Identification of the ligand of Pru p 3, a peach LTP

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Abstract

Key message Pru p 3, a peach LTP, is located in pollinated flower styles and secreting downy hairs, transporting a derivative of camptothecin bound to phytosphingosine. Pru p 3 may inhibit a second pollination and may keep away herbivores until seed maturation.

Abstract The allergen Pru p 3, a peach lipid transfer protein, has been well studied. However, its physiological function remains to be elucidated. Our results showed that Pru p 3 usually carries a lipid ligand that play an essential role in its function in plants. Using ESI-qToF, we observed that the ligand was a derivative of camptothecin binding to phytosphingosine, which is inserted into the hydrophobic tunnel of the protein. In addition, the described ligand displayed topoisomerase I activity inhibition and self-fluorescence, both recognized as camptothecin properties. During flower development, the highest expression of Pru p 3 was detected in the styles of pollinated flowers, in contrast to its non-expression in unpollinated pistils, where expression decreased after anthesis. During ripening, the

expression of Pru p 3 were observed mainly in peel but not in pulp. In this sense, Pru p 3 protein was also localized in trichomes covering the fruit epidermis.

Introduction

Plant non-specific lipid transfer proteins (nsLTPs) are basic proteins with a molecular weight of about 9 kDa (92–94 residues) and amino acid sequence identities ranging from 25 to 95% (Kader 1996; Salcedo et al. 2007; Thoma et al. 1993). Despite the considerable heterogeneity in their primary structure, all LTPs share a compact α -helical fold composed of four α -helices connected by short loops and a non-structured C-terminal tail. This fold is stabilized by eight conserved cysteine residues that form four disulfide bonds (Pacios et al. 2012; Salcedo et al. 2007; Salminen et al. 2016). A characteristic tunnel-like cavity aligned with the long axis of the protein is formed by the space left within the helices. Hydrophobic side chains coat the inner surface of this cavity, which is large enough to accommodate ligands with long chains (Hamilton 2004; Sossountzov et al. 1991; Zachowski et al. 1998). These properties point to a potential role for LTPs in membrane biogenesis, and some members are thought to have a role in defence against pathogens (Molina and Garcia-Olmedo 1993), transport of cutin monomers, and fruit and flower development (Chae et al. 2010; Salcedo et al. 2007; Salminen et al. 2016). The ability of plant nsLTPs to bind a wide variety of lipids enables them to perform various functions (Pacios et al. 2012).

However, given that data on the association between compounds transported by LTPs and their specific functions are scarce in the literature, we expected that time- and tissue-specific expression of LTP genes would shed light on their physiological role in relation to the molecules they carry. Two LTPs, LTP1 (AY792996.1) and LTP2 (AY093699), are expressed in peach (*Prunus persica*) (Bonghi et al. 2011; Botton et al. 2009, 2002). Although LTP1 (Pru p 3 [IPR000528]) is expressed in pollinated flowers, it is poorly expressed in the ovary, and its expression is independent of pollination. LTP2 was highly expressed only in the ovary. By contrast, in fruit, only LTP1 mRNA was detected in the peel (Borges et al. 2006; Botton et al. 2009). Nevertheless, little is known about the physiological role of these LTPs and the specific molecules that they harbour and transport.

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing molecules found in about 20% of plant species. Many of the 16,000 alkaloids, for which structures have been described, participate in the defence of plants against herbivores, microbes, viruses, and competing plants (Wink 2003). Camptothecin is a cytotoxic quinoline alkaloid that was first isolated from the bark and stem of *Camptotheca acuminata* (Happy tree), a tree that is native to China and is used in traditional Chinese medicine. The biosynthesis of this alkaloid in almost all tissues has been described in many species (Lorence and Nessler 2004). Camptothecin is synthesized through a modified terpenoid indole alkaloid pathway in which there is a key enzyme: strictosidine synthase (STR), which is involved in the final step in the formation of the strictosidine backbone (Lorence and Nessler 2004). Gene expression involved in STR synthesis was found in leaves (more active in the young one), flowers (specially in petioles) and fruit and in most plant tissues (Isah and Mujib 2015; Sun et al. 2011; Wen-Zhe 2004; Zhao et al. 2010). It is produced in pre-vacuol compartments before being secreted through glandular trichomes. This mechanism has also been described for other toxic alkaloids such as those in *Cannabis sativa* (Sirikantararnas et al. 2005) and peppermint (Dudareva et al. 2004). Although not yet described in peach, the biosynthesis pathway of camptothecin was predicted from the peach genome (<http://pathways.rosaceae.org>), even though evidence for expression of the final steps of the pathway was lacking.

