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Research article

A low molecular-weight cyclophilin localizes in different cell compartments of *Pyrus communis* pollen and is released *in vitro* under Ca^{2+} depletion

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ABSTRACT

Cyclophilins (CyPs) are ubiquitous proteins involved in a wide variety of processes including protein maturation and trafficking, receptor complex stabilization, apoptosis, receptor signaling, RNA processing, and spliceosome assembly. The ubiquitous presence is justified by their peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, catalyzing the rotation of X-Pro peptide bonds from a *cis* to a *trans* conformation, a critical rate-limiting step in protein folding, as over 90% of proteins contain trans prolyl imide bonds. In Arabidopsis 35 CyPs involved in plant development have been reported, showing different subcellular localizations and tissue- and stage-specific expression. In the present work, we focused on the localization of CyPs in pear (*Pyrus communis*) pollen, a model system for studies on pollen tube elongation and on pollen-pistil self-incompatibility response. Fluorescent, confocal and immuno-electron microscopy showed that this protein is present in the cytoplasm, organelles and cell wall, as confirmed by protein fractionation. Moreover, an 18-kDa CyP isoform was specifically released extracellularly when pear pollen was incubated with the Ca^{2+} chelator EGTA.

1. Introduction

Cyclophilins (CyPs) were first described as intracellular target proteins for the immunosuppressive drug cyclosporin. These proteins play an active role in protein folding insofar as they catalyze the isomerization of peptidyl-prolyl bonds from the *cis*-to the *trans*-conformation (PPIase activity). Consequently, they are included in the protein family of molecular chaperones (Schreiber, 1991). Multiple CyPs in plants have been reported to have different tissue and cellular locations and to be associated with a multitude of functions and regulatory pathways through their foldase, scaffolding, chaperoning or other unknown activities. Many functions of plant CyPs have been proposed, but their physiological relevance in pollen germination or stress responses is still largely unknown (Kumari et al., 2013).

Plant CyPs were first identified in 1990 with the isolation of CyP cDNA sequences from tomato (*Solanum lycopersicum*), maize (*Zea mays*) and oilseed rape (*Brassica napus*) (Gasser et al., 1990). Later, the presence of CyPs was also demonstrated in carrot, pumpkin, raspberry, periwinkle, and rye grass pollen (Cadot et al., 2000, 2006; Marzban et al., 2008; De Canio et al., 2009; de Olano et al., 2010). CyP isoforms are usually encoded by large gene families (*e.g.* 35 genes in the Arabidopsis genome, 28 in rice) and classified according to whether they have a single CyP domain or additional functional domains (Trivedi et al., 2012). Plant CyPs localize in distinct cellular compartments or organelles, such as the cytosol, mitochondria and chloroplasts and their expression is modulated by different abiotic stresses such as heat-, cold-, drought, and salt stress, suggesting a role of these proteins in stress responses (Kumari et al., 2013). The role of CyPs in abiotic stress tolerance is further supported by recent studies demonstrating that the ectopic expression of CyP genes enhance tolerance to multiple abiotic stress conditions (Kaur et al., 2016; Romano et al., 2004). Plant development also requires several and specific CyP isoforms, *e.g.* in Arabidopsis CyP40 is specifically required for vegetative growth, but not for reproductive maturation of the shoot (Berardini et al., 2001), while CyP5 is mainly involved in the coordination of cell polarity along the apical-basal embryo axis (Grebe et al., 2000).

Abbreviations: CyP, Cyclophilin; LC-ESI/MS-MS, Liquid chromatography electrospray ionization-tandem MS; MS, Mass spectrometry; PMF, Peptide mass fingerprinting; PPIase, Peptidyl-prolyl *cis-trans* isomerase; TEM, Transmission electron microscope

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Cadot and co-workers (Cadot et al., 2000) first showed the existence of CyPs in birch pollen and their extractability at alkaline pH, although it is not yet clear whether these CyPs are secreted from pollen grains or are pollen-coated proteins. In pollen, CyPs also localize in the cytosol, vegetative nuclei of grains, and generative cells, suggesting that CyPs are not exclusively pollen-coated proteins.

CyPs have been the subject of considerable scientific interest due to their high biochemical and clinical relevance. Being an IgE-binding protein, CyP is an allergen (Ghosh et al., 2014). CyP has a high homology with Bet v 7, one of the main birch pollen allergens (Cadot et al., 2000), Indeed, during the last decades CyP has been identified as a novel allergen also from olive, carrot, pumpkin, raspberry, periwinkle and rye grass pollen (Cadot et al., 2000, 2006; Fujita et al., 2001; San Segundo-Acosta et al., 2019; Marzban et al., 2008; de Olano et al., 2010; De Canio et al., 2009). Thus, CyP has been confirmed to be a pan-allergen and found to be cross-reactive across species, including humans (Fluckiger et al., 2002).

Numerous metabolic and cytosolic processes sustain the tip growth of pollen tubes (Cheung and Wu, 2008), among which the maintenance of a tip-focused cytosolic Ca^{2+} gradient, which is generally supported by an influx of Ca^{2+} through the apical plasma membrane (Steinhorst and Kudla, 2013). The tip-focused Ca^{2+} gradient is indispensable for pollen tube tip growth and orientation, as it regulates actin organization, protein kinase activities and exocytosis (Cardenas et al., 2008). In fact, the oscillatory increase of Ca^{2+} influx follows tube elongation but precedes the fusion of secretory vesicles (Coelho and Malho, 2006). While investigating the release of the extracellular Ca^{2+} signal transducer calmodulin (CaM) (Shang et al., 2005), Yokota and colleagues (Yokota et al., 2004) found that a 21-kDa CyP was specifically released into the extracellular medium when lily pollen was incubated in the presence of EGTA or at low concentrations of Ca^{2+}. However, the release mechanism of CyP from pollen grains and their role once released remains to be elucidated, even if an involvement in signal transduction during pollen tube growth in the style was hypothesized (Ghosh et al., 2014).

In spite of all this evidences regarding the role of CyPs in pollen tube growth, the distribution of CyPs in pollen remains to be elucidated. Therefore, in this work, we investigated for the first time, the localization of CyPs in pear pollen using several microscopy techniques and the different distribution of specific isoforms in grains and pollen tubes by chromatographic, electrophoretic, and immunological approaches.

2. Materials and methods

2.1. Plant material and pollen germination

Mature pollen of pear (*Pyrus communis* cv. Williams) was collected from plants grown in experimental plots at the University of Bologna (Department of Agricultural and Food Sciences). Pollen handling, storage, hydration and germination were performed as previously reported (Aloisi et al., 2015). After 1 h of germination, the medium was supplemented with different concentrations of EGTA (0.4 mM, 1 mM and 5 mM) up to 2 h. In control samples, pollen was allowed to germinate up to 2 h without any EGTA supplementation. For germination tests, pollen grains were gently washed with germination medium or HEMS buffer (25 mM Hepes pH 7.5, 3 mM EDTA, 2 mM $MgCl_2$, 15% sucrose) supplemented or not with EGTA and allowed to germinate in standard conditions for 2 h. Pollen was visualized under a light microscope (Nikon Eclipse E600) equipped with a digital camera (Nikon DXM1200). At least 100 pollen grains were counted to determine pollen tube length and percentage germination.

