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ORIGINAL ARTICLE

# Polyamines and transglutaminase activity are involved in compatible and self-incompatible pollination of *Citrus grandis*

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**Abstract** Pollination of pummelo (*Citrus grandis* L. Osbeck) pistils has been studied *in planta* by adding compatible and self-incompatible (SI) pollen to the stigma surface. The pollen germination has been monitored inside the pistil by fluorescent microscopy showing SI altered morphologies with irregular depositions of callose in the tube walls, and heavy callose depositions in enlarged tips. The polyamine (PA) content as free, perchloric acid (PCA)-soluble and -insoluble fractions and transglutaminase (TGase) activity have been analyzed in order to deepen their possible involvement in the progamic phase of plant reproduction. The conjugated PAs in PCA-soluble fraction were definitely higher than the free and the PCA-insoluble forms, in both compatible and SI pollinated pistils. In pistils, pollination caused an early decrease of free PAs and increase of the bound forms. The SI pollination, showed highest values of PCA-soluble and -insoluble PAs with a maximum in concomitance with the pollen tube arrest. As TGase mediates some of the effects of PAs by covalently binding them to proteins, its activity, never checked before in *Citrus*, was examined with two different assays. In addition, the presence of glutamyl-PAs confirmed the enzyme assay data and excluded the possibility of a misinterpretation. The SI pollination caused an increase in TGase activity, whereas the compatible pollination caused its decrease. Similarly to bound PAs, the glutamyl-PAs and the enzyme activity peaked in the SI pollinated pistils in concomitance with the observed block of the pollen tube growth, suggesting an involvement of TGase in SI response.

**Keywords** *Citrus* · Pollen · Polyamines · Reproduction · Self-incompatibility · Transglutaminase

**Abbreviations**

| | |
|---|---|
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| DMC | *N*′,*N*′-dimethyl casein |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetraacetic acid |
| EGTA | Ethylene glycol tetraacetic acid |
| GSI | Gametophytic self-incompatible |
| HPLC | High-performance liquid chromatography |
| PAs | Polyamines |
| PBS | Phosphate-buffered saline |
| PMSF | Phenyl methyl sulfonyl fluoride |
| PCA | Perchloric acid |
| PCD | Programmed cell death |
| PU | Putrescine |
| SI | Self-incompatible |
| SD | Spermidine |
| SM | Spermine |
| SSI | Sporophytic self-incompatible |
| TCA | Trichloroacetic acid |
| TGase | Transglutaminase |
| tTGase | Tissue transglutaminase |
| UP | Unpollinated |

## Introduction

The polarized apical growth of the pollen grain gives rise to the pollen tube that transport sperms to the female

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gametophytes and indeed it is an essential requisite for the sexual reproduction of the flowering plants. Pollen tubes grow rapidly at their tips reaching extended lengths; this process, known as pollen germination, is characterised by the continuous rebuilding of the cell wall, by an elaborate and dynamic actin cytoskeleton, by a highly active membrane trafficking system and an apical migration of the cytoplasm that together provide the driving force and the secretory activities needed for growth (Cheung and Wu 2008). Pollen tube elongation involves the integration of many signals occurring in the stigma and stylar tissues (McClure and Franklin-Tong 2006) and in recent years, an increasing number of molecules involved in specific events during pollen–pistil interaction were identified in several model plants (Wheeler et al. 2009). A general consensus regulating a common programme of cellular interaction, however, has not been established. How pollen–pistil interactions occur and who are the actors of this cross talk involved in regulation of pollen tube growth inside the pistil, represent an intriguing aspect of plant reproductive biology.

The self-incompatibility (SI) response in plant reproduction is a species-specific and genetically controlled mechanism. SI is the most important system used to prevent inbreeding and promote out-breeding; from an evolutive point of view, it is thought that the wide success of the angiosperms is due, at least in part, to the arrival of SI mechanism to control the reproductive process. 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 controlled by a single S locus that has multiple *S*-alleles. Up to date two major classes of SI at the genetic level are known: 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. *Malus domestica* and *Pyrus communis*) and Papaveraceae the SI is of GSI type; in Papaveraceae SI is reported to be a type of programmed-cell death (PCD), which is accompanied by a cascade of events resulting in the arrest and death of pollen tubes (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-RNase) activity that enter into 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-RNases inside the pollen tube are activated and a degradation of pollen RNA takes place determining the arrest of pollen tube growth; anyway, other genes still to be identified are involved in the regulation of this molecular mechanism. In *Papaver rhoeas*, self-incompatibility proteins encoded by the stigma trigger $Ca^{2+}$-dependent signals after interaction with incompatible pollen (Thomas and Franklin-Tong 2004). A key target for the signaling network triggered by $Ca^{2+}$ increase is the actin cytoskeleton, whose altered dynamics drive pollen tubes into PCD (Thomas et al. 2006). SI also triggers rapid changes in the dynamics of cortical microtubules (Poulter et al. 2008).

Many studies have shown the effect of polyamines (PAs) on cell growth in animals and plants and in their reproductive processes (Pohjanpelto et al. 1981, Galston and Kaur-Sawhney 1990). Chibi et al. (1994) pointed out that PA synthesis is required for the maturation of *Nicotiana tabacum* pollen. PA synthesis occurs also during in vitro pollen tube emergence and elongation, but, despite PA biosynthesis, a concomitant decrease in PA levels occurred, being released in the external space (Bagni et al. 1981). Simultaneously with the decrease in free PAs, changes in the levels of PAs bound to low- and high-mass molecules take place not only inside the pollen tube (Chibi et al. 1994; Antognoni and Bagni 2008), but also in the growth medium (Di Sandro et al. 2010). When pollen germinates in vivo, its external space consists of the extracellular matrix (ECM) where there are components of either pollen and pistil origin. PAs in vivo could have a role also in SI as the RNase activity can be controlled by PAs as reported in *Malus domestica* pollen (Speranza et al. 1984).

