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ORIGINAL ARTICLE

Compatible and self-incompatible pollination in *Pyrus communis* displays different polyamine levels and transglutaminase activity

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Abstract The polyamine (PA) content and the transglutaminase (TGase) activity have been investigated in *Pyrus communis* pollination with compatible and self-incompatible (SI) pollen in order to deepen their possible involvement in the progamic phase of plant reproduction. The PA distribution as free, perchloric acid (PCA)-soluble and PCA-insoluble fractions in ungerminated (UGP), germinating pollen (GP), styles and pollinated styles with compatible and SI pollens is discussed in the light of a possible role during pollination. Generally, the conjugated PAs both in PCA-soluble and PCA-insoluble fractions were higher than the free form. Within the conjugated PAs, the PCA-insoluble ones were the highest with the exception of the not pollinated styles. As TGase mediates some of the effects of PAs by covalently binding them to proteins, the activity of this enzyme, never checked before in styles and pollinated styles, was examined. In the SI styles, the TGase activity is higher in comparison to style-pollinated with compatible pollen, and high molecular mass cross-linked products were formed, suggesting an involvement of TGase in SI response. This is the first evidence on the presence of this enzyme activity in not pollinated and pollinated styles.

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Abbreviations

PAs	Aliphatic polyamines
PU	Putrescine
SD	Spermidine
SM	Spermine
TGase	Transglutaminase
GP	Germinating pollen
UGP	Ungerminated pollen
SI	Self-incompatibility
PCA	Perchloric acid

Introduction

Sexual reproduction of flowering plants depends on delivery of the male gamete to the ovule, which occurs through a long, polarized apical growth of the pollen grain, giving rise to the pollen tube. It grows exclusively at its tip, and this growth is distinguished by very fast rates and reaches extended lengths. This process known as pollen germination is characterized by the continuous rebuilding of the cell wall and apical migration of the cytoplasm sustained by cytoskeleton re-organisation. An intriguing aspect of plant reproductive biology is how pollen–pistil interactions occur and who are the actors of this cross talk, involved in regulation of pollen tube growth inside the pistil.

It is known that PAs are essential for cell growth in animals and plants; in fact they are important in the cycling of plant cells (Serafini-Fracassini 1991) and it has been

observed that PA-deficient animal cells cannot undergo a normal cytodieresis due to lack of organization of the cytoskeleton (Pohjanpelto et al. 1981). However, little information is available on the role of growth substances, among them PAs, during the apical growth of pollen in the styles. Despite activation of PA biosynthesis during pollen tube emergence and elongation, a concomitant decrease in PA levels has been observed, most likely because of their release in the external space (Bagni et al. 1981; Speranza et al. 1984). The role of PA inside and outside the pollen tube has not been clarified, but it appears to be related to the metabolism of different RNAs and to the control of RNAse activity during tube emergence and growth (Speranza et al. 1984). Simultaneously with the decrease in free PAs, changes in the levels of PAs bound to low- and high-mass molecules take place inside the pollen tube (Chibi et al. 1994; Antognoni and Bagni 2008).

TGases (EC 2.3.2.13) are enzymes able to post-translationally modify proteins with different reactions (Lorand and Graham 2003); one of the best-known reaction consists in forming bridges between proteins throughout the conjugation of glutamine to lysine residues or PAs. In the latter case modifying the protein substrate by "cationisation" or by forming inter- or intra-molecular bridges through polyamines of different length (Serafini-Fracassini et al. 2009). Due to their effects on the physical and chemical properties of proteins, they have many biotechnological applications particularly in the food processing industry, in medicine and in cosmetics, increasing the demand for an inexpensive, efficient and safe source of recombinant enzymes (Capell et al. 2004).

The presence of TGase can explain the increase in bound putrescine (PU) levels during *Malus* pollen germination previously detected by Bagni et al. (1981). Moreover, the observed decrease of free PAs during pollen germination could be related not only to their release into the medium, but also to their binding to heavy-mass proteins, which subtracts them from the free PA pool. Thus, we can hypothesize that TGase-mediated PA binding may also be important for the rapid rearrangement and translocation of the cytoskeleton elements, essential during pollen tube elongation (Cai et al. 2000) occurring in pollinated styles.

