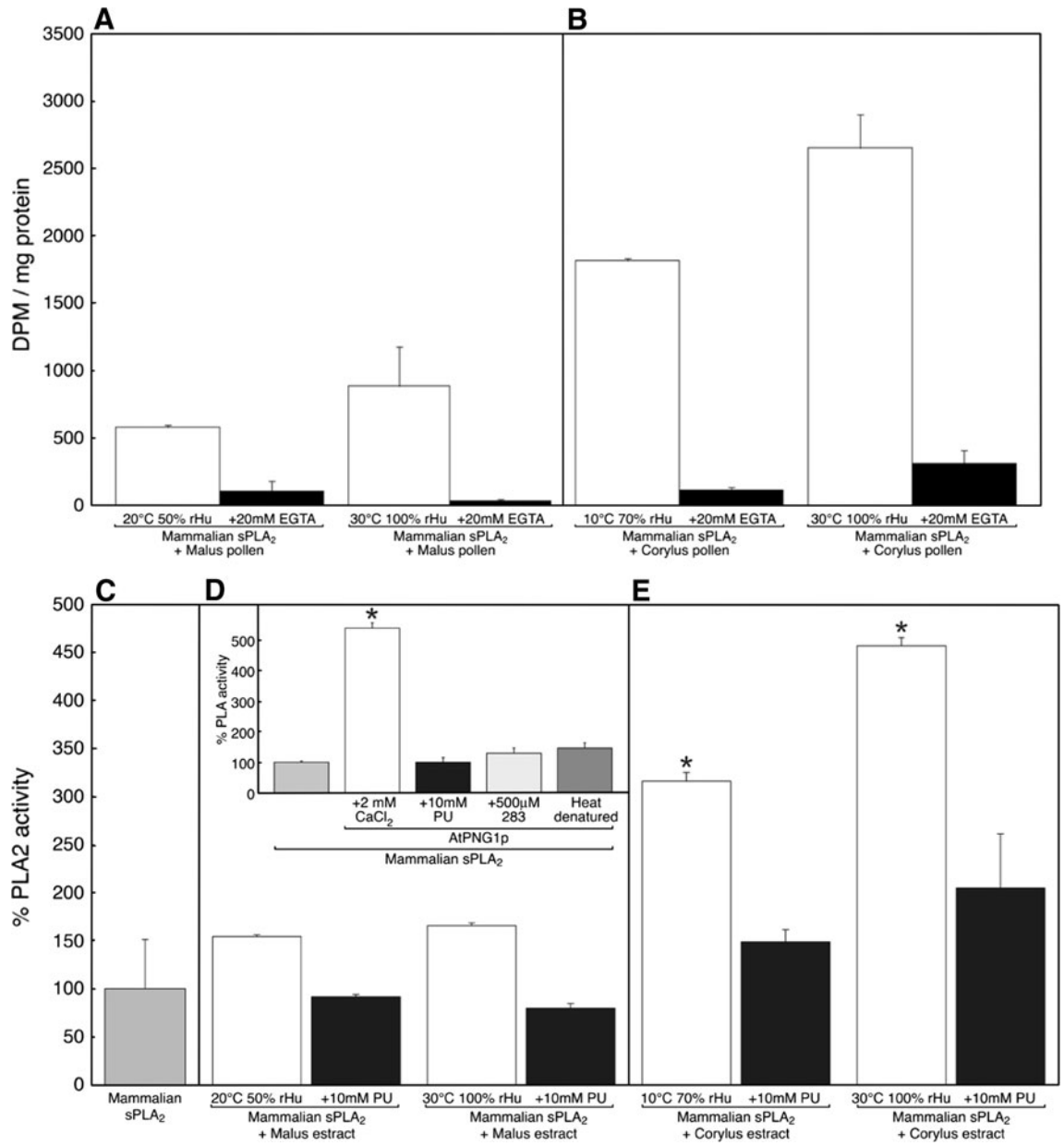

Fig. 8 Effect of environmental conditions on *M. domestica* and *C. avellana* pollen activity in conjugating polyamines to $sPLA_2$ and in stimulating $sPLA_2$ enzymatic activity **A** and **B**. The TGase activity was measured after pre-incubation of *M. domestica* (**A**) and *C. avellana* (**B**) pollen grains exposed to natural conditions (20°C–50% rHu o/n and 10°C–70% rHu o/n, respectively) or to simulated high temperature and humidity conditions (30°C–100% rHu o/n) in buffer containing 5 µg $sPLA_2$ and 0,2 µM of [1.4(n)^3 H]-PU]. As positive control, 5 mM $CaCl_2$ was added or 20 mM EGTA as negative control. Data were expressed as DPM/mg protein. Each data point represents the mean of three independent experiments undertaken in triplicate. **C**, **D** and **E** The activity of 10U/ml of mammalian $sPLA_2$ (**C**) was measured after incubation at 37°C for 30 min with 250 µg of protein extracts of *M. domestica* (**D**) and *C. avellana* (**E**) pollen, treated as reported above, in a buffer containing 2 mM Ca^{2+} (without DTT and EDTA/EGTA which alter $sPLA_2$ activity). Pollen protein extracts were treated with 10 mM PU as negative control. *Insert* The activity of mammalian $sPLA_2$ (10 U/ml) was measured after pre-incubation with 10 µg of AtPng1p recombinant protein, treated with 2 mM Ca^{2+}, 10 mM PU, 500 µM 283 or heat denatured and then similarly incubated with $sPLA_2$ enzyme. Data (A405 nm) were expressed as percentages of the activity values of standard $sPLA_2$, which were given the value of 100, after subtraction of background values given by the extracts. Each data point represents the mean of three independent experiments undertaken in triplicate. The significance of each sample was calculated with respect to standard $sPLA_2$ (**c**) (* $p \leq 0.05$ with the Student's *t* test [two-tailed distribution, two-sample equal variance)]

conditions (20°C–50% rHu o/n and 10°C–70% rHu o/n, respectively, for *M. domestica* and *C. avellana*). The conjugation of PU to $sPLA_2$ was greatly reduced or nullified by EGTA. These finding showed that mammalian $sPLA_2$ may function as a substrate also for extracellular pollen TGase activity. To verify if a plant TGase activity can also affect