2.2. Fluorescence and confocal microscopy

Indirect immunofluorescence microscopy in pollen tubes was performed according to standard procedures (Cai et al., 2011). Briefly, germinated pollen samples were fixed with 3% paraformaldehyde in PM buffer (50 mM Pipes, pH 6.9, 1 mM EGTA, 0.5 mM $MgCl_2$) for 30 min, then cell wall digestion was performed with 1.5% cellulase. After two washes with PM buffer, samples were incubated with the primary antibodies. The anti-CyP CYP-18 antibody (Kielbowicz-Matuk et al., 2007) was used at 1:100 dilution, and incubated at 4 °C overnight. Following two washes with PM buffer, samples were incubated with a goat anti-rabbit Alexa Fluor 488 nm (Invitrogen) as secondary antibodies for 45 min in the dark at 37 °C. After two washes in PM buffer, samples were placed on slides and covered with a drop of Citifluor and observations were made using a Zeiss Axio Imager fluorescence microscope equipped with a 63x objective. Images were captured with an MRm AxioCam video camera using AxioVision software. Reconstruction of tube sections was done from Z-series images obtained with the Zeiss Apotome (0.5 μm between each Z image) using the LOCI Import filter and the Reslice command of ImageJ software (https://imagej.nih.gov/ij/). As a control, fixed pollen was directly incubated with a secondary antibody.

2.3. Immunogold electron microscopy

Immunogold labelling on pear pollen tubes was performed according to the protocol described in (Li et al., 1995). The anti-CyP antibody was used at the dilution of 1:300 in 50 mM Tris–HCl pH 7.6, 0.9% NaCl, 0.1% Tween-20, 0.2% BSA. The goat anti-rabbit secondary antibody, diluted 1:20 for 45 min at room temperature, was conjugated with 15 nm gold particles (BioCell). Images were captured with a transmission electron microscope (TEM) Philips Morgagni 268 D set at 80 kV and equipped with a MegaView II CCD Camera (Philips Electronics, Eindhoven, The Netherlands). Samples were incubated with 5% normal goat serum (Invitrogen) for 20 min at room temperature to prevent binding to unspecific sites. Sections were incubated with the primary antibody for 1 h and then washed (3–4 times) in 50 mM Tris–HCl pH 7.6, 0.9% NaCl, 0.1% Tween 20 for 30 min. After drying, samples were incubated with the gold-conjugated secondary antibody for 15 min at room temperature. After washing for 30 min as described above and for further 10 min with dH_2O (distilled H_2O), sections were counterstained with 2% uranyl acetate in H_2O for 10–20 min, carefully washed in dH_2O for 15 min and then counterstained with lead citrate for 5–10 min. Scaling was done using the scale bar generated by the microscope software (AnaliSYS). For each experimental condition, at least 50 pollen tubes and grains were analyzed.

2.4. Extraction of proteins from cytosol, membrane, and cell wall fractions

Sequential fractionation and isolation of subcellular proteins from germinated pollen was performed as described by Parrotta et al. (2016) with some modifications. Briefly, after hydration, pollen tubes were incubated for 90 min in growth medium, while treated samples were supplemented with 3 mM EGTA after 45 min of incubation. Pollen tubes from each sample (control and supplemented with EGTA), were collected by low speed centrifugation and washed with HM buffer (50 mM Hepes pH 7.5, 2 mM $MgCl_2$) containing 10% sucrose. Then, pollen was lysed in a cold room (4 °C) using a Potter-Elvehjem homogenizer (40 strokes); the lysis buffer was HM supplemented with 0.1% Triton. After centrifugation at 500*g* for 10 min (4 °C), the supernatant was removed and centrifuged at high speed (100,000 g for 45 min at 4 °C). The resulting pellet (Mem-Org fraction) was resuspended in 1 M Tris HCl pH 7.4; samples were then centrifuged at 15,000 g for 5 min in a microfuge and the supernatant was directly used. The supernatant from the high-speed centrifugation (cytosolic fraction) was then resuspended in suitable buffers for either 1-D or 2-D electrophoresis. The pellet from the initial low-speed centrifugation (cell wall fraction) was washed several times with HM buffer to remove contaminating proteins. The last pellet was resuspended in 1 M Tris HCl pH 7.4. Protein concentration was determined using a commercial kit (BCA Protein Assay, Thermo Fisher),

according to the manufacturer's instructions. Each sample was analyzed in three replicates.

2.5. 1-D and 2-D electrophoresis

Separation of proteins by 1-D electrophoresis was performed on 15% mini gels using a Mini-Protean cell (Bio-Rad) equipped with a Power Pac Bio-Rad 300 at 200 V for approximately 50 min. Forty μg of proteins were loaded per lane. Gels were stained with Bio-Safe Coomassie blue (Bio-Rad).

For 2-DE analysis, 11-cm IPG Strips with a 6–11 pH gradient (Bio-Rad) were used in combination with 18% Criterion XT gels (Bio-Rad). Strips were rehydrated in the solubilization buffer (40 mM Tris, 8 M urea, 2 M thiourea, 2% CHAPS, and traces of bromophenol blue) to which 18 mM DTT and 20 μL/mL IPG buffer were added. Samples were dissolved at 1 mg/mL in the solubilization buffer. Strips were rehydrated overnight in Immobiline Dry Strip Reswelling Tray (GE HealthCare) and covered with the Dry Strip Cover PlusOne (GE HealthCare). Strips were run using a Protean IEF Cell (Bio-Rad) through six different steps:

- From 0 to 500 V for 30 min.
- From 500 V to 1000 for 30 min.
- From 1000 to 8000 V for 3 h.
- 8000 V until a total of 15,000 Vhr (Volts h^{-1}).
- From 8000 to 500 V for 10 min.
- Hold step of 500 V until use of strips.

Strips were stored at −80 °C or used immediately. In both cases, they were equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8 containing 6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 10 mg/mL DTT). Proteins were then separated in the second dimension based on a Bis-Tris buffer system. Molecular weight standards of the Precision series (Bio-Rad) were run in parallel.

2.6. Western blotting and image analysis

Electroblotting of proteins to nitrocellulose membrane was performed using a Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer's instructions. Quality of blotting was determined by checking the transfer of precision pre-stained molecular standards (Bio-Rad). After blotting, membranes were blocked overnight at 4 °C in 5% Blocking-Grade Blocker (Bio-Rad) in TBS (20 mM Tris pH 7.5, 150 mM NaCl) plus 0.1% Tween-20. After washing with TBS, membranes were incubated with the primary antibody (anti CYP-18 diluted at 1:1000) for 1 h. A goat anti-rabbit IgG (Bio-Rad) diluted 1:3000 was used as secondary antibody. After rinsing the membranes with TBS, the immunological reactions were visualized with ImmunStar (Bio-Rad). Images of gels and blots were acquired using a Fluor-S apparatus (Bio-Rad) and analyzed with the Quantity One software (Bio-Rad). Exposure times were 30–60 s for blots and 5–7 s for Coomassie-stained gels.

All blots were developed using identical conditions, from substrate incubation to exposure time. All images were processed correspondingly using the Autoscale command (to improve the quality of gels and blots) and the Background Subtraction command (to remove the background noise). The relative intensity of single spots was calculated with the Volume tool of Quantity One software. Blots were performed in triplicate.