Part of the bound PA could be conjugated to proteins by TGases (EC 2.3.2.13), which are widespread enzymes able to modify proteins with different reactions (Lorand and Graham 2003; Beninati et al. 2009; Serafini-Fracassini et al. 2009). Among them, the crosslink of proteins throughout the conjugation of glutamine to lysine residues or the amine incorporation into proteins was reported. In the latter case, the protein substrate is modified by "cationisation" or by forming inter- or intra-molecular bridges through PAs of different length. They have many biotechnological applications (i.e. 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). TGase was detected in pollen of *Malus* and *Pyrus* and also in the pollen external medium (Del Duca et al. 2010; Di Sandro et al. 2010); it could be responsible for the increase in the levels of some bound PA during pollen germination previously detected (Bagni et al. 1981; Del Duca et al. 2010). We observed 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. 1997; Del Del Duca et al. 1997, 2009). Consequently, pollen TGase may take part in the mechanism of cytoskeleton regulation during pollen tube growth and also during its PCD. However, the cytoskeleton involvement in SI phenomena is so far solely reported in incompatible *Papaver* tubes (Thomas et al. 2006).

TGases are a significant factor of PCD in animals and are considered as markers of apoptosis. Although the relationship between TGases and apoptosis is not well defined in plants, an acropetal wave of TGase activity precedes the PCD wave of the corolla cells in tobacco flower (Della Mea et al. 2007a, b).

When the germination occurs in planta inside the style, the secreted TGase could have a potential role in the adhesion of pollen tube to stylar cells, similar to the role of mammalian tTGase in cell–ECM interactions (Di Sandro et al. 2010), thus allowing style anchorage and subsequent tube migration.

All these data suggest that PAs and also TGase could be involved in the GSI. In the present paper, we focused on the analysis of PAs pattern and TGase activity by comparing compatible and SI pollination in pummelo (*Citrus grandis* L. Osbeck) which is considered a true species and ancestor of several other genotypes (Deng et al. 2007). In contrast to model species, the SI reaction in *Citrus* has been poorly studied, but it is assumed to be gametophytically determined since it occurs after the pollen has covered some distance through the gynoecium (Soost 1965); it is genetically controlled as some genes are differentially expressed in cross and self-pollinated styles, among which genes related to actin-binding proteins, phenylpropanoid pathway, $Ca^{2+}$, hormone-signalling and stress response (Distefano et al. 2009).

The aim of this work was to shed more light on the physiological role of PAs and TGase on pollen–pistil interactions in *Citrus* and to verify if the proposed involvement of PAs and TGase found in *Rosaceae* (Del Duca et al. 2009, 2010) is similar also in a genus belonging to *Rutaceae* family and thus be a potentially widespread mechanism.

## Materials and methods

### Plant material

Pistils of pummelo (*Citrus grandis* L. Osbeck) were obtained from plants grown at the "Primosole" experimental farm of Catania University. Several groups of flowers were emasculated just before anthesis from three trees of pummelo, hand-pollinated and bagged in cotton tissue.

### In vivo pollen germination: pollination of pistils

In vivo pollen germination was performed by gently brushing the stigmatic surface of the pistils with a small paintbrush preventively put in contact with pollen. In the compatible pollination model, pistils of pummelo (P) have been pollinated with pollen of 'Fortune' mandarin (F) (*C. clementina* Hort *ex* Tan. × *C. reticulata* Blanco) and were indicated as P × F; in the self-pollination, pistils of P have been pollinated with pollen of P and were indicated as P × P.

The pollen was let to germinate inside the pistils for 2, 4, 6 and 8 days. After each of these time periods, the pistils were collected from flowers and immediately frozen with liquid $N_2$ and stored at −80°C. Also pistils not pollinated (UP) were collected. In some case, pistils (not pollinated, cross and self-pollinated) were fixed in FAA (5 ml formalin, 5 ml glacial acetic acid, 90 ml ethanol 70%) and stored at 4°C for microscope observation.

### Fluorescence microscopy analysis

Pistils fixed in FAA were washed with water three times—1 h per wash—and left in 5% sodium sulphite overnight. The pistils were softened in 5% sodium sulphite in a microwave for 45 s. Prior to squashing, pistils were cut into two parts (stigma with style, ovary) and these were further cut longitudinally and split into two. The preparations were observed by fluorescence microscope (Leica DM 2500 of Leica Microsystems GmbH using I3 filter, Wetzlar, Germany) following staining with 0.1% aniline blue in 0.1 N $K_3PO_4$ and squashing. A pollen grain was considered germinated when the pollen tube was longer than the pollen grain diameter. The length of the longest pollen tube was recorded as a percentage of style length. The number of pollen tubes reaching the base of the style was also recorded.

### HPLC for PAs analysis

Free and conjugated PAs content in UP, cross- and self-pollinated pistils were determined by HPLC after extraction with 4% perchloric acid (PCA), as described by Scaramagli et al. (1995). PAs content was expressed on UP or pollinated pistils fresh weight basis.