mammalian $sPLA_2$ activity, the recombinant plant TGase AtPng1p (Fig. 8D insert) was utilised. Recombinant TGase significantly activated $sPLA_2$ in the presence of calcium and its action was inhibited by either the addition of a competitive inhibitor of TGase activity (PU), an irreversible TGase inhibitor (283) or by heat denaturation of the recombinant TGase (Fig. 8D insert). To investigate whether $sPLA_2$ could be activated by pollens, it was incubated with extracts from pollen grains exposed to natural and climate change conditions. The activity of the $sPLA_2$ was significantly enhanced by incubation with protein extract of *C. avellana* pollen exposed to natural conditions. Protein extracts of pollen treated with environmental stressors (30°C–100% rHu o/n) increased $sPLA_2$ activity even more (Fig. 8E). On the contrary, proteins extracted from *M. domestica* pollen were much less effective in stimulating $sPLA_2$ enzyme activity (Fig. 8D) and the exposure to stress conditions did not lead to a significant change.

Discussion

The comparison of the properties of pollens from *Corylus avellana* (hazel), known to be allergenic, and *Malus domestica* (apple), the allergenicity of which has not been classified, constitutes the necessary pre-requisite to verify the hypotheses that pollen TGase might have a role in respiratory allergies and be one of the factors by which meteorological and pollutant conditions affect these pathologies.

Allergenicity

The first step of our research was aimed to verify whether some important apple allergen genes, known to be expressed in the fruit and to provoke allergic reactions after ingestion (Pagliarani et al. 2009; Fernandez-Rivas et al. 2006), were also expressed in apple pollen. In particular, *Mal d 3.01*, which encodes for an apoplastic lipid transfer protein (nsLTP), was expressed to a higher extent during the entire germination, whereas *Mal d 1* sub I and *Mal d 2* that encode for a putative PR-10 protein and an apoplastic thaumatin-like protein, respectively, were expressed only in pollen after the initial phase of the germination. These results suggest that apple pollen could be potentially allergenic.

Although some of these allergens are cytoplasmic proteins (Mal d 1 and Mal d 4), they need to be externalised to access the human mucosae cells of the immune system. In contrast, Mal d 3 and Mal d 2 are apoplastic; their release could perhaps be easy and responsible for the rare cases of respiratory allergy to *M. domestica* pollen reported in the literature (Herrmann et al. 1995). These assumptions were confirmed also by the recognition of proteins easily released from grain wall by serum of patients allergic to apple fruit. Tentatively, the high molecular weight of these proteins should result from aggregation as previously reported for olive pollen allergens (Castro et al. 2007).