2.7. CyP purification and mass spectrometry identification

The supernatant obtained after washing with HEMS buffer containing 1 mM EGTA was collected for gel filtration chromatography using a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with 25 mM Tris-HCl pH 7.5, 1 mM PMSF, 1 mM DTT, 3 mM EDTA, and 1 mM PMSF, before loading the protein sample. Elution was performed at a constant flow rate of 0.5 mL/min; a UV detector at 280 nm was used to check fractions eluted from the column. Eluted fractions showing protein peaks were separated by SDS-PAGE (15% acrylamide) and/or blotted on nitrocellulose and stained by iodine-starch staining (Kumar et al., 1985).

Protein identification was performed as previously described (Hellman et al., 1995; Soskic et al., 1999). The electrophoretic lane of interest was manually excised and processed as reported elsewhere (Aloisi et al., 2016). Acquisition of mass spectra was performed using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, United States) in reflector positive mode. Spectra were analyzed by Flex Analysis software v. 3.0. Peptide mass fingerprinting (PMF) database searching was carried out in NCBInr and/or Swiss-Prot databases using Mascot (Matrix Science Ltd., London, UK, http://www.matrixscience.com) on-line available software. The search settings were as follows: mass tolerance was set at 100 ppm, trypsin as the digestion enzyme with one allowed missed cleavage and oxidation of methionine as a variable modification. In order to accept identifications, the number of matched peptides, the extent of sequence coverage, and the probabilistic score were considered. Peptide digests that did not give unambiguous identifications were subjected to peptide sequencing by tandem MS. MS/MS analysis was performed on the Ultraflex III MALDI-TOF/TOF instrument. Two to three PMF peaks showing a high intensity were CID (Collision Induced Dissociation) fragmented using Argon as collision gas, and MALDI-TOF/TOF tandem MS was performed in LIFT mode by software-controlled data acquisition. Fragmented ions were analyzed using the Flex Analysis software v. 3.0. The MS/MS database search was carried out in NCBInr and/or Swiss-Prot databases using the on-line MASCOT MS/MS ion search software. The following parameters were applied for the database search: trypsin specificity, one missed cleavage allowed, peptide precursor mass tolerance: ± 100 ppm, fragment mass tolerance ± 0.6 Da, peptide precursor charge state +1, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine as a possible modification. Protein identification was considered significant based on Mascot ion score, peptide coverage by “b” and “y” ions, and expected value.

Liquid chromatography electrospray ionization-tandem MS (LC-ESI/MS-MS) was performed with a Micro-HPLC Pump Phoenix 40 (Thermo Finnigan, San Jose, CA, USA) equipped with the LCQ DECA IT mass spectrometer (Thermo Finnigan). The TurboSEQUEST algorithm (Thermo Finnigan) analyzed spectra. Using the online MASCOT MS/MS ion search software, an MS/MS database search was carried out in the NCBI nr or Swiss-ProtKB databases. Only peptides with individual ion scores of less than 0.05 ($p < 0.05$) were considered significant.

2.8. Bioinformatic analysis

A bioinformatic annotation of CyP genes from pear was performed by comparing the FASTA sequence of CyP from *Phaseolus vulgaris*, present in NCBI databases, to the pear genome (Jung et al., 2019). We performed a tblastn (search translated nucleotide databases using a protein query) on *Pyrus Communis* cv. Bartlett DH Genome v2.0 transcripts. mRNA sequences of the corresponding genes were blasted on protein databases available online at NCBI (blastx; search protein databases using a translated nucleotide query), in order to confirm that the identified sequences correspond to CyPs. Alignments, target name, E-Value, % of identity and chromosomal coordinates for putative CyP genes are reported.

2.9. Statistical analysis

Pollen germination rate, pollen tube length and band intensity analysis were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/index.html). Differences between sample sets were determined by analysis of variance (one-way ANOVA, with a threshold *P*-value of 0.05) using R.

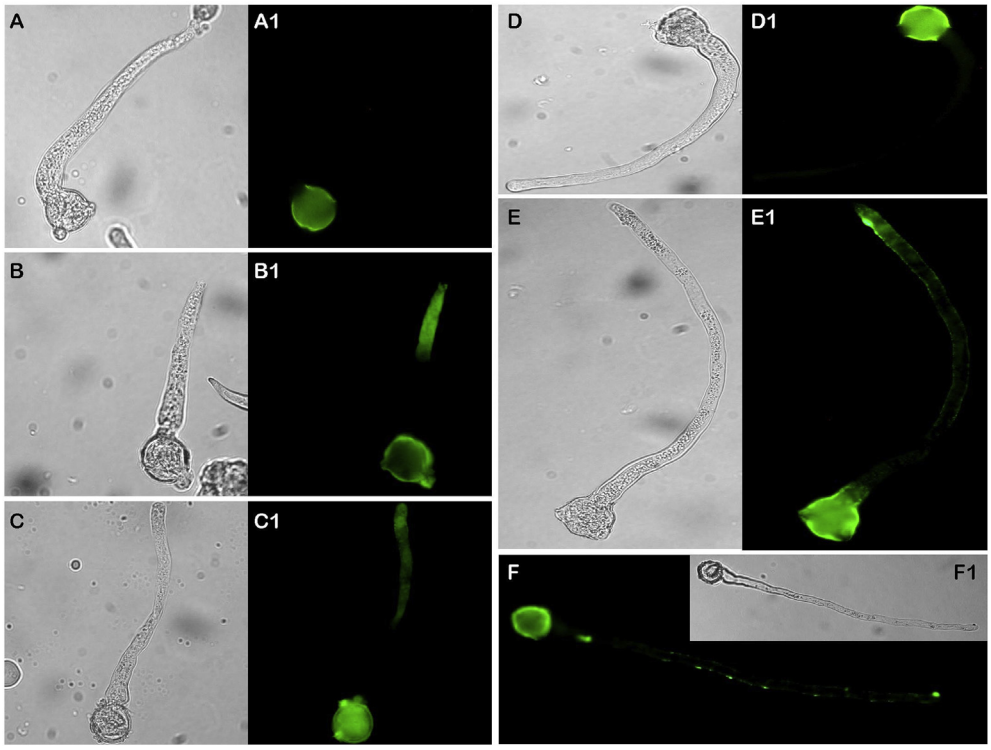

Fig. 1. CyP localizes in the cytoplasm and in the pollen tube cell wall. CyP immunolocalization by fluorescence microscopy of pollen tubes probed with CYP-18 antibody and a secondary Alexa Fluor 488 antibody, after cell wall digestion (**B1, C1**) and without cell wall digestion (**E1**, **F**). **B**, **C**, **E** and **F1** bright field images of the corresponding germinated pollen reported in the fluorescence images. Autofluorescence is visible in the pollen grain. Aspecific signal of primary antibody was not detected along the tube, either in pollen whose cell wall was digested (**A1**), nor in pollen whose cell wall was not digested (**D1**). **A** and **D**, control pollen, respectively with digested and not digested cell wall, in bright field.