### Protein extraction

Pollinated pistils proteins were solubilised 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 were removed from the total homogenate by centrifugation at 10,000*g* for 10 min at 4°C, as described by Cai et al. (1997). Protein concentration was estimated on the supernatant by a modified bicinchoninic acid method (BCA) with bovine serum albumin (BSA) as the standard protein (Brown et al. 1989).

### Transglutaminase assay

For biotin-labeled cadaverine incorporation TGase assay, pistils and pollinated pistils were homogenized in three volumes of 50 mM Tris–HCl, pH 8, containing 1 mM PMSF, 1 mM EDTA and 2 mM DTT, and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was dialyzed for 3 h at 4°C against 1,000 volumes of Tris–HCl 10 mM pH 8, containing 1 mM mercaptoethanol, and then TGase activity was checked. The assay was carried out according to Lilley et al. (1998) with EGTA instead of EDTA as a negative control.

The in vitro radiometric assay for TGase activity was performed on pistils and pollinated pistils as previously described by Del Duca et al. (1997), with the following modifications. The incubation mixture included 100 µg of protein extract, 20 µl of 500 mM Tris buffer pH 8 containing 10 mM DTT, 20 mM $CaCl_2$ or 50 mM EDTA and 1 mM PU. As radioactive tracer, 148 kBq [1, 4(n)-$^3$H]-PU (0.55 TBq mmol$^{-1}$; GE HealthCare) was added to the assay mixture and the volume adjusted to 200 µl with MilliQ 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). The pellet was washed at least three times with anhydrous diethyl ether and resuspended for proteolysis according to Folk et al. (1980); before adding digestive enzymes, an aliquot of sample (20 µl) was checked for radioactivity that was counted in a Beckman LS 6500 counter.

### HPLC identification of γ-glutamyl PA derivatives

After proteolytic digestion, products were separated by ion exchange chromatography using a Jasco HPLC system (4.5 × 90 mm column, packed with Ultropac 8 resin, $Na^+$ form; Jasco Europe, Milan, Italy) and the five-buffer system previously described by Folk et al. (1980). The identity of the PA derivatives was determined by comparison with the corresponding retention times of glutamyl PA standards obtained by 2 µg of purified guinea pig liver TGase assayed in the presence of 50 µg dimethyl casein (DMC). The standards were proteolytically digested and analyzed like the pistils samples. The identity of conjugated PAs was determined after release of free PAs by acid hydrolysis (6 M HCl) of the ion exchange chromatographic fractions corresponding to the predicted retention times.

## Results

### Histological analysis

Stigmas of *Citrus grandis* L. (Pummelo) flowers were cross pollinated with Fortune mandarin (P × F, compatible cross) or self pollinated with Pummelo (P × P, self incompatible system, SI) and the tube germination inside the stigma, style and ovary were checked using a fluorescence microscope. No differences in germination rate were observed in the stigmas of the compatible and self-incompatible crosses (Fig. 1a, b), while drastic morphological modifications were recorded in the pistils between both types of crosses. The P × P showed less pollen tubes grown in the style (Fig. 1c) and they were arrested or followed twisted directions, showing altered morphologies with irregular depositions of callose in the tube walls, and heavy callose depositions at the pollen enlarged tube tips (Fig. 1c, e). In the compatible P × F combination pollen tubes grew straight without twisting and formed regular-sized callose plugs at regular intervals (Fig. 1d), reaching the ovary locule (Fig. 1f).

To compare the kinetics of pollen tube growth, self- and cross-pollinated pistils were sequentially evaluated. Figure 2 shows a different pollen tube development in self-pollinated P × P and cross-pollinated P × F pistils. In P × P pollen tube elongation stopped between 4 and 6 days after pollination, whereas in cross-pollinated pistils P × F, pollen tube reached the ovary in 8 days (Fig 1f). In self-incompatible condition pollen tubes arrested in the first half of the style and practically none reached the base of the style (Figs. 1e, 2), as compared with cross-pollinated P × F, where more than 50 pollen tubes/pistil reached the ovary.

### Polyamine levels

PAs have been extracted from pistils pollinated as reported in Fig. 1 and separated in three fractions: free and bound PAs, the latter being separated as PCA-soluble and -insoluble PAs. In Fig. 3a free PAs levels in not pollinated (UP) and in pollinated pistils of Pummelo are reported. In general pollinated pistils show an abrupt decrease in free PAs content when compared to UP ones in which putrescine (PU) and spermidine (SD) levels are similar, spermine (SM) being much lower; among pollinated pistils, the self-pollinated ones P × P show the highest free SD and PU content, respectively, after 2 and 4 days from pollination. In detail, at day 4 the level of PU is similar to that reported for UP pistils and about four times higher when compared to cross-pollinated ones P × F. At day 2, the level of SD in self-pollinated pistils is about three times higher when compared to the value observed in cross-pollinated ones.