Moreover as GP was found in the mucus of allergic patients (Accorsi et al. 1982), it cannot be excluded that pollen tube emission could be a way to release allergens, such as the PR-10 expressed during germination; some of these PR-10 proteins from other species have been reported to have a ribonuclease activity (Zubini et al. 2009), which is related to the pollen incompatibility response in *Rosaceae*. However, *M. domestica* pollen is not classified among the relevant species in pollinosis. This observation could be related to the fact that apple pollination is entomophilous and thus pollen, which is produced in small amount, is sticky and reaches with difficulty the human airways. The data obtained from semi-quantitative RT-PCR analyses on allergen genes suggested that apple pollen could potentially be as allergenic as the fruit, although other factors, such as a different organisation of the pollen cell wall (Hesse 1979) and the presence or a different release of activating factors of the allergenic cascade, should also be considered.

Transglutaminase

Among the possible activating factors of the allergenic cascade, the TGase enzyme was examined in the two types of pollens and some differences were found in terms of location and activity. Data on the existence of an extracellular TGase activity were already available for *M. domestica* GP in optimal conditions, and in this situation the enzyme remained visible around the grain also after cell wall digestion, probably because sporopollenine protected this part of the pollen (Iorio et al. 2008; Di Sandro et al. 2010). The present research established that the enzyme was mainly expressed at the beginning of germination, when it was particularly active, but also continued to be expressed during the entire germination period. In addition, the extracellular activity, determined in vivo in GP, was not significantly different in the two pollens, at least at the end of the germination periods. By comparing the localisation of the enzyme itself and its products of catalysis in the two pollens, these were both detected mainly around the pollen grains and also along the shank of the pollen tubes. However, the fluorescence signals were much more evident around the pollen grain wall of *M. domestica* than that of *C. avellana*.

The specific TGase inhibitor 281 was very effective on the extracellular TGase activity of both pollens, as shown in Fig. 4A, B. It has been previously established that 281 blocks apple pollen tube growth and inhibits TGase activity

in a dose-dependent way (Di Sandro et al. 2010). The present data, obtained by confocal microscopy (Fig. 3Cb, Db), suggest that the inhibitor accumulated into tubes of both pollens, as well as in hazel grain, where the fluorescence due to TGase was not visible; the inhibitor probably could not permeate the cell wall of apple grain, as the TGase products remained clearly detectable therein.

The presence of an active TGase in the *C. avellana* grain is confirmed by the data reported in Fig. 4; the finding that highly soluble proteins of *C. avellana* UGP, being more easily dispersed into the germination/assay medium, showed an activity greater than that of *M. domestica* suggesting that the two grains behaved in a different way.

It is known that the cell walls of anemophilous and entomophilous pollens grains have a different composition and organisation (Pacini and Hesse 2005), which could justify an easier or earlier release of various molecules, including allergens, extracellular TGase and its products by the anemophilous pollens, which are probably more permeable to external compounds.

Moreover, *M. domestica* and *C. avellana* pollens also differ in their incompatibility mechanisms. *M. domestica* is gametophytic, while *C. avellana* is sporophytic, with the pollen/style recognition times more rapid in hazel, whose incompatibility glycoproteins are exposed to and immediately recognised by stigma cells. TGase is reported to be part of the factors involved in gametophytic incompatibility of Rosaceae, being particularly active in self-incompatible pollen, as shown by Del Duca et al. (2010). In *Malus* germinating pollen, TGase was found to be released extracellularly (Di Sandro et al. 2010). It is intriguing to note that genes involved in cell–cell recognition (as allergens, resistance genes and F-boxes) are usually organised in clusters in the *Maloideae* genomes; this phenomenon can represent an evolutive conserved strategy for the cells to recognise pathogens or self/non-self pollen. As a consequence, the incompatibility response and allergenicity could have some common mechanism at the molecular/cytologic level and TGase could represent one of the factors that plays a role in both mechanisms. These hypotheses would be a subject of future studies.