3. Results

3.1. CyP localizes in several pollen compartments

In order to investigate the localization of CyP, several microscopy techniques were used. First, in order to discriminate whether pollen CyP localizes within the cytoplasm or in the cell wall, germinated pollen was treated or not with cell wall-degrading enzymes and CyP localization was performed by immunofluorescence. Immunolocalization of CyP in pollen treated with cell wall degrading enzymes showed a diffuse and uniform signal, mostly localized in the apical and subapical region of the pollen tube (Fig. 1B and 1C); when the pollen cell wall was not digested, cells showed a regular distribution of CyP along the cell wall, mostly with a dot-like appearance (Fig. 1E and F). Detection of CyP in pollen with both digested and undigested cell walls suggested that CyP localizes both in the cytoplasm and in the cell wall of pear pollen. No unspecific signal was detected, either in pollen with undigested cell wall nor in pollen with digested cell wall (Fig. 1A–D). In order to confirm the localization of CyP in the cell wall, confocal microscopy was performed in germinated pollen not treated with cell wall-degrading enzymes. A distribution of CyP all along the outer layer of the tube was observed (arrows in Fig. 2A). Along the pollen tube, the signal exhibited mainly a dot-like appearance and looked more intense in the apical region, confirming the results obtained by fluorescence microscopy (Fig. 2A–B). The distribution of CyP was also analyzed by a tube sections reconstruction of the cross sections in two different points of the pollen tube (indicated by dotted lines), which highlighted the distribution of CyP along the cell edge, suggesting its association with the cell wall (Fig. 2B, inserts). A similar distribution was found in pollen grains (Fig. 2C). The distribution of CyP was further analyzed by immunoelectron microscopy. In the apex/subapex region of pollen tubes, gold particles were found mostly in association with the plasma membrane and in the cell wall, but gold particles were also found in the cytosol, presumably associated to organelles or vesicles. The signal was evident in association with the cell wall at the tube apex (Fig. 2D, arrows). A similar distribution was found along the pollen tube. Again, CyPs were mainly associated with the plasma membrane, the cell wall and the cytosol, probably in association with organelles and vesicles (arrow in Fig. 2E). No unspecific signal was detected, neither in fluorescence acquisition nor in TEM analysis (Fig 1s).

The localization of CyPs in different cellular compartments was also analyzed by pollen sub-fractionation followed by western blotting. Sub-fractionation and isolation of subcellular proteins was performed in order to isolate proteins bound to the cell wall (**CW**), soluble cytosolic proteins (**CYT**) and transmembrane proteins with strong interactions to the intracellular membrane-bound organelles (**Mem-Org**). Results indicated that CyP accumulated in all compartments to different levels, as highlighted by western blot data (Fig. 3A), and confirmed after quantification of single band intensities. Analysis of band intensity indicated that EGTA increased CyP accumulation in this fraction (Fig. 3B). In the Mem-Org fraction as well as in the cytosol fraction, control and EGTA-treated samples did not show any statistically relevant differences (Fig. 3C and D). This result was made even more evident after a similar electrophoretic separation followed by coomassie blue staining Fig. 2s. CyP antibody recognized a single band with a molecular weight of *ca.* 18 kDa (Fig. 3E), while 2-D electrophoretic separation followed by immunodetection revealed two different spots in pollen tubes grown in standard condition (Fig. 3F); spots differed for the pI value (pH range 8.5/8.7), suggesting the presence of at least two protein isoforms in pear pollen.

3.2. Ca^{2+}-depletion induces the release of CyP from pear pollen

In order to understand the effect of EGTA on the release of CyP in pollen growth medium, the tube length of pollen grown in the presence of 0.4 mM, 1 mM and 5 mM EGTA was analyzed. The presence of EGTA, even at the lowest concentration, significantly reduced the growth rate of pollen tubes; no significant differences between tube growth in the presence of 1 mM and 5 mM of EGTA were observed (Fig. 4A). The presence of EGTA also affected germination, since washing pollen grains with HEMS buffer supplemented with EGTA decreased the germination rate from 80% (no EGTA) to 25%. The washing step also reduced the germination rate in the absence of EGTA, but to a