Previous results from animal cells suggested the involvement of TGase in the modification of cytoskeleton proteins showing that actin is a substrate of TGase (Lorand and Graham 2003). Recently, Robinson et al. (2007) summarized the cytoskeleton substrates of TGase 2, one of the nine TGases identified in animals. In plants, the crude extract of *Malus domestica* pollen contained TGase activity and catalyzed the incorporation of PAs into proteins with molecular mass of 43 kDa and 52–58 kDa, identified as actin and α-tubulin. Moreover, actin of germinating pollen gave rise to cross-linked products of high molecular mass that incorporate PAs (Del Duca et al. 1997).

SI is the most important system used to prevent inbreeding and promote outbreeding; from an evolutive point of view, it is thought that the wide success of the Angiosperms is due, at least in part, to the arrive, during reproduction, of SI mechanism. It involves a pollen–pistil interaction and a cell–cell recognition system, which regulates the acceptance or rejection of pollen landing on the stigma of the same species, so that incompatible pollen is selectively inhibited at a specific stage during pollination, preventing self-fertilization. SI is a species-specific and genetically controlled mechanism: it is in fact controlled by a single S locus that has multiple S-alleles. Up to date are known two major classes of SI at the genetic level: gametophytic SI (GSI) and sporophytic SI (SSI). GSI is so-called because the incompatibility phenotype of the pollen is determined by its haploid (gametophytic) genome, whereas with SSI the pollen exhibits the incompatibility phenotype of its diploid (sporophytic) parent. In Rosaceae (e.g. *M. domestica* and *Pyrus communis*) and Papaveraceae the SI is of GSI type (McClure and Franklin-Tong 2006).

Up to date scarce information is available on the role of PAs and their glutamyl-derivatives, during the in vivo pollen germination. In the present paper, we focused on the analysis of PAs pattern and TGase activity by comparing compatible and SI pollination in *P. communis*. The aim of the work was to shed more light on the physiological role of PAs and TGase on pollen–pistil interactions.

Materials and methods

Plant material

Mature pollen of pear (*P. communis* cv Abbé Fètel and cv Williams) was obtained from plants grown in experimental plots (Dipartimento di Colture Arboree, Bologna, Italy), collected as previously described (Bagni et al. 1981); the pollen was stored at −20°C with NaOH pellets to maintain it dry until use. Styles were collected from flowers of *P. communis* cv Abbé Fètel at balloon stage and immediately frozen with liquid N_2 and stored at −80°C.

HPLC PA analysis

Free and conjugated PA content in GP and in UGP, as well as in styles and in pollinated styles were determined by HPLC after extraction with 4% perchloric acid (PCA), as described by Scaramagli et al. (1995). PA content was expressed on fresh weight basis; in case of pollen, the fresh weight was the weight of grains before incubation in the germination medium.

In vitro and in vivo pollen germination

For in vitro germination, pollen was rehydrated at 30°C and 100% relative humidity for 30 min. Germination was performed for 90 min in a liquid medium as described by Bagni et al. (1981).

In vivo pollen germination was performed by gently brushing the stigmatic surface of the styles with a small paintbrush preventively put in contact with pollen. In the compatible pollination model, styles of cv Abbé Fétel have been pollinated with pollen cv William and were indicated as A × W; in the SI pollination styles of cv Abbé Fétel have been pollinated with pollen cv Abbé Fétel and were indicated as A × A. The pollen was let to germinate inside the styles for 48 h and the germinated tubes were checked randomly under microscope after each style was longitudinally sectioned in four parts. The styles pollinated with self (incompatible) and cross (compatible) pollen were collected and stored at −80°C, until analysis were done.

TGase radiometric assay

The extraction of samples and the in vitro activity of TGase were performed on the GP, not pollinated and pollinated style as previously described by Del Duca et al. (1997), with the following modifications. The incubation mixture included 100 µg of extract, 20 µl of 500 mM Tris buffer pH 7 containing 10 mM DTT, and 50 mM $CaCl_2$ or 50 mM EDTA. As radioactive tracer, 148 kBq [1, 4(n)-^{3}H]-PU (0.55 TBq mmol^{-1}; GE Healthcare, Milano, Italy) was added to the assay mixture and the volume adjusted to 200 µl with Milli-Q water. After 120 min of incubation at 30°C, the reaction was stopped and repeatedly pelleted with 5% (w/v) TCA as described by Del Duca et al. (1997) and radioactivity counted in a Beckman LS 6500 counter.