Environmental criticalities

The second step of our work was devoted to verify if the environmental conditions such as temperature, humidity alone or in combination with some pollutants (copper and acid rain) could have an effect on the viability and germination of both pollens. *C. avellana* appeared to be more resistant to temperature and humidity changes than *M. domestica*, even with a 20°C increase above the natural conditions, to which anemophilous pollen can be subjected to during pollination. A prolonged treatment at high temperature and % rHu caused pollen death of *M. domestica* and not of *C. avellana*, as revealed by the respiratory and cytoplasmic enzymes activity. However, germination resulted in a more sensitive parameter of the damage, as it was dramatically affected by long exposure to extreme temperature and humidity conditions in both pollens and even more influenced by copper and acid rain treatment. Bellani et al. (1997) reported that the environmental stressors, and in particular acid rain, repeatedly supplied to apple trees, act primarily by altering the function of cytoplasmatic enzymes and affecting organelles, including mitochondria, plastids and endoplasmic reticulum of pollen.

Simulated environmental conditions caused an increase in extracellular TGase activity, which was far more marked in *C. avellana* pollen than *M. domestica* pollen, and in both pollen, it was found to be enhanced by pollution conditions. In apple pollen, the TGase expression and activity in vivo and in vitro was not significantly affected by temperature and humidity increases. By contrast, in hazel pollen exposed to increasing temperature and humidity, TGase activity was found to be significantly increased compared to the level in simulated natural conditions. In vivo immunofluorescence with the polyclonal antibody AtPng1p showed that the enzyme was released into the medium, probably due to a leakage from pollen cell wall or membrane. This release might allow the enzyme, when the entire pollen grains were assayed in vivo in the presence of high Ca^{2+} concentration, necessary to allow germination, to more freely reach the substrate immobilised on the plate well surface and to catalyse a large amount of products. Copper pollution and acid rain significantly increased the extracellular TGase activity of both pollens, possibly through a process related to a damage of pollen membrane or even cell wall. The enzyme itself undergoes possible events of degradation, especially after acid rain treatments of both pollens. Under copper pollution in *C. avellana* pollen, the more intense fluorescence of the 55-kDa AtPng1p immunoreactive band, previously observed in the apple pollen and tobacco corolla cell walls (Di Sandro et al. 2010; Della Mea et al. 2007a, b), suggests that this apoplastic enzyme could be responsible for the intense extracellular activity. Other isoforms have been detected before in *M. domestica* pollen, mainly the 70 kDa (Del Duca et al. 2009), whereas other isoforms have been previously reported (Serafini-Fracassini et al. 2009).

The environmental conditions including variations in T and rHu, and copper and acid rain pollution, differently affected the two pollens, which responded with a different behaviour. Mainly in *C. avellana*, an extracellular TGase activity is stimulated by environmental changes and pollution. As a consequence, it may be effective also on cross-linking substrates located outside the pollen.

Factors of inflammation

The third step of this work has shown that $sPLA_2$, known to be involved in the inflammation response and to be released from the inflammatory cells into the airway mucosae, is also a substrate of plant TGases; the modification of $sPLA_2$ via TGase caused the stimulation of its in vitro activity. We have shown that a recombinant plant TGase, AtPng1p, greatly activates mammalian PLA_2 in the presence of Ca^{2+}, whereas 283, TGase heat-denaturation and a high amount of PU caused a significant activity reduction. This inhibitory effect occurred when an excess of competitive PA amine donors caused the formation of mono-glutamyl derivatives prevalently. This could saturate the PLA_2 sites of isopeptide Gln-Lys bridge formation, necessary to allow the dimerisation of PLA_2 in its active conformation, as suggested by Cordella-Miele et al. (1990). The same authors reported that mammalian tTGase caused the formation of an isopeptide bond between a Gln and a Lys causing an intramolecular conformational change, which allows the formation of a non-covalently bound transient dimer responsible for tenfold $sPLA_2$ activity stimulation with respect to the monomer. When different amines or PAs at optimal concentration were incubated with $sPLA_2$, tTGase catalysed the production of a high percentage of *N*-mono(γ-glutamyl)-PA and caused an increase in PLA activity; *N*,*N*-bis(γ-glutamyl)-PA could also be formed (Cordella-Miele et al. 1993).

Similarly, pollen of both plants exposed to natural or stress conditions were able to modify by polyamination (using [^{3}H]PU as tracer) the mammalian $sPLA_2$, suggesting that a pollen TGase is active also on mammalian PLA. It is known that pollen releases PAs (Bagni et al. 1981), which in turn can be a substrate of TGase. The polyamination of $sPLA_2$ was much evident when pollen grains of *C. avellana* were assayed compared to *M. domestica,* especially if the stressors were high temperatures and humidity. This finding is in agreement with the data obtained on extracellular TGase activity, which was much higher in hazel pollen than in apple pollen.