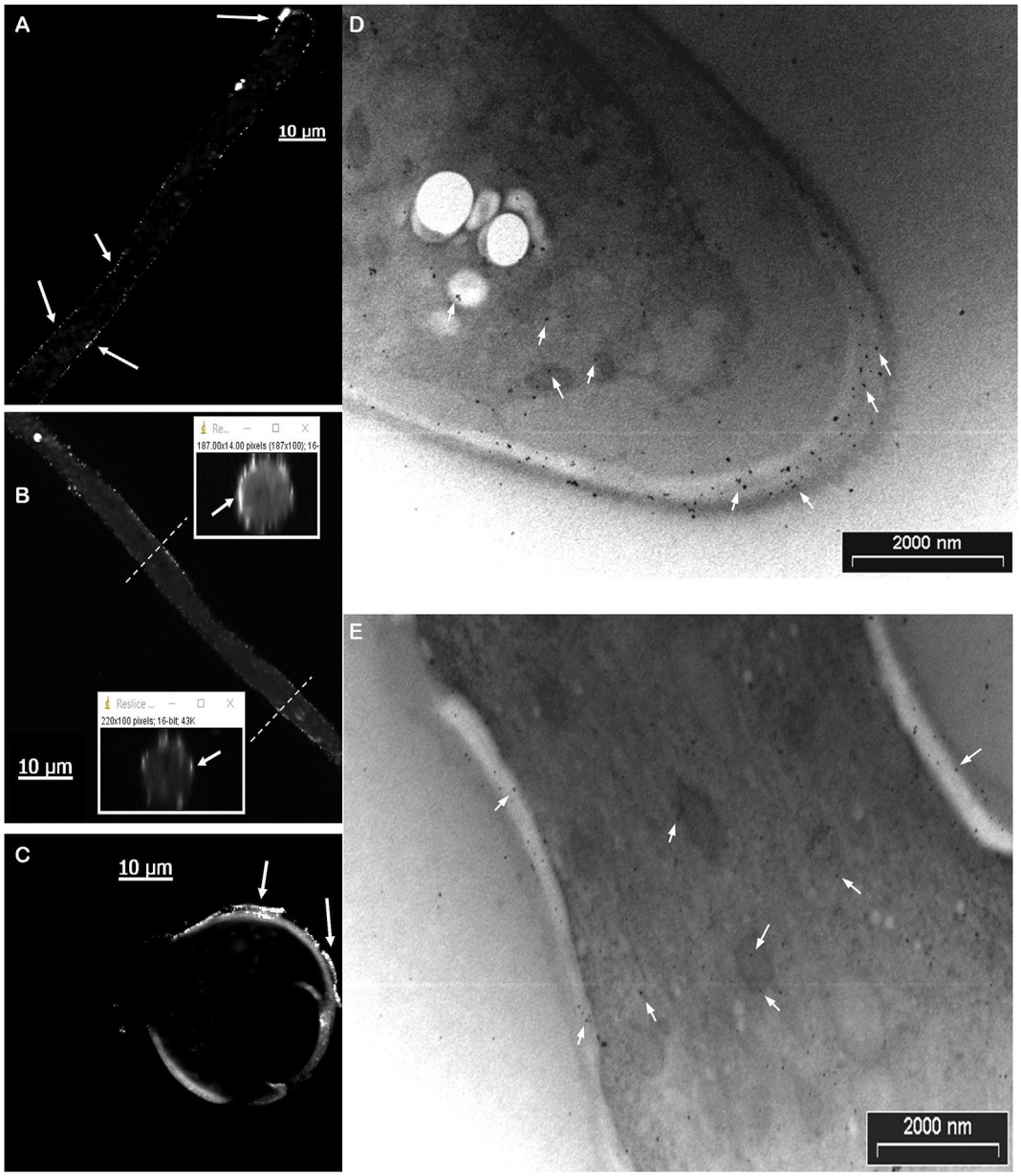

Fig. 2. Distribution of CyP within the pollen tube. Confocal microscopy of germinated pollen not treated with cell wall degrading enzymes (**A-C**). Arrows highlight distribution of CyP all along the outer layer of pollen tube (**A**). Cross sections (inserts) at two different points of the pollen tube (indicated by dotted lines) highlight the distribution of CyP in the cell edge (**B**). A similar distribution was found in pollen grains (**C**). CyP distribution detected by immunoelectron microscopy in the apex/subapex region of pollen tubes (**D**) and in the pollen tube shank (**E**). Gold particles were found mostly in association with the plasma membrane, in the cell wall and in the cytosol, presumably associated with organelles or vesicles (arrows).

significantly lesser extent (from 80% to 60%) (Fig. 4B). The distribution of CyP was analyzed by immunoelectron microscopy after treatment with EGTA showing that the density of gold particles was lower than in the controls (Fig. 4C). In particular, we found that gold particles were predominantly associated with the plasma membrane and the inner layer of the cell wall, and not with the cytoplasm (Fig. 4D).

The 2-D electrophoretic separation followed by immunodetection revealed two isoforms in pollen grains (WPG - Washed Pollen Grains) and tubes (WPT - Washed Pollen Tubes). After washing with HEMS buffer containing 1 mM EGTA, tubes (TPT - Total Pollen Tubes) and grains (TPG - Total pollen Grains) revealed only one spot with a pI value around 8.7 (Fig. 4E and its quantification 4F) suggesting the preferential release of only one CyP isoform in the germination medium.

To confirm the EGTA-induced release of CyP, proteins present in the supernatant obtained after washing with HEMS buffer containing 1 mM EGTA were processed for purification and then analyzed by MS. After gel filtration chromatography, SDS-PAGE separation, and transfer of proteins to nitrocellulose followed by iodine-starch staining, we found five fractions (fractions 33–37) showing mainly one band of *ca.* 18 kDa (Fig. 5). These five fractions were collected and electrophoretically separated, thus the 18 kDa band has been cut from the gel and analyzed by MS.

The 18-kDa protein was demonstrated to be a CyP by MALDI-TOF (Table 1) and confirmed by LC-MS/MS (Table 2 reports the comparison by Mascot software). This confirmed the release of one CyP isoform into the growing medium under Ca^{2+}-depletion conditions.

A bioinformatic analysis of the NCBI database showed that CyPs are not annotated for *Pyrus communis*. For this reason, we compared the FASTA sequence of *Phaseolus vulgaris* to the entire genome of *P.*

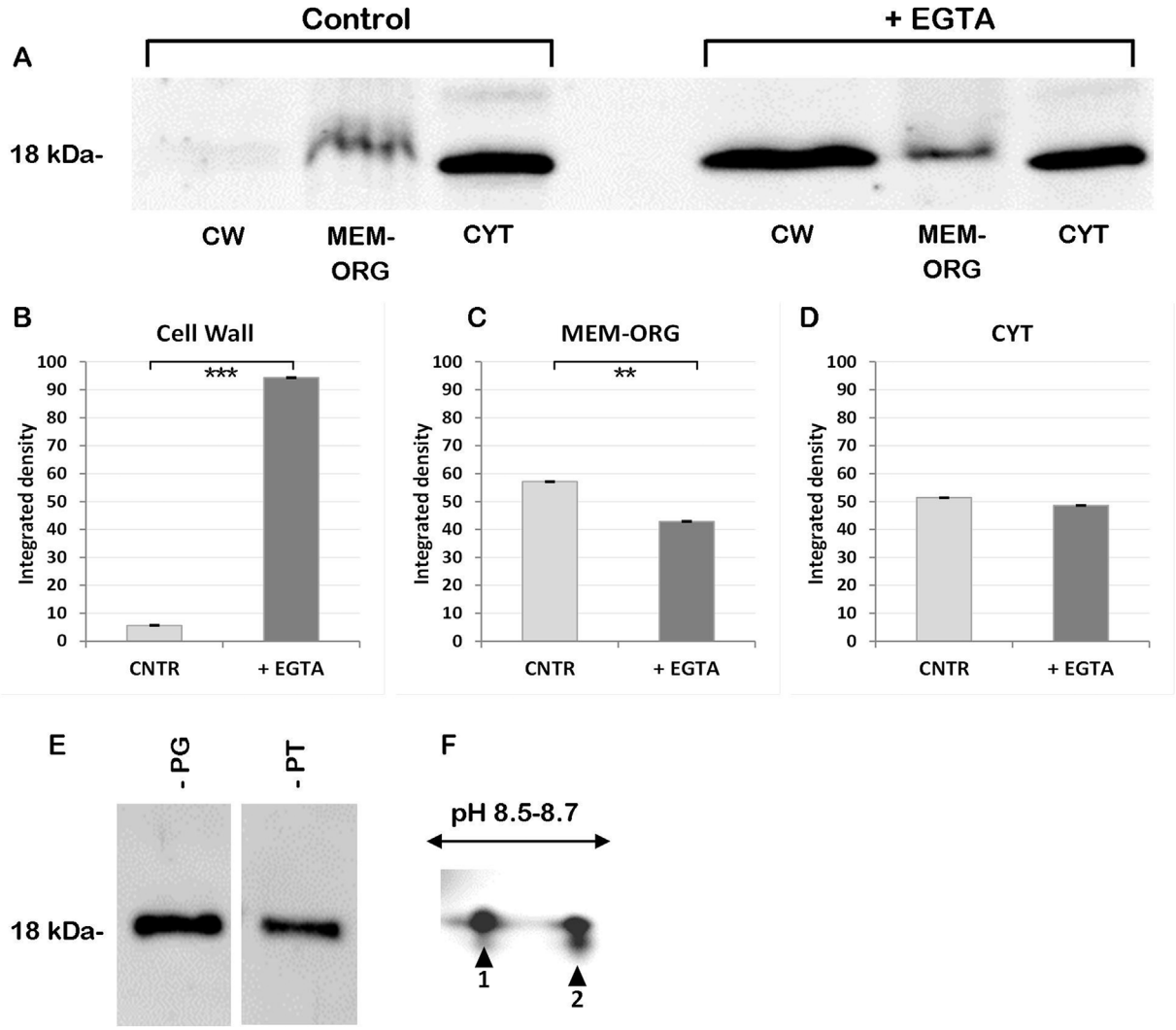

Fig. 3. Western blotting analysis of CyP confirms its localization in several cell compartments and the presence of two different isoforms. Sub-fractionation of pollen cell compartments followed by western blotting (**A**) and relative quantification (**B, C and D**). Three compartments (cell wall, cytosol and membrane-organelles) are shown by representative images. Values are mean ± SD of three replicates analyzed in duplicate. Means were compared by one way ANOVA. ** = $p \leq 0.01$;*** = $p \leq 0.001$. The cell wall compartments showed the marked significant differences between control and EGTA treated samples. Immunoblot analysis of CyP in pollen grains (PG) and pollen tubes (PT) (**E**). 2D electrophoresis followed by immunoblot analysis of proteins extracted from pollen tubes (**F**).

communis.