Statistics

Each determination was repeated at least three times. All values were means with standard errors. The Student's *t* test was used to compare means, as reported in the figure legends.

Fluorescence microscopy analysis

Samples were observed with a Zeiss Axiophot optical microscope equipped with a 63× objective. Images were captured with a MRc5 AxioCam video camera using AxioVision software. Callose was stained with decolorized aniline blue as previously reported (Persia et al. 2008).

Proteins extraction and Western blotting

Pollinated styles proteins were solubilized at 4°C in extraction buffer (10 mg ml^{-1}) containing 100 mM Tris–HCl pH 8.5, 2 mM dithiothreitol (DTT), 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.2 mM phenylmethylsulphonylfluoride (PMSF) in a Potter–Elvehjem homogenizer. Large cell debris was removed from the total homogenate by centrifugation at 10,000 *g* for 10 min at 4°C, as described by Cai et al. (2000). Protein concentration was estimated on the supernatant by a modified bicinchoninic acid method with bovine serum albumin as the standard protein (Brown et al. 1989). A total of 100 µg of proteins/lane were electrophoresed on 12% (w/v) polyacrylamide SDS–PAGE slab gels and electroblotted as previously reported (Del Duca et al. 1997). For immunoblotting 81D4 monoclonal anti–N^{ε}(γ-glutamyl)lysine Ab (Covalab, Lyon, France) was diluted at 1:200 in Tris buffer 20 mM pH 7.5 containing 150 mM NaCl and let at 4°C overnight then proteins were finally detected by an anti-mouse immunoglobulin conjugated to peroxidase and revealed using the ECL system (GE Healthcare)

Results

Free and bound PA determinations

In Fig. 1a free PAs levels in UGP, GP and styles of *P. communis* cv Abbé Fétel were reported. In UGP, the levels of the three PAs were very similar, whereas in GP, SM was the highest followed by SD and PU. On the contrary, an opposite pattern was observed in style tissue, being PU the most abundant and SM the lowest one. In GP a significant decrease of all three PA in respect to UGP occurred: the most dramatic decreased regards PU level (about 90%), whereas the level of SD and SM were reduced of about 50 and 30%, respectively. In Fig. 1b, the free PAs content in pollinated styles was reported. A × W indicates styles of cv Abbé Fétel pollinated with pollen cv William and represent the compatible pollination model, whereas A × A indicates styles of cv Abbé Fétel pollinated with pollen cv Abbé Fétel representing the SI pollination. In compatible pollinated styles (A × W) the PU and SM were similar and about more than twofolds higher in respect to SD, whereas in SI pollinated styles PU was the highest. In the compatible pollinated styles (A × W) all the three PAs showed a higher content in respect to the SI pollinated styles (A × A), with a ratio of 1,3 for PU, 2 for SD and 3,3 for SM.

In Fig. 2a PCA-soluble PAs levels were reported. The PAs conjugated to low molecular weight compounds showed the following trend: in UGP, SD was by far the most abundant PAs found reaching levels definitely higher