In the presence of *C. avellana* pollen extract, the activity of the $sPLA_2$ increased by more than three and four times, respectively, under natural or stress conditions. In both cases, $sPLA_2$ activity was significantly reduced by competition with an excess of PU. On the contrary, *M. domestica* extract was poorly effective. This increase in $sPLA_2$ activity is likely to be due to the TGase present in the extract, consistent with similar raises in sPLA2 activity by the recombinant plant TGase AtPng1p and by previous work with mammalian tTGase (Cordella-Miele et al. 1993). However, it cannot rule out the possibility that a PLA activity, reported to be present in *Arabidopsis thaliana* (Ishiguro et al. 2001), is also present in pollen of *C. avellana,* amplifying the $sPLA_2$ activity detection.

On the bases of the present data, it can be summarised that mammalian $sPLA_2$ can be activated by plant TGases. In particular, *C. avellana* pollen greatly stimulated $sPLA_2$ activity, especially when stressed by environmental conditions that are likely to facilitate the release of the enzyme by this anemophilous pollen, thus favouring its allergenicity. Naturally also other molecules, from allergens to other factors of the inflammatory cascade, might also be easily released by the same process.

These data on the whole are in agreement with the hypothesis that pollen TGase might have a role in respiratory allergies and be one of the factors by which environmental conditions and pollution affect these pathologies.

Acknowledgments We acknowledge "Fondazione del Monte di Bologna e Ravenna" which supported Dr. R.A. Iorio with a research contract on "Cambiamenti climatici e inquinanti atmosferici dell'area urbana di Bologna: influenza sulle allergie crociate"; Italian Ministero dell'Università for the grant PRIN 2007 and Bologna University for the grant Progetto strategico di Ateneo 2006 "Crossallergenicity" to D. Serafini-Fracassini. We are also grateful to the British–Italian partnership project 2005–2006 to Dr. A. Di Sandro and Dr. E. Verderio for partially supporting the work performed at the NTU. We would like to thank Nottingham Trent University for the RAE QR (U12) fund to E. Verderio. We wish to thank Dr Graham Ball for advice on environmental simulations and Mrs D. Baker (both from Nottingham Trent University) for help with the environmental chamber. We also acknowledge the "Dottorato di Biologia e Fisiologia cellulare" of the Bologna University for supporting visits at NTU of Dr. R. A. Iorio during her PhD course. We thank Prof. M. Griffin of the School of Life and Health Sciences, Aston University (Birmingham) for producing and providing TGase inhibitor 281 and 283, and Prof. R. Botta, University of Torino, who kindly donated pollen of *Corylus avellana*. We thank Mr. N. Mele for the image elaboration.

References

Accorsi CA, Bandini Mazzanti M, Fano RA, Forlani L, Lolli F, Rivasi F, Trevisan G, Zucchi L (1982) Granuli pollinici in strisci di secreto bronchiale. Estratto dalla Rivista di Patologia e Clinica della Tubercolosi e di Pneumologia 53:759–812

Adhikari A, Reponen T, Grinshpun SA, Martuzevicius D, LeMasters G (2006) Correlation of ambient inhalable bioaerosols with particulate matter and ozone: a two-year study. Environ Pollut 140:16–28

Alexander MP (1969) Differential staining of aborted and nonaborted pollen. Stain Technol 44:117–122

Bagni N, Adamo P, Serafini-Fracassini D, Villanueva VR (1981) RNA, proteins and polyamines during tube growth in germinating apple pollen. Plant Physiol 68:727–730

Baumgartner W, Golenhofen N, Weth A, Hiiragi T, Saint R, Griffin M, Drenckhahn D (2004) Role of transglutaminase 1 in stabilisation of intercellular junctions of the vascular endothelium. Histochem Cell Biol 122:17–25

Beggs PJ (2004) Impacts of climate change on aeroallergens: past and future. Clin Exp Allergy 34:1507–1513

Bellani LM, Rinallo C, Muccifora S, Gori P (1997) Effects of simulated acid rain on pollen physiology and ultrastructure in the apple. Environ Pollut 95:357–362

Botta R, Gianotti C, Richardson DG, Suwanagul A, Sanz CL (1994) Hazelnut variety organic acids, sugars and total lipids fatty acids. Acta Hortic 351:693–700