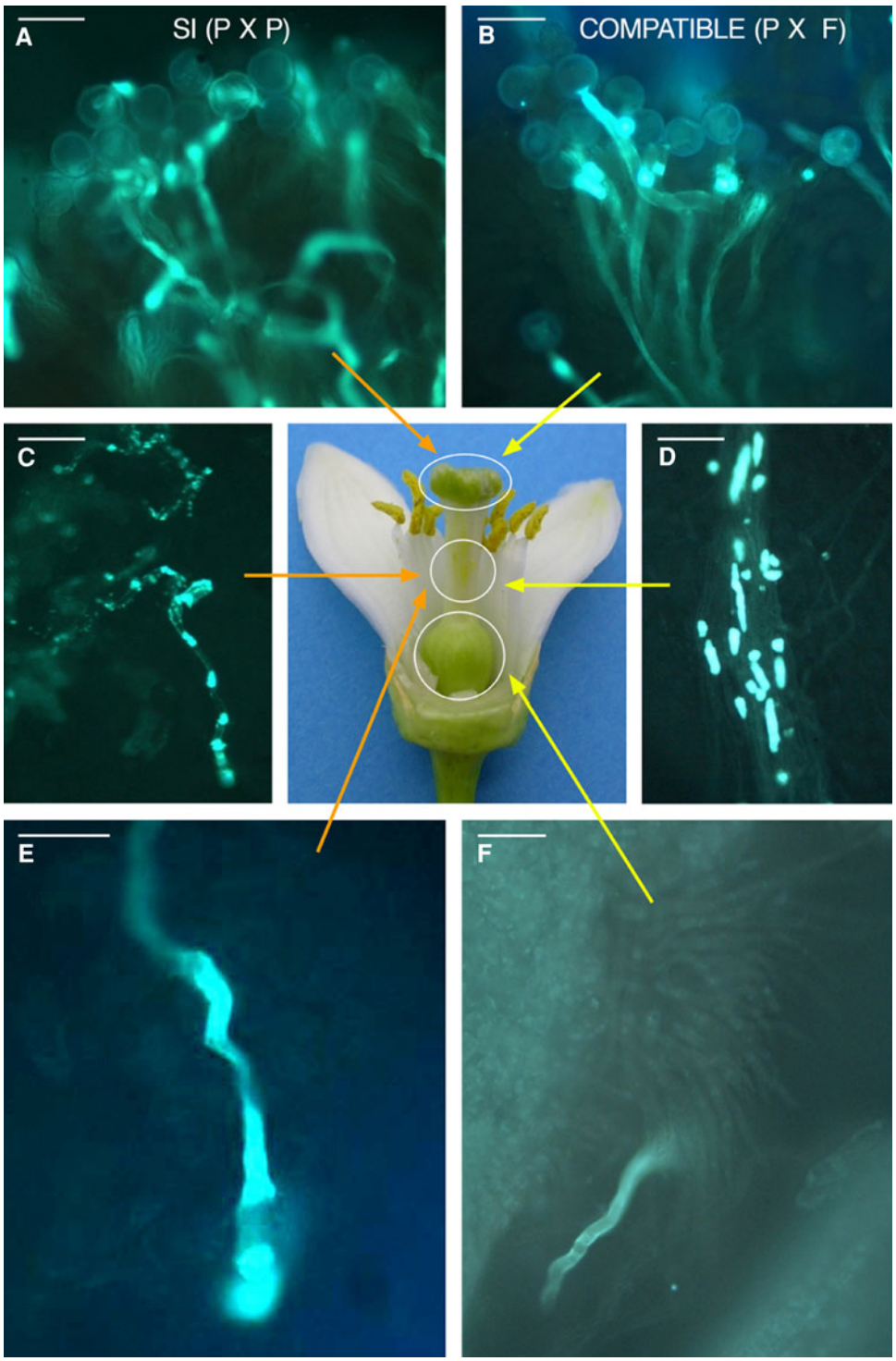


**Fig. 1** Pollinated pistils of pummelo (*Citrus grandis* L. Osbeck*)* at the fluorescence microscopy. Pollen tube germination (**a**, **b**), pollen tube elongation (**c**, **d**), pollen tube arrest (**e**) and pollen tube inside the ovary (**f**), in SI P × P (**a**, **c**, **e**) and compatible P × F (**b**, **d**, **f**) pollination (*scale bar* = 50 µm). P × P indicates pistils of pummelo pollinated with pollen of pummelo representing the SI pollination, whereas P × F indicates pistils of pummelo pollinated with pollen of 'Fortune' mandarin and represent the compatible pollination model. The presence of callose plugs along the tube and at the apical region of the tube in self-incompatible (SI) is labeled by aniline blue

At day 8 in SI, PU strongly decreased, SD showed no marked differences.

In Fig. 3b, PCA-soluble PA levels were reported. In UP pistils, among the three PAs, PU was the highest being four times with respect to SD whereas SM was present only in traces. For pollinated pistils in both compatible and SI, PU concentration was the highest being three to six times higher than SD in all samples and SM being the lowest. In SI pollinated pistils all three PA levels were higher compared to cross-pollinated ones, except for PU which in P × F peaked at day 2, whereas in P × P, peaked at day 6; SD and SM did not show significant variations.