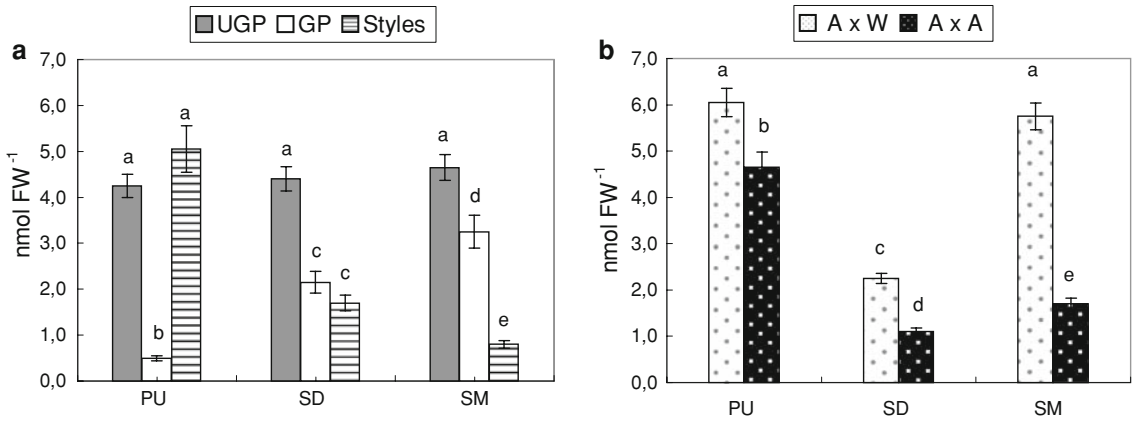

Fig. 1 Free PA content in UGP, GP, styles (**a**) and in pollinated styles (**b**) of *Pyrus communis* cv Abbé Fétel. *A* × *W* indicates styles of cv Abbé Fétel pollinated with pollen cv William and represent the compatible pollination model, whereas *A* × *A* indicates styles of cv Abbé Fétel pollinated with pollen cv Abbé Fétel representing the SI pollination. Values are the mean ($n = 3$) ± SD. *Different letters* (*a*, *b*, *c*, *d*, *e*) indicate means that are significantly different according to Student's *t* test at the 1% level

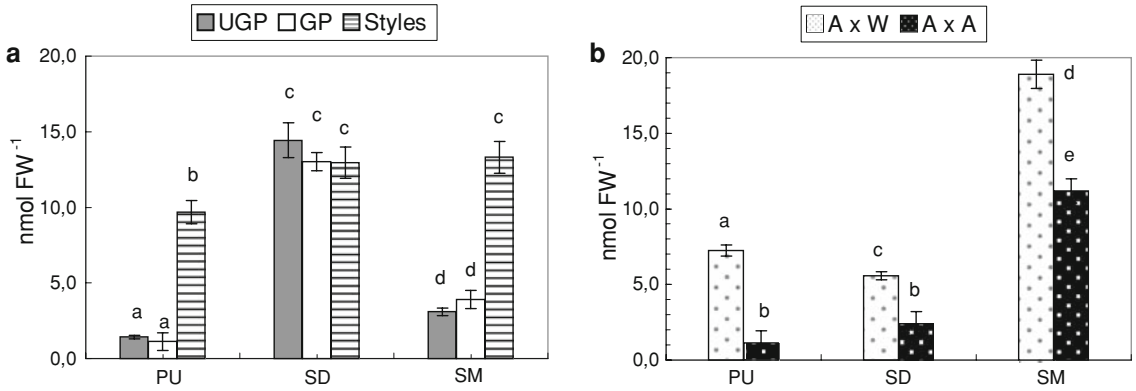

Fig. 2 PCA-soluble PA levels in UGP, GP, styles (**a**) and in pollinated styles (**b**) of *Pyrus communis* cv Abbé Fétel. Pollination was performed as reported in Fig. 1. Values are the mean ($n = 3$) ± SD. *Different letters* (*a*, *b*, *c*, *d*, *e*) indicate means that are significantly different according to Student's *t* test at the 1% level

than SM and PU and free SD. The same pattern was observed in GP, whereas in style the differences among the three PAs were much less pronounced. As regards pollinated styles (Fig. 2b) in both compatible and SI pollinated styles, SM was more than three folds higher than PU and SD. Also in this case, in compatible pollinated styles all three PAs levels were higher compared to SI ones being PU 6,3, SD 2,3 and SM 1,7 times, respectively, higher in the compatible pollinated styles.

Concerning PCA-insoluble PAs (Fig. 3a, b), UGP showed SD level much higher than PU and SM (3- and 20-folds, respectively); a similar trend was observed in GP. It is worth observing that SD bound to high molecular mass compounds represents the richest SD fraction found in UGP. In the styles, the levels of the three conjugated PAs were lower in respect to UGP and GP being the SD the predominant one. Nevertheless, it was about 28- and 15-folds, respectively, less than SD content in UGP and GP. Changes in the PCA-insoluble PAs in UGP and GP are dependent on the length of the polyamine considered: the PU content did not significantly change among UGP and GP, whereas SD and SM decreased in GP 1,9 and 5,1-fold, respectively (Fig. 3a).

In pollinated styles, the level of PCA-insoluble PAs were higher (a part for PU in A × W) in respect to the free and PCA-soluble ones. The SI-pollinated styles A × A showed a PU and SM content almost two fold higher in respect to their levels in compatible fertilized style A × W, whereas the SD content was lower of about 26% (Fig. 3b).