Burney P, Summers C, Chinn S, Hooper R, van Ree R, Lidholm J (2010) Prevalence and distribution of sensitization to foods in the European community respiratory health survey: a EuroPrevall analysis. Allergy 65:1182–1188

Cai G, Faleri C, Del Casino C, Emons AM, Cresti M (2011) Distribution of callose synthase, cellulose synthase and sucrose synthase in tobacco pollen tube is controlled in dissimilar ways by actin filaments and microtubules. Plant Physiol 155:1169–1190

Calzoni GL, Speranza A, Bagni N (1979) In vitro germination of apple pollens. Scientia Hortic 10:49–55

Castro AJ, Alchè JD, Calabozo B, Rodriguez-Garcia MI, Polo F (2007) Pla l 1 and Ole e 1 pollen allergens share common epitopes and similar ultrastructural localization. J Investig Allergol Clin Immunol 17:93–99

Cecchi L, D'Amato G, Ayres JG, Galan C, Forastiere F, Forsberg B, Gerritsen J, Nunes C, Behrendt H, Akdis C, Dahl R, Annesi-Maesano I (2010) Projections of the effects of climate change on allergic asthma: the contribution of aerobiology. Allergy 65:1073–1081

Cordella-Miele E, Miele L, Mukherjee AB (1990) A novel transglutaminase-mediated post-translation modification of phospholipase A2 dramatically increases its catalytic activity. J Biol Chem 265:17180–17188

Cordella-Miele E, Miele L, Beninati S, Mukherjee AB (1993) Transglutaminase-catalyzed incorporation of polyamines into phospholipase A2. J Biochem (Tokyo) 113:164–173

D'Amato G, Cecchi L (2008) Effects of climate change on environmental factors in respiratory allergic diseases. Clin Exp Allergy 38:1264–1274

D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, Behrendt H, Liccardi G, Popov T, van Cauwenberge P (2007) Allergenic pollen and pollen allergy in Europe. Allergy 62:976–990

Del Duca S, Bregoli AM, Bergamini C, Serafini-Fracassini D (1997) Transglutaminase-catalyzed modification of cytoskeletal proteins by polyamines during the germination of *Malus domestica* pollen. Sex Plant Reprod 10:89–95

Del Duca S, Serafini-Fracassini D, Bonner PLR, Cresti M, Cai G (2009) Effects of post-translational modifications catalyzed by pollen transglutaminase on the functional properties of microtubules and actin filaments. Biochem J 418:651–664

Del Duca S, Cai G, Di Sandro A, Serafini-Fracassini D (2010) Compatible and self-incompatible pollination in *Pyrus communis* displays different polyamine levels and transglutaminase activity. Amino Acids 38:659–667

Della Mea M, Caparros-Ruiz D, Claparols I, Serafini-Fracassini D, Rigau J (2004) AtPng1p. The first plant transglutaminase. Plant Physiol 135:2046–2054

Della Mea M, Serafini-Fracassini D, Del Duca S (2007a) Programmed cell death: similarities and differences in animals and plants. A flower paradigm. Amino Acids 33:395–404

Della Mea M, De Filippis F, Genovesi V, Serafini-Fracassini D, Del Duca S (2007b) The acropetal wave of developmental cell death of tobacco corolla is preceded by activation of transglutaminase in different cell compartments. Plant Physiol 144:1211–1222

Di Sandro A, Del Duca S, Verderio E, Hargreaves A, Scarpellini A, Cai G, Cresti M, Faleri C, Iorio RA, Hirose S, Furutani Y, Coutts IGC, Griffin M, Bonner PLR, Serafini-Fracassini D (2010) An extracellular transglutaminase is required for apple pollen tube growth. Biochem J 429:261–271

Fernandez-Rivas M, Bolhaar S, Gonzalez-Mancebo E, Asero R, van Leeuwen A, Bohle B, Ma Y, Ebner C, Rigby N, Sancho AI, Miles S, Zuidmeer L, Knulst A, Breiteneder H, Mills C, Hoffmann-Sommergruber K, van Ree R (2006) Apple allergy across Europe: how allergen sensitization profiles determine the clinical expression of allergies to plant foods. J Allergy Clin Immunol 118:481–488

Griffin M, Casadio R, Bergamini CM (2002) Transglutaminases: nature's biological glues. Biochem J 368:377–396