Concerning PCA-insoluble PAs (Fig. 3c), in the UP pistils, the three conjugated PAs were present only in traces. In pollinated pistils the level of PCA-insoluble PAs was definitely higher in P × P with respect to P × F, in

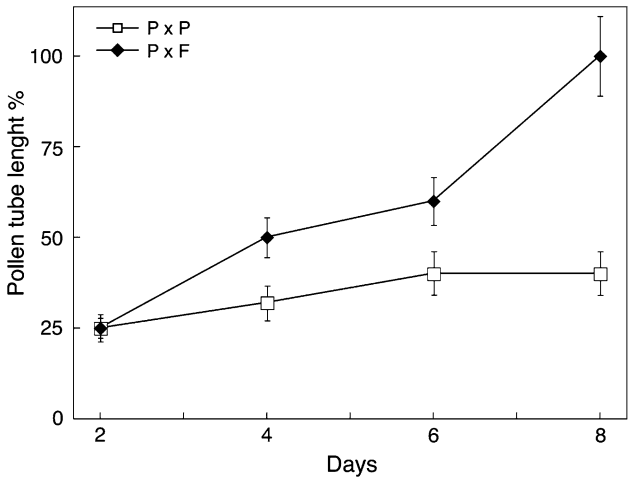


**Fig. 2** Kinetics of pollen tube growth in the pistils of *Citrus grandis* SI (P × P) and cross-pollinated (P × F) flowers at day 2, 4, 6, 8. The tube length percentage of P × F at day 8 has been considered as 100

which their levels were very low and some PA remained undetectable; also in case of PCA-insoluble PAs, the SI pollinated pistils at 6 days from pollination showed the highest PU level, whereas SD, decreased after 2 days onwards.

## Transglutaminase activity

The TGase activity has been checked in the pollinated pistils by the microplate assay (Fig. 4a) and by radiometric test (Fig. 4b), resulting in $Ca^{2+}$ dependence. The first assay performed by checking the incorporation of biotin-cadaverine in dimethylcasein showed that in P × F pistils, the TGase activity tends to decrease from 2 to 6 days after pollination (Fig. 4a). Differently, in P × P pistils at day 6 the activity described a sharp increase resulting about fourfold higher with respect to the P × F one, indicating that self-pollination enhance TGase activity in a definite stage of pollination when the pollen tube arrests its growth. A similar trend has been observed by the radiometric test performed in the presence of PU as reported in Fig. 4b, highlighting that endogenous pollen proteins were substrates for pollen TGase during pollination of the pistils.

## Identification of polyamine derivatives as γ-glutamyl-PAs

The analysis of the digested TCA-insoluble product of the TGase obtained by the radiometric assay as reported in Fig. 4b showed that both mono- and bis-glutamyl-PU were comparatively detected by the radioactive elution profile. Figure 5a reported the profiles at day 6 from pollination, chosen as the most representative ones. Both P × P glutamyl-derivatives were produced in higher amount compared to P × F products.

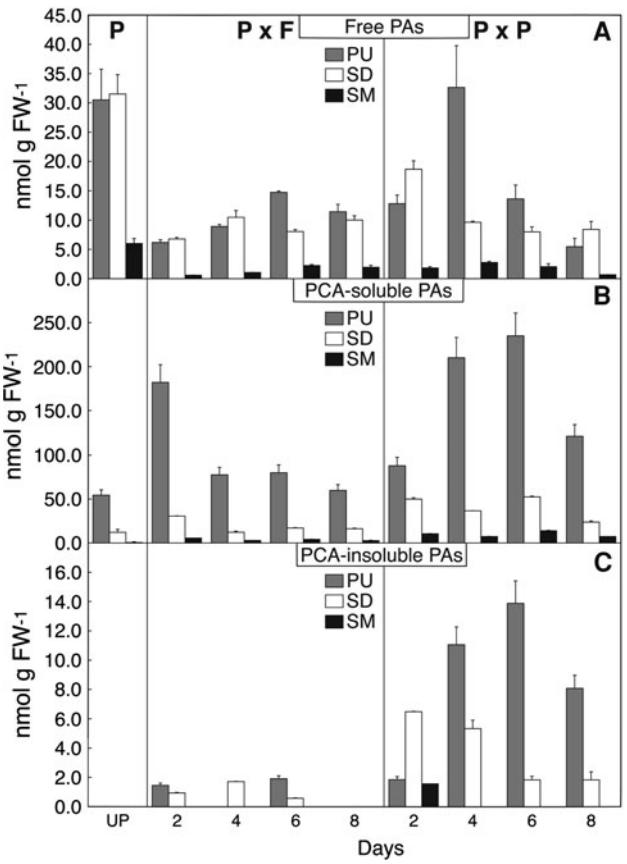


**Fig. 3** Polyamines (PAs) (PU, SD, SM) content in not pollinated (UP) and in pollinated pistils P × F and P × P of *Citrus grandis* at different days of pollination as reported in Fig. 2. Free PAs (**a**), PCA-soluble PAs (**b**), PCA-insoluble PAs (**c**) content. Values are the mean ($n = 3$) ± SD. The free PAs values in P × F and P × P samples were significantly different from the respective control in P except for PU at 4 days P × P, according to Student's *t* test at the 1% level. The PCA-soluble PAs values were not significantly different except for PU in P × F at 2 days and in P × P at 4, 6 and 8 days; SD values were significantly different only at 2 and 6 days in P × P. The PCA-insoluble PAs detectable in all pollinated samples were significantly different, as in P they were present only in traces

The covalent linkages of PU occurred via a $Ca^{2+}$-dependent TGase, as demonstrated by the elution profile of the P × P samples treated with EGTA during the TGase assay (Fig. 5a). By examining the entire pollination period, bis-glutamyl-PU was almost always prevalent and, whereas in P × F both glutamyl-derivatives gradually decreased during the progression of pollination, in the incompatible P × P both peaked at day 6 from pollination (Fig. 5B) confirming the data obtained by both TGase activity assays.