TGase activity in pollinated styles and immunoblotting of its products

The TGase activity checked in the pollinated styles showed an increase in respect to the activity measured in GP and not pollinated styles. The activity resulted Ca^{2+}-dependent, as 5 mM EDTA inhibited it almost completely. In A × A fertilized style the activity resulted about twofold higher in

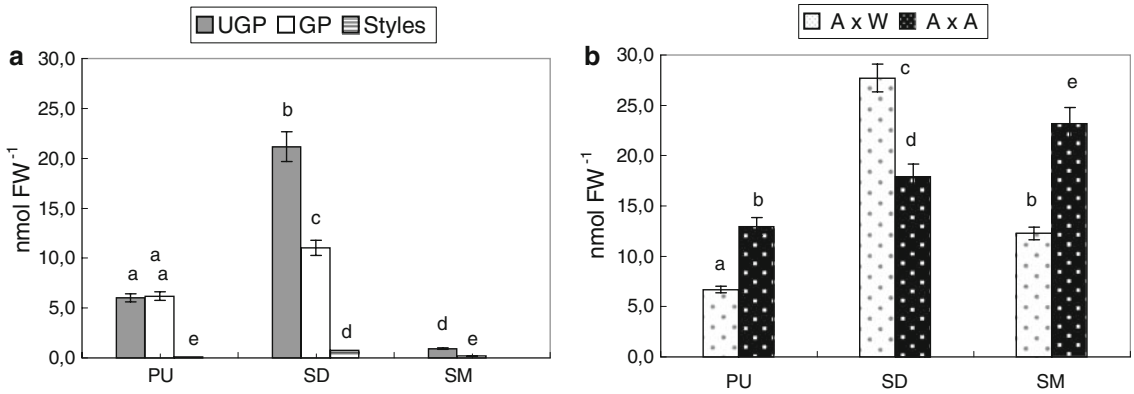

Fig. 3 PCA-insoluble PA content in UGP, GP, styles (**a**) and in pollinated styles (**b**) of *Pyrus communis* cv Abbé Fétel. Pollination was performed as reported in Fig. 1. Values are the mean ($n = 3$) ± SD. *Different letters* (*a*, *b*, *c*, *d*, *e*) indicate means that are significantly different according to Student's *t* test at the 1% level

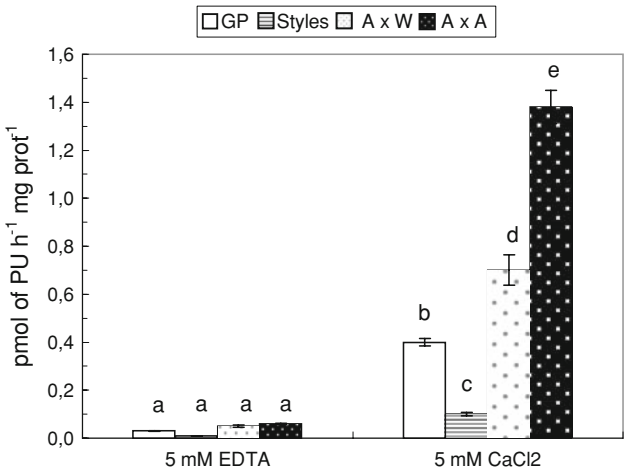

Fig. 4 TGase activity (pmol of PU h^{-1} mg $prot^{-1}$) in GP, not pollinated styles, self- and cross-pollinated styles**.** Pollination was performed as reported in Fig. 1. Values are the mean ($n = 3$) ± SD. *Different letters* (*a*, *b*, *c*, *d*, *e*) indicate means that are significantly different according to Student's *t* test at the 1% level

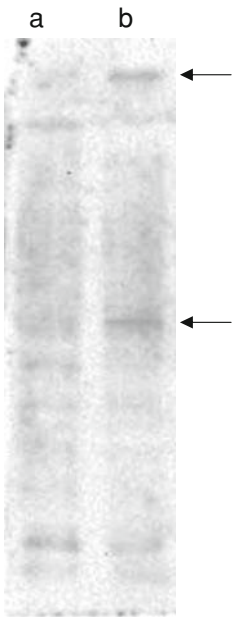

Fig. 5 Western blotting analysis on pollinated style extracts probed with the 81D4 monoclonal anti-N^{ε}(γ-glutamyl)lysine (Covalab, Lyon, France). Pollination was performed as reported in Fig. 1. **a** Extract of styles pollinated with compatible pollen ($A \times W$). **b** Extract of styles pollinated with SI pollen ($A \times A$). *Arrows* indicate the presence of two main labeled bands in SI-pollinated style, the upper band representing possibly cross-linked proteins with a molecular mass too high to enter in the stacking gel

respect to the A × W one, indicating that SI enhance TGase activity (Fig. 4).