Results of Blast analysis highlighted the presence of 33 putative genes corresponding to CyPs (Table 3). Each sequence was analyzed, the corresponding mRNA sequence downloaded and reported on NCBI database to confirm the correspondence to CyPs already annotated in other plant species.

4. Discussion

Although the pear genome has been sequenced, to date there are no CyPs annotated in the NCBI database. A bioinformatic analysis based on the draft of the pear genome (Chagne et al., 2014), allowed the identification of 33 possible genes that correspond to CyPs in the NCBI database. The number of putative CyPs was similar in other plant species, such as Arabidopsis (35 genes) and in rice (28 genes), where CyPs constitute a family of highly conserved proteins encoded by multigenic families. The phylogenetic analysis of rice and Arabidopsis CyP family members indicate high sequence variation in the proteins (Trivedi et al., 2012), however CyPs can be classified according to whether they have a single CyP domain or they possess additional functional domains (Romano et al., 2004). In general low molecular-weight CyPs possess peptidyl-prolyl *cis-trans* isomerase activities thought to play an important role in protein folding and processing (Yokota et al., 2004), however a higher molecular weight CyP (*ca.* 40 kDa) in *Spinacia oleracea* was also possess a isomerase activity (Fulgosi et al., 1998).

In this work, we analyzed the localization of CyP in pollen grains and tubes of pear, first using fluorescent microscopy that indicated a dot-spot localization along the cell edge, probably in association with the plasma membrane or the cell wall; the signal appeared more intense in the apical region. These evidences were confirmed by subsequent analysis with immunogold electron microscopy. In this case, gold particles specifically localized in the cytosol, the plasma membrane and the cell wall. Distribution of CyP in different compartments was confirmed by protein differential extraction followed by immunoblot detection; those tests highlighted specific accumulation of CyP isoforms. CyPs localized mainly in the membrane/organelle fraction, followed by the cytosol compartment. CyPs were less abundant in the cell wall fraction. However, the increase in CyP accumulation in cell wall fraction after the addition of EGTA in the growth medium, indicated that CyPs were targeted towards the cell wall compartment in the presence of EGTA. These data are in agreement with the localization of CyPs in other plant

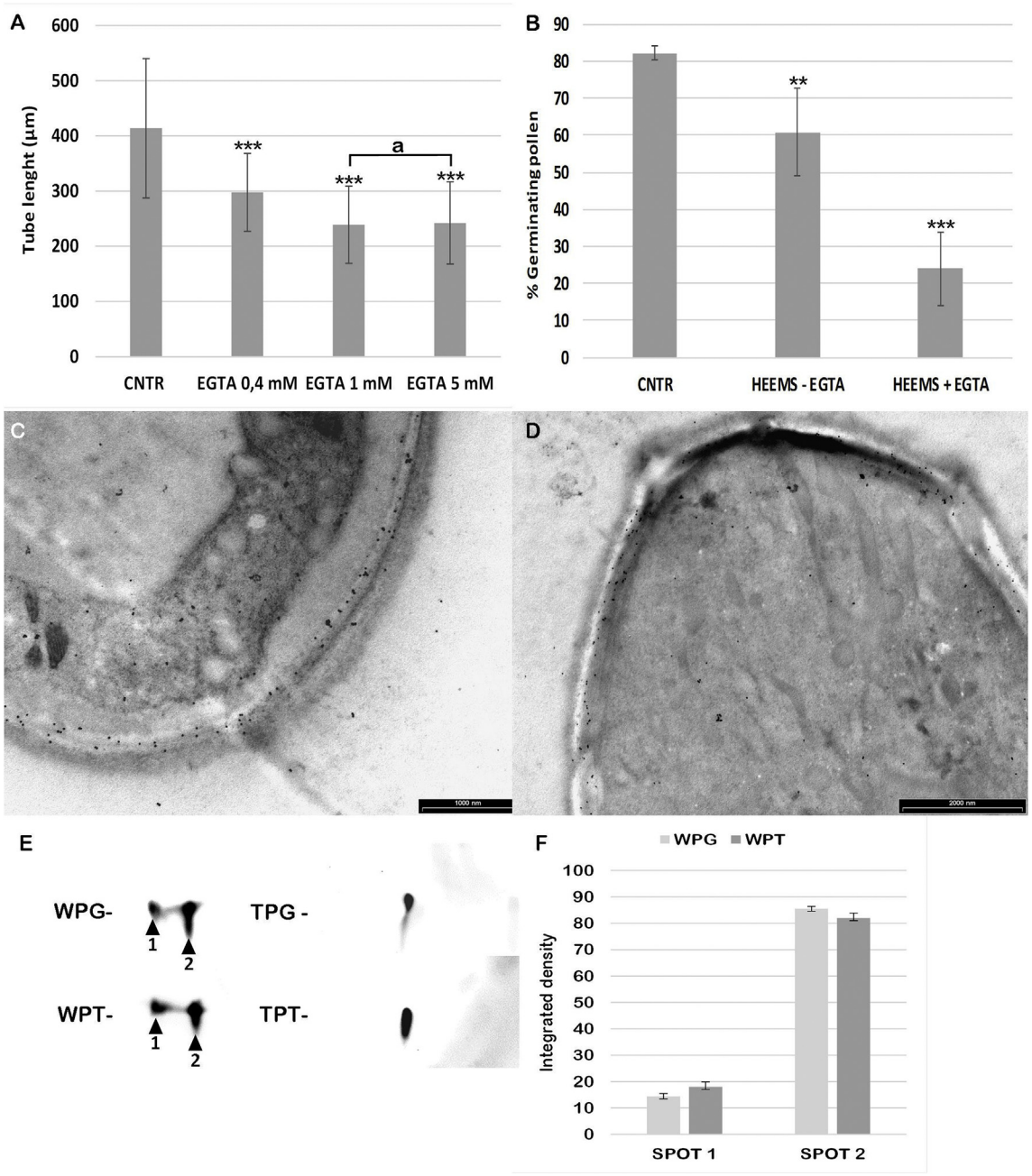

Fig. 4. EGTA inhibits pollen germination and pollen tube elongation and determines the release of CyP. EGTA (0.4 mM, 1 mM and 5 mM) arrested pollen tube elongation in a dose-dependent manner. The means of tube length derive from at least 100 measurements in three independent experiments. Means of treated and not treated (control) samples after 2 h germination by one way ANOVA. ** = $p \leq 0.01$;*** = $p \leq 0.001$. Bars indicate standard deviation. Samples marked with "a" are not significantly different (**A**). Analysis of germination of pollen after EGTA treatment. Pollen was gently washed with HEMS buffer supplemented or not with 3 mM EGTA and allowed to germinate in standard medium for 2 h. Means derive from at least 100 measurements and the experiment was repeated three times. Means of samples were compared with control sample after 2 h germination by one way ANOVA. ** = $P < 0.01$; *** = $P < 0.001$. Bars indicate standard deviation (**B**). Distribution of CyP by immunoelectron microscopy in control pollen (**C**) and after EGTA, showing a lower density of gold particles (**D**). 2-D electrophoretic separation followed by immunodetection. Two isoforms were detected in pollen grains (WPG - Washed Pollen Grains) and tubes (WPT - Washed Pollen Tubes), after EGTA washing one isoform was detected in tubes (TPT - Total Pollen Tubes) and grains (TPG - Total pollen Grains) (**E**). Relative quantitation of the two CyP spots of Fig. 4E. No significant differences were detected (**F**).

cells, where they are known to be targeted to different subcellular compartments such as the cytoplasm, nucleus, endoplasmic reticulum, mitochondria and chloroplasts (Kielbowicz-Matuk et al., 2007; Romano et al., 2005; Vasudevan et al., 2015).