## Discussion

The present investigation shows that a correlation exists between pollination of pistils, TGase activity and PA

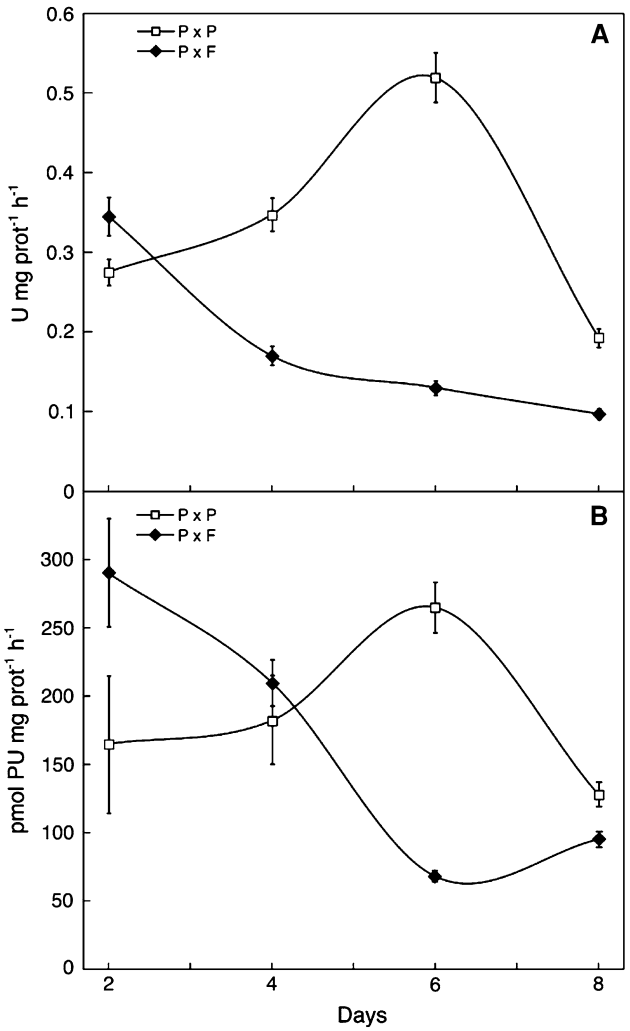


**Fig. 4** Transglutaminase activity in pollinated pistils P × F and P × P of *Citrus grandis* at different days of pollination. The activity has been analyzed by a microplate colorimetric assay (**a**) and by a radiolabeled polyamine incorporation assay in the presence of [$^3$H]-PU as tracer (**b**). Values are the mean ($n = 3$) ± SD

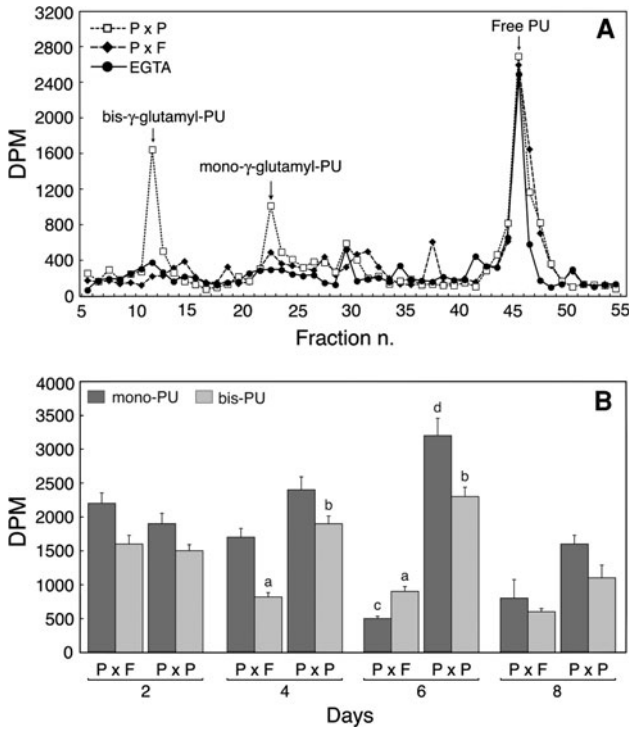


**Fig. 5** HPLC analysis of labeled mono- and bis-γ-glutamyl PU catalyzed by TGase of pollinated pistils P × F and P × P of *Citrus grandis*. **a** Elution profile of the products of TGase catalysis performed in the presence of $Ca^{2+}$ (P × P and P × F) or EGTA (P × P) and [$^3$H]-PU as tracer at day 6 from pollination. A marked amount of radioactivity eluted in the fractions corresponding to the retention time for free PU behaving as internal standard. **b** Incorporation of [$^3$H]-PU at different days of pollination. Data points represent means ± SD of three replicates. Samples indicated with *a* and *b* were significantly different each other; similarly for samples indicated with *c* and *d* according to Student's *t* test at the 1% level

content, whose patterns change during pollen germination according to the type of pollination, compatible P × F or SI P × P, the type of polyamine and their free/bound fraction considered.

Histological analysis shows that the behavior of *Citrus* pollen looks like a GSI type as the pollen tube growth is drastically reduced and arrested in the upper or middle style; practically none reached the base of the style as compared with compatible pollination in which a significant number of pollen tubes reached the ovary; moreover, SI caused an altered morphology in pollen tubes which look twisted with irregular callose deposition that is related to the interruption of the pollen tube growth along the style. The measurement of the pollen tube length clearly showed that in SI, already in the very early phases, the growth velocity is slower with respect to the compatible pollination and that at day 6 the growth is blocked. Thus this is a critical point in which pollen completed its PCD.