Evidence that endogenous pollen proteins were substrates for pollen TGase during pollination of the styles was obtained by immunostaining with monoclonal antibody 81D4, which is specific for the products of TGase catalysis (Fig. 5). The SI sample showed two main labeled bands: the upper band represented possibly cross-linked proteins with a too high molecular mass to enter in the stacking gel, whereas the lower band had an estimated MW of about 50 kDa.

Fluorescence microscopy analysis

The self-fertilized styles were stained with aniline blue for callose reaction 48 h after pollination. Tubes showed a clear reaction with the staining, indicating the occurrence of callose plugs in the region of the tube apical part (Fig. 6). These callose formations were related to the interruption of the pollen tube growth in incompatible fertilization. In this system, pollen tubes penetration into the *gynoecium* reached only the upper first third of the style length, whereas in the compatible system the tube growths up to the ovule without any sign of apical callose plugs.

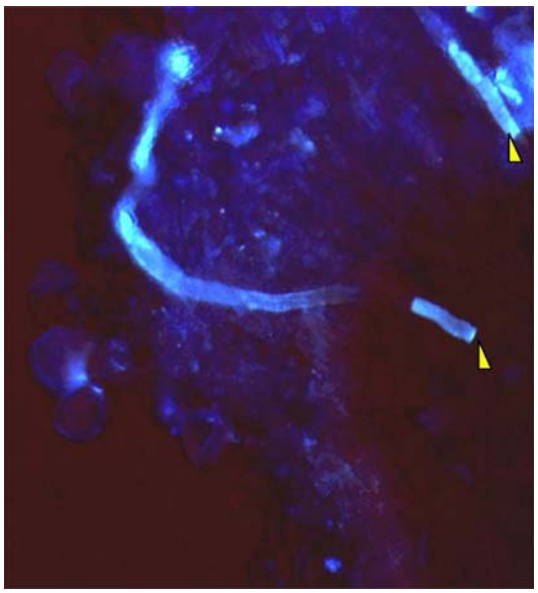

Fig. 6 Morphology of incompatible pollinated styles at the fluorescence microscopy. Callose labeling by aniline blue of Abate SI pollen tubes germinated in styles for 48 h. *Arrows* indicate the presence of callose plugs in the apical region of the tube

Discussion

An interesting aspect of higher plant reproduction is the question of how the pollen–pistil interaction could regulate the pollen tube growth. The present investigation shows that a correlation exists between pollination of styles and PAs content.

Our results show that PAs patterns change during pollen germination according to the type of polyamine and the polyamine fraction considered. The decreasing trend of free PAs observed in *Pyrus* after germination are in agreement with those obtained in *M. domestica* pollen (Bagni et al. 1981) and also in *Actinidia deliciosa* pollen (Antognoni and Bagni 2008). These results could be due to their release in the external space suggesting a possible role of these compounds in the progamic phase of the fertilization process.

During pollination of the styles with compatible pollen, PU and SM content increase in respect to PAs content in GP and styles not pollinated, whereas SD content does not change. All three PAs are higher in styles pollinated with compatible pollen in respect to SI pollinated styles. These results are in agreement with the possible inhibition of RNase activity by PAs as it is known that an inhibition of RNase activity occurs in GSI (McClure and Franklin-Tong 2006). In Rosaceae, GSI is dependent on mechanisms blocking pollen growth at the level of the upper third of the style. A key role in this SI is attributed to the stylar *S* locus, which encodes for glycoproteins showing ribonuclease (*S*-RNases) activity that enter to the pollen tube. There, they are inactivated and degraded in compatible pollen allowing the pollen tube to growth. On the contrary, in SI pollinated styles the pistil S-RNase inside the pollen tube are activated and a degradation of pollen RNA take place determining the arrest of pollen tube growth; anyway, other genes still to be identified, are involved in the regulation of this molecular mechanism. The inhibitory effect of RNase activity by PAs in plant has been reported. In *M. domestica* pollen the RNase activity was highly sensitive to the inhibitory effect of PAs: at 1 mM PU and spermidine (SD) reduce the RNase activity at 60% whereas the same concentration of spermine (SM) reduces the activity more than 50% (Speranza et al. 1984). The protective effect of polyamines on RNA has been reported also in other plant tissue i.e. in tuber of *Solanum tuberosum*. Potato tuber discs evinced a dramatic rise in RNase activity 24 h after excision and this rise was completely blocked by SD and SM in a concentration-dependent manner. Polyamines inhibited both the wound-induced RNase and the rise in RNase activity, which may result from other senescence-linked events (Altman 2006).