Griffin M, Mongeot A, Collighan R, Saint RE, Jones RA, Coutts IG, Rathbone DL (2008) Synthesis of potent water-soluble tissue transglutaminase inhibitors. Bioorg Med Chem Lett 18:5559–5562

Hallstrand TS, Wurfel MM, Lai Y, Ni Z, Gelb MH, Altemeier WA, Beyer RP, Aitken ML, Henderson WR (2010) Transglutaminase 2, a novel regulator of eicosanoid production in asthma revealed by genome-wide expression profiling of distinct asthma phenotypes. PLoS One 5:e8583

Herrmann D, Henzgen M, Frank E, Rudeschko O, Jäger L (1995) Effect of hyposensitization for tree pollinosis on associated apple allergy. J Investig Allergol Clin Immunol 5:259–267

Hesse M (1979) Entwiklungsgeschichte und ultrastruktur von pollenkitt und exine bei nahe verwandten entomophilen und anemophilen sippen der *Oleaceae, Scrophulariaceae, Plantaginaceae* und *Asteraceae*. Plant Syst Evol 132:107–139

Iorio RA, Di Sandro A, Scarpellini A, Del Duca S, Serafini-Fracassini D, Verderio E (2008) Visualisation of transglutaminase-mediated cross-linking activity. Plant Biosyst 142:360–366

Ishiguro S, Akiko Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The defective in anther dehiscence gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell 13:2191–2209

Jones RA, Nicholas B, Mian S, Davies PJ, Griffin M (1997) Reduced expression of tissue transglutaminase in a human endothelial cell line leads to changes in cell spreading, cell adhesion and reduced polymerisation of fibronectin. J Cell Science 110:2461–2472

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lorand L, Graham RM (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4:140–156

Metha K, Fok JY, Mangala LS (2006) Tissue transglutaminase: from biological glue to cell survival cues. Frontiers Biosci 11:163–185

Miele L (2003) New weapons against inflammation: dual inhibitors of phospholipase A2 and transglutaminase. J Clin Investig 111:19–21

Motta AC, Marliere M, Peltre G, Sterenberg PA, Lacroix G (2006) Traffic-related air pollutants induce the release of allergen containing cytoplasmic granules from grass pollen. Int Arch Allergy Immunol 139:294–298

Pacini E, Hesse M (2005) Pollenkitt—its composition, forms and functions. Flora 200:399–415

Pagliarani G, Paris R, Tartarini S, Sansavini S (2009) Cloning and expression of the major allergen genes in apple fruit. J Hort Sci Biotech Isafruit Special Issue 176–181

Peden DB (2002) Pollutants and Asthma: Role of Air Toxics. Environ Health Perspect 110:565–568

Rogers CA, Wayne PM, Macklin EA, Muilenberg ML, Wagner CJ, Epstein PR, Bazzaz FA (2006) Interaction of the onset of spring and elevated atmospheric CO_2 on ragweed (*Ambrosia artemisiifolia* L.) pollen production. Environ Health Perspect 114:865–869

Serafini-Fracassini D, Della Mea M, Tasco G, Casadio R, Del Duca S (2009) Transglutaminases: widespread cross-linking enzymes in plants. Amino Acids 36:643–657

Shea KM, Truckner RT, Weber RW, Peden DB (2008) Climate change and allergic disease. J Allergy Clin Immunol 122:443–453

Sohn J, Kim TI, Yoon YH, Kim JY, Kim SY (2003) Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis. J Clin Investig 111:121–128

Verderio E, Nicholas B, Gross S, Griffn M (1998) Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachment, and cell death. Exp Cell Res 239:119–138

Verderio EM, Johnson T, Griffin M (2004) Tissue transglutaminase in normal and abnormal wound healing. Review article. Amino Acids 26:387–404

Weber RW (2002) Mother Nature strikes back: global warming, homeostasis, and implications for allergy. Ann Allergy Asthma Immunol 88:251–252

Ziska LH, Caulfield FA (2000) Rising CO_2 and pollen production of common ragweed (*Ambrosia artemisiifolia*), a known allergy inducing species: implications for public health. Aust J Plant Physiol 27:893–898

Zubini P, Zambelli B, Musiani F, Ciurli S, Bertolini P, Baraldi E (2009) The RNA hydrolysis and the cytokinin binding activities of PR-10 proteins are differently performed by two isoforms of the Pru p 1 peach major allergen and are possibly functionally related. Plant Physiol 150:1235–1247