CyP was localized in the cytosol and vegetative nuclei of pollen grains and generative cells, indicating that it is not a pollen-coated protein. It was suggested that CyPs released from rice pollen is involved in signal transduction during pollen tube growth (Dai et al., 2006). The release mechanism and the role of the pollen-released protein remain both to be elucidated. Moreover, it is not clear whether pollen contains several isoforms; the only evidence of several isoforms in pollen derive from studies in *Oryza sativa*, where three different CyPs were identified with a proteomics approach (Dai et al., 2006). In our study, we found at least two different isoforms in pear pollen after 2-D electrophoretic separation followed by immunoblot detection in control pollen tubes.

In the present study, we showed that the Ca^{2+} chelator EGTA causes

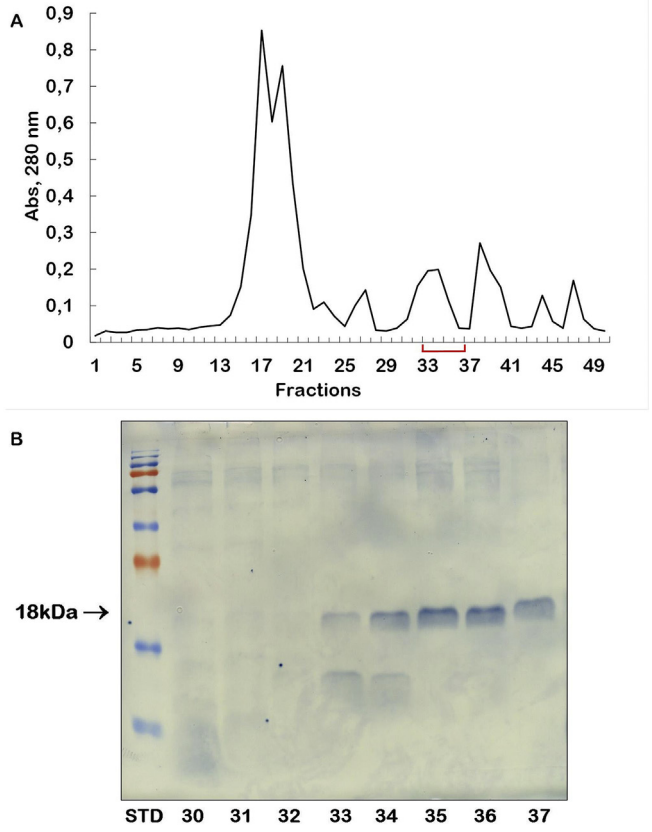

Fig. 5. Chromatographic separation of pollen proteins released after washing pollen grains with HEMS buffer containing EGTA.
Chromatogram of gel filtration chromatography using a Superdex 75 10/300 GL column. Fractions have been eluted with HEMS buffer supplemented with 1 mM EGTA and checked for absorbance (Abs) at 280
nm. Red bar indicate a peak of Abs at 280
nm (A); when these fractions were run on SDS-PAGE (15%) and transferred to nitrocellulose, after iodine starch staining, they appeared definitely enriched in one band with 18
kDa (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

a significant reduction of pear pollen tube length, even at low concentration (< 0.4 mM), while concentrations above 1 mM totally blocked tube growth, in agreement with earlier work (Picton and Steer, 1983) and with our recent data (Aloisi et al., 2017). Washing grains with EGTA also significantly reduced pollen germination rate. Apart from the cessation of tube elongation, no other morphological changes were detected. EGTA also induced protein release into the medium, among which a low molecular-weight CyP was abundant. This 18-kDa CyP was homologous to a CyP of *Phaseolus vulgaris* (identified with MALDI-TOF analysis). This finding was confirmed by subsequent analysis with LC-MS/MS spectrometry, which highlighted homology with CyPs from *Arabidopsis thaliana* and *Glycine max*. The role of Ca^{2+} and, in particular, of the tip-focused cytosolic Ca^{2+} gradient (supported by an influx of Ca^{2+} through the apical plasma membrane) is well known to be essential for the growth and orientation of pollen tubes (Franklin-Tong, 1999). Yokota and coworkers showed that a 21-kDa CyP was released from pollen grains into the extracellular medium *in vitro* at low concentrations of Ca^{2+}. This was confirmed for pollen of lily, *Nicotiana tabacum* and *Tradescantia,* while CyP release from pollen of the gymnosperm *Cryptomeria japonica* required alkaline pH besides Ca^{2+} depletion (Yokota et al., 2004). Several articles report a quick release of cytoplasmic proteins upon pollen hydration. This release can happen in the atmosphere after contact with air humidity, in aqueous media (during fertilization or in-vitro pollen rehydration) but also after the interaction of pollen with human mucosa (Morales et al., 2008; Aloisi et al., 2018; Zaidi et al., 2012). Among proteins released, also pollen allergens belonging to different protein families were found in different pollen (Alche et al., 2004; Grote et al., 1993; Vega-Maray et al., 2006; Vrtala et al., 1993) and CyPs are not an exception (Zaidi et al., 2012). For this reason, pollen allergy can be triggered not only by whole pollen grains, but also by sub-particles and fragments. Pollen CyPs have been speculated to serve a protective role during pollen dehydration and to be involved in signal transduction during pollen tube growth in the style once released in the extracellular medium (Dai et al., 2006). Moreover, plant CyPs belong to the group of stress-induced proteins. They are involved in responding to various environmental stimuli including wounding and exposure to chemicals (Zaidi et al., 2012), Ca^{2+} depletion induced by EGTA, could have favored CyP release in the extracellular medium. EGTA could, on the other hand, loosen the pollen tube cell wall thus favoring the release of cytoplasmic material from the pollen tube. In fact Ca^{2+} ions are needed to cross-link acid pectins at the subapex edge of the pollen tube (Rockel et al., 2008) where they bind Ca^{2+}, thereby contributing to strengthen the cell wall (Palin and Geitmann, 2012; Wolf and Greiner, 2012). If Ca^{2+} ions are not present, newly synthetized ductile methyl-esterified pectins are secreted at the tube apex, where they are chemically converted into acid pectins, but do not cross-link and form a stiff net.