The accumulation of PCA-soluble PAs in reproductive organs of plants has been associated with fertility (Martin-Tanguy et al. 1982). Hydroxycinnamic acid (HCA)-PAs have been found in the pollen of various plant species (Bokern et al. 1995), thus suggesting a general role for these phenylpropanoid compounds in pollen function. The triferuloyl-SD is a component of the tryphine, which constitutes the pollen coat containing also a range of lipids, glycolipids, flavonoids and proteins; besides other functions, the pollen coat is involved in pollination and in pollen–stigma interaction. Microscopic observation have shown some defects in the pollen KO for *sht* gene whose product, the SD hydroxycinnamoyl transferase, catalyzes the conjugation of hydroxycinnamoyl CoA to SD, having a possible role in the organization of the cell wall (Grienenberger et al. 2009). HCA are able to bridge, through ester–ether linkages, different cell wall polymers, especially lignins and hemicelluloses (Lam et al. 1992), thus playing a role in modulating the rigidity of the cell wall. It is well known that most of the cellular SD pool is localized in the cell wall compartment, as reported in carrot cells (Bagni and Pistocchi 1990), kiwifruit anthers at the stage of mature pollen (Biasi et al. 2001) and *Quercus dentata* pollen (Bokern et al. 1995). In the light of PAs interaction with cell wall molecule, it is possible that PCA-soluble PAs influence pollen germination via a structural effect on the cell wall; its strength could be necessary to allow the tube to penetrate inside the style. Particularly PCA-soluble SD reached a level similar to that of UGP (it did not occur neither for free nor for PCA-insoluble SD), allowing to hypothesize a relevant involvement in cell wall organization of GP. A role in the cell wall organization could

explain also the higher amount of PCA-soluble PAs in compatible pollinated styles in respect to SI-pollinated ones. In fact, in A × W the pollen tubes grow for longer time in respect to A × A in which the tube does not reach the ovule arresting its growth in the upper third of the style length. Besides, the interactions with cell wall components, PCA-soluble PAs could play an important role in the regulation of the PA pool inside the cell (Bagni and Pistocchi 1990).

Concerning the styles, the high content of PCA-soluble PAs in respect to the free PAs, could be possible due to the protective role of PAs, toward ovary development. It has been observed that PAs causes a reduction of necrosis in in vitro culture of ovaries of *A. deliciosa* and it has been proposed that this protective effect could be due to a membrane protection or to their role as anti-stress compounds (Biasi et al. 1997).

PCA-insoluble PAs represent quantitatively the most important PAs fraction at least in pollen. The PCA-insoluble derive from the binding of PAs to high molecular mass partner; i.e. by the binding of PCA-soluble PAs (e.g. diferulic acid-PAs) to cell wall molecules as hemicelluloses and/or lignin, giving rise to the PCA-insoluble fraction of PAs (Antognoni and Bagni 2008); alternatively, PAs are covalently conjugated to proteins via TGase (Serafini-Fracassini et al. 1988). In some cases, the importance of PAs in cell wall is related to the presence of TGase in the same compartments as reported in the alga *Chlamydomonas reinhardtii,* where TGase activity has been shown to affect cell wall glycoproteins. In the beginning of cell wall formation, an early TGase catalyses a cross-linking reactions which lead to the formation of a "soft envelope". Then, this structure organises the self-assembly of glycoproteins, and finally, an oxidative cross-linking reaction (isodityrosine cross-linking) renders the wall insoluble. *Chlamydomonas* secretes an extracellular 72 kDa-TGase, the maximal activity of which precedes the insolubilization of the assembled Hyp-rich glycoprotein. The addition of cadaverine, SM, SD and PU at high concentrations disrupts the normal process of wall assembly. In the cell wall, PAs are linked to several proteins and salt-soluble glycoproteins (Waffenschmidt et al. 1999). A clear confirmation of TGase presence in higher plant cell wall was obtained, in isolated walls of *Nicotiana* petals, by detection of TGase immuno-reactivity by western blotting and catalytic activity (Della Mea et al. 2007a).