## Free polyamines

During pollination of the pistils with compatible pollen P × F, free PA content decreased with respect to that of UP pistils, whereas in SI P × P, after an initial decrease, an increase of free PAs takes place, with a maximum occurring at day 4 in concomitance with the slowing down of pollen tube growth. The decreasing trends of free PAs in compatible pollination P × F are in agreement with those obtained in *Pyrus communis* (Del Duca et al. 2010); this decrease could be due to PA degradation or linkage to other molecules as suggested also by the PAs concomitant marked increase of the PCA-soluble. During germination in vitro of *Malus domestica* (Bagni et al. 1981) and *Actinidia deliciosa* (Antognoni and Bagni 2008) pollen, a release of free PAs was observed in the external space (which in vivo is

represented by the ECM) thus it could be related to the pollen/pistil interaction.

The increase of free PAs in SI pollination P × P could be related to their effect as ROS scavengers (Ha et al. 1998). In *Citrus reticulata* SM pre-treatment caused accumulation of higher endogenous PAs and accordingly to more effective ROS scavenging (Shi et al. 2010; Velikova et al. 2000). Tip-localized ROS were needed to sustain the normal rate of pollen tube growth (Foreman et al. 2003; Potocký et al. 2007). ROS are an inevitable consequence of aerobic metabolism and in plant cells, there are many potential ROS sources. In cells without chloroplasts, such as pollen tubes, plasma membrane NADPH oxidases are also considered potential sources of ROS (Mittler 2002; Neill et al. 2002). In *Pyrus pyrifolia* SI a NADPH decrease blocked plasma membrane ROS formation, and finally resulted in disruption of ROS cell wall. S-RNase activity particularly lowered tip-localized ROS of incompatible pollen tubes through arresting the ROS formation in mitochondria or cell walls. ROS disruption induced arrest of $Ca^{2+}$ currents, depolymerization of actin cytoskeletons and degradation of nuclear DNA driving the pollen to PCD (Wang et al. 2010). Thomas et al. (2006) found that actin depolymerization was sufficient to induce PCD in the SI pollen of poppy (*Papaver rhoeas*).

## Bound polyamines

The SI-pollination caused an increase of PCA-soluble and -insoluble PAs, with respect to their content in cross-pollinated pistils, in particular of PU which peaked at day 6 when tube arrested its growth.

Among PCA-soluble PAs, which are the fraction quantitatively more relevant, either phenylpropanoid or low MW proteins conjugates catalyzed by TGase, are known. The phenylpropanoid PA conjugates have been reported in flowers of many plant species and their accumulation in reproductive organs of plants has been associated with fertility (Martin-Tanguy et al. 1982). Their presence in *Arabidopsis thaliana* has only been recently established in flower buds and pollen grains (Fellenberg et al. 2009; Grienenberger et al. 2009). The triferuloyl-SD is a component of the tryphine, a constituent of the pollen coat involved in pollination and in pollen-stigma interaction. Microscopic observations 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). Hydroxycinnamic acids 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 kiwifruit anthers at the stage of mature pollen (Biasi et al. 2001) and in *Quercus dentata* pollen (Bokern et al. 1995). In the light of PAs interaction with cell wall molecules, it is possible that PCA-soluble PAs influence pollen germination via a structural effect on the cell wall, so PAs could contribute in enhancing the strength of the cell wall and this could be important in SI pollen tube where apical plugs are formed.

The PCA-insoluble PAs derive from the binding of free PAs or PCA-soluble PAs to high molecular mass partners as i.e. diferulic acid-PAs to cell wall molecules as hemicelluloses and/or lignin (Antognoni and Bagni 2008). The SI-pollination causes an increase of PCA-insoluble PAs, with respect to their content in cross-pollinated pistils, in particular of SD and PU, the latter peaking at day 6 when tube arrest its growth similarly to PCA-soluble ones.

The bound PA contents have been analyzed also in the pollinated styles of *Pyrus* (compatible and SI), where an increase of PCA-insoluble PAs is also evident in SI, thus suggesting a possible role of bound PAs in the progamic phase of the fertilization process. However not all the present data are easily comparable with those reported for *Pyrus*, probably due not only to the different plant family but also as only the cutted styles of *Pyrus* were considered (Del Duca et al. 2010) instead of the entire pistil analyzed in *Citrus*.

Even though the pattern of total PA is a significant parameter to evaluate stress responses (abiotic and biotic), it does not provide sufficient information to be ascribed only to one specific event, as they are involved in several different cell physiological functions.

## Transglutaminase

Among the bound PA fractions there are those covalently conjugated to proteins by TGase. In *Citrus* pollination P × F the decreasing enzyme activity paralleled the PCA-soluble PU decreasing level. By contrast, in P × P pollination, in which callose plugs were formed at the tip of the tube (a typical feature occurring in SI) (Fig. 1e), and the tube does not reach the ovule arresting its growth in the first half of the style length, a progressive increase of TGase activity occurred and exhibited a significant peak at day 6 after pollination. A similar result has been observed in *Pyrus communis*, where in SI pollination, when tube arrested its growth (48 h after pollination), TGase activity was higher (about the double) in comparison to compatible pollination, high molecular mass and a new 50 kDa-band cross-linked products were formed, suggesting an involvement of TGase in pear SI response (Del Duca et al. 2010).