This specific localization was apparently similar in control samples (pollen tubes grown in the absence of EGTA) and in EGTA-washed pollen tubes. EGTA caused a decrease of protein content mostly in the cytosolic compartment of the pollen tube; Ca^{2+}-depletion did not release cell wall-associated CyP. This evidence was confirmed by the presence of only one isoform in pollen after treatment with EGTA. Currently, we can only speculate that EGTA washing caused the release of only one isoform. We can hypothesize that the isoform indicated by "1" in Fig. 4E was released completely into the growth medium. This evidence confirms earlier data showing that lily pollen grown in an EGTA-containing medium releases CyP, mainly from the cytoplasm, while CyPs were not released from the vegetative nucleus and the generative cell (Yokota et al., 2004).

The cellular function of CyP remains essentially unknown, although a role as molecular chaperones or folding catalysts was suggested, especially under stress condition. The role of CyPs in abiotic stress tolerance is further supported by recent studies which demonstrate that the ectopic expression of CyP genes enhance tolerance to multiple abiotic stress conditions (Kaur et al., 2016; Romano et al., 2004). In plants, their presence has been reported in almost all organs studied (*e.g.* roots, leaves, stems, buds, and anthers) and have been hypothesized to be involved in intracellular signaling pathways by reacting with calcineurin, a calcium-binding protein (Cadot et al., 2000). However, only a few studies have focused on the presence of CyPs in pollen (Cadot et al., 2000). Because of the poor knowledge of the cellular function of CyPs, it is difficult to predict their role in pollen. CyPs may enhance the tolerance capacity of pollen against environmental stress

Table 1
EGTA-released proteins identified by MALDI-TOF MS.

Description	Accession number	Database	Peptide	Organism
CyP	gi|829119	NCBInr	HVVFGQVVEGLDVVK	*Phaseolus vulgaris*
CyP	gi|829119	NCBInr	VFFDMTIGGQPAGR	*Phaseolus vulgaris*

Table 2
EGTA-released proteins identified by LC-MS/MS.

Description	Accession number	Database	Peptide	Organism
Peptidyl-prolyl *cis-trans* isomerase CYP18-3 (CyP)	CP18C_ARATH	Swiss-Prot	K.HVVFGQVVEGLDVVK.A	*Arabidopsis thaliana*
Peptidyl-prolyl *cis-trans* isomerase CYP19-2 (CyP)	CP19B_ARATH			
Peptidyl-prolyl *cis-trans* isomerase 1 (Cyclophilin)	CYP1_SOYBN	Swiss-Prot	K.HVVFGQVIEGLNVVK.D	*Glycine max*
Peptidyl-prolyl *cis-trans* isomerase CYP18-3 (CyP)	CP18C_ARATH	Swiss-Prot	K.HVVFGQVVEGLDVVK.A	*Arabidopsis thaliana*
Peptidyl-prolyl *cis-trans* isomerase CYP19-2 (CyP)	CP19B_ARATH			

Table 3
Putative CyPs identified in *Pyrus communis* cv. Bartlett based on DH Genome v2.0 transcripts.

N°	Target Name	E-Value	Identity	Chromosome alignment
1	pycom05g11090	4.69409E-95	156/172 (90.7%)	Chr5:14565526..14566044 +
2	pycom10g10310	1.83384E-88	150/172 (87.21%)	Chr10:13611493..13612254 +
3	pycom12g22510	3.87554E-72	130/171 (76.02%)	Chr12:23381723..23382987 +
4	pycom04g20270	8.96782E-68	121/155 (78.06%)	Chr12:23381723..23382987 +
5	pycom11g06130	5.04152E-64	117/170 (68.82%)	Chr11:4728747..4731377 +
6	pycom03g05580	4.91214E-62	114/170 (67.06%)	Chr3:4421561..4423606 +
7	pycom01g17040	1.55409E-59	111/170 (65.29%)	Chr1:16437708..16439668-
8	pycom16g10580	4.07188E-54	112/171 (65.5%)	Chr16:7251810..7254139-
9	pycom15g24270	1.40401E-53	104/174 (59.77%)	Chr15:18478393..18479270 +
10	pycom17g28180	2.44746E-52	102/168 (60.71%)	Chr17:26204064..26206132 +
11	pycom02g13470	3.78116E-52	103/174 (59.2%)	Chr2:10000668..10003665 +
12	pycom05g06840	3.94678E-52	104/174 (59.77%)	Chr5:9651006..9654480 +
13	pycom07g03370	3.25961E-51	101/171 (59.06%)	Chr7:2786574..2789287 +
14	pycom02g23890	7.04685E-51	103/175 (58.86%)	Chr2:21983699..21986377 +
15	pycom13g10750	2.79206E-50	105/159 (66.04%)	Chr13:7241405..7243784-
16	pycom07g20020	7.02564E-50	103/175 (58.86%)	Chr7:22220925..22222910-
17	pycom03g20370	4.1466E-48	100/141 (70.92%)	Chr3:20862051..20866253-
18	pycom10g10540	1.85793E-45	94/171 (54.97%)	Chr10:13910258..13913098 +
19	pycom15g10880	1.05727E-44	89/169 (52.66%)	Chr15:7309999..7314262 +
20	pycom05g11340	4.05245E-44	93/171 (54.39%)	Chr5:14882526..14885427 +
21	pycom08g12230	5.74429E-44	87/173 (50.29%)	Chr8:10534103..10540454 +
22	pycom11g24550	1.39269E-29	60/90 (66.67%)	Chr11:27467528..27470983-
23	pycom02g16470	1.01918E-24	69/143 (48.25%)	Chr2:13622162..13623645 +
24	pycom14g02830	3.64173E-24	68/148 (45.95%)	Chr14:2163415..2166024-
25	pycom12g02890	5.94354E-23	72/161 (44.72%)	Chr12:2613417..2615911-
26	pycom02g15080	6.48339E-21	64/132 (48.48%)	Chr2:11798030..11802120 +
27	pycom15g26020	1.19076E-20	64/132 (48.48%)	Chr15:20657508..20661646 +
28	pycom11g21670	1.42082E-15	61/111 (54.95%)	Chr11:24744737..24745960 +
29	pycom03g09380	2.00947E-14	35/56 (62.5%)	Chr3:7892245..7892819 +
30	pycom15g05220	1.55074E-13	53/150 (35.33%)	Chr15:3121075..3122688-
31	pycom07g05380	3.18915E-13	46/67 (68.66%)	Chr7:4652891..4653133-
32	pycom07g05330	3.18915E-13	46/67 (68.66%)	Chr7:4612297..4612539-
33	pycom05g20510	5.25035E-10	49/140 (35%)	Chr5:23291697..23297603 +

because their synthesis in maize and bean has been shown to be up-regulated in response to some selective stress conditions, such as heat, chemical exposure, and infection by pathogens (Marivet et al., 1994).

Author contributions

L.P., I.A., C.S., C.F. and L.B. performed the investigation and methodological analysis; L.P. and I.A. wrote the original draft; C.S. G.C. A.KM. and S.D.D. design the research, reviewed and edited the text. All authors read and approved the manuscript.

Declaration of competing interest

All authors of the manuscript declare that they have no potential sources or conflict/financial interest. The research involves neither human participants nor animals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.045.

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