In the present paper, we found a high amount of PCA-insoluble PU and SD in UGP and GP, whose presence could be related not only to the building of cell wall, but also to the organization of cytoskeleton. The pollination of the styles, in particular the SI-pollination, cause an increase of PCA-insoluble PAs in respect to their content in GP and styles not pollinated. These data are in agreement with TGase activity that resulted higher in SI-pollinated styles in which the arrest of pollen tube growth is confirmed by the callose plugs formation at the tip of the tube (Fig. 6).

The importance of TGase in pollen tube growth and indeed in pollination of the style is related, at least in part, to its action on cytoskeleton proteins. In *M. domestica* pollen, PA-mediated post-translational modification of actin and tubulin occurs by a Ca^{2+}-dependent TGase activity, causing the formation of high mass aggregates of actin (Del Duca et al. 1997). Recently, the enzyme has been partially purified and tested on isolated cytoskeleton proteins showing that it is able to induce the formation of high molecular mass aggregates of actin and tubulin. Moreover, TGase controls different properties of the pollen tube cytoskeleton (including the ability of actin and tubulin to assemble and their interaction with motor proteins) and consequently regulates the development of pollen tubes (Del Duca et al. 2009). The cytoskeleton involvement in SI phenomena is so far solely reported in incompatible *Papaver* tubes, where after pollen–stigma interaction an high Ca^{2+} influx take place and then F-actin foci are formed by a still uncharacterized cross-linking mechanism that blocks tube elongation, causing GP programmed cell death (PCD) (McClure and Franklin-Tong 2006).

In animal cells, it has been reported that following stress or insult, up-regulation of TGase often occurs (Ientile et al. 2007; Kotsakis and Griffin 2007). The enhanced activity of TGase in cells can lead to massive intracellular (both nuclear and cytoplasmic) cross-linking, resulting in cell death if Ca^{2+} homoeostasis in these cells is suddenly perturbed (Griffin et al. 2002).

Lilley et al. (1998) detected TGase activity in root and shoot tissues of dicotyledonous (pea and broad bean) and monocotyledonous (wheat and barley) plants. In both root and leaf TGase exhibited Ca^{2+}-dependence and Ca^{2+} concentration affected the type of linkage produced. The incorporation of PAs into proteins was activated by 20 nM Ca^{2+}; this means that the resting level of cytosolic Ca^{2+} could be responsible for the intracellular role of TGase, i.e. the attachment of primary amines to proteins with consequent effects on their charge and/or conformation. On the other hand, the plant TGase protein cross-linking function, resulting in the formation of the ε-(γ-glutamyl)-lys isodipeptide bonds, was activated by Ca^{2+} at mM concentrations. Thus, TGase could catalyse protein cross-linking reactions in high Ca^{2+} environments. As an high Ca^{2+} influx takes place in incompatible *Papaver* pollen during SI response, this could explain the enhanced TGase activity observed in A × A in respect to A × W. Moreover, in these conditions, TGase could cross-link cytoskeleton proteins generating aggregates (Del Duca et al. 1997, 2009) as the actin aggregates observed in *Papaver*.

The immunostaining with monoclonal antibody 81D4 showed that endogenous pollen proteins form high molecular mass aggregates during SI pollination (Fig. 5). This antibody specifically binds TGase-mediated protein–protein and amine-protein crosslinks, reacting with bis-PA derivatives > gln-lys isodipeptide linkage > mono-PA derivatives but not with their free counterparts (Thomas et al. 2004). The formation of high mass aggregates are related to the arrest of pollen tube growth and possibly its PCD in *P. communis*. We know that also in plant TGase is involved in PCD (Della Mea et al. 2007b), but to elucidate if also in *P. communis* this phenomena occurs in SI further work is needed.

In conclusion, although it is not possible to define a causal relationship between PAs and TGase from one side and the SI response on the other, their role seems to be consistent at least as a part of an integrated multifactors responding system occurring after pollen–pistil interactions.

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