Both methods of TGase activity determination performed with *Citrus* pollinated pistils gave similar trends;

the microplate assay allows to check the transamidation activity by incorporation of the biotin-cadaverine having a unique primary amino group available into the exogenous substrate *N*′,*N*′-dimethylcasein. The radiometric TGase assay allows to detect the radioactivity incorporation of PU as mono- and bis-derivatives into TCA-precipitated endogenous proteins. Amine incorporation into dimethylcasein is still the main way of detecting TGase activity, but when screening crude plant extracts, the possibility of interference by enzymes such as diamine oxidases should be verified, as they are able to cause the incorporation of PAs into dimethylcasein in a $Ca^{2+}$-independent reaction via Schiff base formation (Siepaio and Meunier 1995). For these reasons, the glutamyl-PAs were identified at four different stages of pollination in P × P and P × F, as the isolation of these PA conjugates provides unequivocal proof of a catalytically active TGase (Folk et al. 1980). The trend of the amount of these compounds, whose largest formation occurs at day 6, was in accordance to that of TGase activity and of bound PAs (Figs. 3, 4) confirming that SI strongly affects PAs metabolism with a maximum when pollen tube growth is arrested.

In relationship to their role, mono-PU can confer to proteins additional positive charges, thus causing changes in their conformation. It is also conceivable that the free terminal primary amino group of mono-PU can interact non-covalently with several molecules, for example negatively charged ones, including hydroxycinnamic acids, to form complex aggregates. Bis-PU, always produced in larger amount, can form intermolecular cross-links; a complex high molecular mass net can thus be formed.

One of the roles of TGase in pollen tube growth has been studied in *Malus domestica* where PA-mediated post-translational modification of actin and tubulin occurs by a $Ca^{2+}$-dependent TGase activity (Del Duca et al. 1997). 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 in the presence of relatively high concentration of PU. Consequently TGase was able to control some properties of the pollen tube cytoskeleton (including the ability of actin and tubulin to self-assemble and their interaction with motor proteins) regulating 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 takes place and then F-actin foci are formed by a still uncharacterized cross-linking mechanism that blocks tube elongation, causing pollen PCD (Thomas and Franklin-Tong 2004).

In animal cells, it has been reported that following stress or insult, up-regulation of TGase often occurs leading to massive intracellular cross-linking, resulting in cell death if $Ca^{2+}$ homoeostasis is suddenly perturbed (Griffin et al. 2002). In plants, 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. The TGase protein cross-linking function, resulting in the formation of the $\varepsilon$-($\gamma$-glutamyl)-lysine isodipeptide bonds, was activated by $Ca^{2+}$ at mM concentrations, allowing to conclude that TGase could catalyze protein cross-linking reactions in high $Ca^{2+}$ environments (Lilley et al. 1998). The different TGase products as a function of $Ca^{2+}$ concentration could occur also in pollen tube where $Ca^{2+}$ is about 100-fold more concentrated in the apical tip when compared to the region behind the tip, the clear zone (Cheung and Wu 2008). If a high $Ca^{2+}$ influx also takes place in *Citrus,* as observed in incompatible *Papaver* pollen during SI response, this could explain the enhanced TGase activity observed in SI P × P with respect to cross-pollinated pistils P × F. In the former, TGase could cross-link cytoskeleton proteins generating aggregates (Del Duca et al. 1997, 2009) similarly to the actin aggregates observed in *Papaver* (Thomas et al. 2006).

In addition, there are evidences that TGase could also be involved in the building of the cell wall. A clear confirmation of TGase presence also in higher plant cell wall was obtained in isolated walls of *Nicotiana* petals (Della Mea et al. 2007b). Recently, it has been observed that TGase could be secreted from the apical tube of *Malus* pollen catalyzing its reaction in the extracellular space and along the cell wall where the enzyme and the products of its catalysis have been immuno-co-localised, possibly playing a role in strengthening the pollen cell wall (Di Sandro et al. 2010). In the cell wall of the alga *Chlamydomonas reinhardtii,* TGase activity has been shown to be involved in its organization. The addition of PU, cadaverine, SD and SM at high concentrations disrupts the normal process of wall assembly (Waffenschmidt et al. 1999), possibly by forming mono-PAs derivatives instead of bis-PAs which, when lower PA concentrations are assayed, could form bridges among proteins.

The SI response is caused by the interaction between pollen and pistil. The latter, when not pollinated, is rich in free PU and SD, either in *Citrus* and in *Pyrus*. Also ungerminated pollen is rich in free PAs, as shown in *Malus* and *Pyrus* (Bagni et al. 1981; Del Duca et al. 2010), which decreased at germination. It was shown that free PAs of *Malus* pollen were released and conjugated in the external medium, which in vivo is represented by ECM, that is the site of the cross-talk between pollen and pistil.

Their conjugation occurring at pollination either in *Pyrus* and *Citrus*, could be catalyzed, at least in part, by the active pollen TGase also released, as suggested by the

immunorecognition of the enzyme in in vivo pollination of *Malus*, where the cells of the style are poorly decorated by the TGase ID10 antibody, but clearly evident around the pollen tube (Di Sandro et al. 2010). At present it is difficult to shed light on the contribution of the single partner to the complex scenario of the PAs and TGase in SI response, where also the ECM, without doubt, plays a role.

Present results obtained by analysing the entire time course of pollination in *Rutaceae* mirrored the involvement of TGase in the response pathway of SI pollination reported for *Rosaceae* family (Del Duca et al. 2010). This suggests that the underlying molecular mechanism could be a general feature of SI response.

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