In this manuscript, we have characterized the ligand of Pru p 3 as a derivative of camptothecin bound to a long hydrophobic tail identified as phytosphingosine. By monitoring the expression of the *Pru p 3* gene and genes of key enzymes in the biosynthesis pathway of both camptothecin and phytosphingosine at different stages of flower and fruit development, we found that the ligand of Pru p 3 may play a role in those processes. We propose that the Pru p 3–ligand complex could help prevent double pollination

and defend against herbivores until the embryo (seed) has fully matured.

Materials and methods

Isolation of Pru p 3

Pru p 3 was purified as previously described (Diaz-Perales et al. 2002). Briefly, peach peel was separated by cation exchange chromatography on a SepPakR AccellPlus CM cartridge (Waters Corp, Milford, MA, USA) with 20 mM formic acid, pH 4.0. The cationic retained fraction was then eluted with 0.75 mM NaCl in the same buffer (1 mL/min). This fraction was further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Nucleosil 300-C4 column (7×250 mm; particle size 5 µm; Tecknokroma, Barcelona, Spain) to obtain the purified HPLC fraction. The fraction was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% for 5 min and 10–100% over 150 min; 1 mL/min).

Protein fractions were quantified using the bicinchoninic acid assay (Pierce, Cheshire, UK), and purity was measured using SDS-PAGE. Replicates were electrotransferred to PVDF membranes, and incubated with Pru p 3 polyclonal antibodies after blocking. The binding was revealed by HRP-chemiluminescence.

N-terminal amino acid sequencing was performed with an Applied Biosystems 477A gas-phase sequencer (Applied Biosystems), and mass spectrophotometric analysis was performed with a Biflex III Spectrometer (Bruker–Franzen Analytik, Bremen, Germany). Fingerprinting analysis was performed using standard methods after trypsin digestion.

Circular dichroism analysis

The samples (cationic retained and HPLC purified fraction) were desalted using MicroSpin G25 Columns (GE Healthcare, United Kingdom). Circular dichroism measurements were performed in water on a J-810 spectropolarimeter (Jasco, Canada) using 1-mm path-length quartz cells equilibrated at 20 °C. Spectra were recorded from 190 to 260 nm with 0.5 nm resolution at a scan speed of 50 nm/min. Final spectra were baseline-corrected by subtracting the corresponding solvent spectra obtained under identical conditions. Data were fitted using the DichroWeb analytical tool. Results were expressed as the mean residue ellipticity (Θ) for a given wavelength.

Isolation of the ligand of Pru p 3

The ligand was separated from the retained cationic fraction by size-exclusion LH-20 chromatography using

methanol:water (70:30) as the mobile phase. Fractions were checked using thin layer chromatography on silica gel-coated plates (Merck, USA) and developed with methanol:water (70:30 v/v) in a saturated chromatography chamber for about 20 min. The plates were visualized under UV light (254 nm) and stained with vanillin (1% in ethanol) and dichlorofluorescein (0.1% in ethanol-NaOH 2.5 mM) (Sigma-Aldrich, Germany). Fluorescent fractions were pooled and re-purified by HPLC as previously described (Kulkarni et al. 2010), with slight modifications. Isocratic analytical HPLC was performed using an RP-C18 column (Nucleosil 120 C18, 5 μ m, 250 $\times$ 4 mm, Scharlab, Spain). The mobile phase for alkaloid elution was acetonitrile:water (85:15) at a flow rate 0.5 mL/min for 150 μ L of sample. Sample peaks were detected using UV light at 254 nm. All reagents were HPLC grade.

Pru p 3-ligand-binding assays

To study the binding affinity of Pru p 3 for its ligand, increasing amounts of the ligand (0.0001–1 μ g) were dotted onto a nitrocellulose membrane. After blocking, the membrane was incubated with a solution of Pru p 3 (1 μ g/ μ L), and binding was revealed by immunodetection using specific antibodies. After extensive washing, dot intensity was calculated using a Bio-Rad Pharos FXTM Plus Molecular Image. Binding affinity was expressed as the dissociation constant $K_d = [\text{Pru p 3}][\text{Ligand}]/[\text{Complex}]$, where [Complex] was measured as the Pru p 3-bound form using a pattern curve with known amounts of the protein. All tests were performed in three independent assays.

Mass spectrometry analysis of the ligand of Pru p 3

The ligand of Pru p 3 was analysed using the HPLC system. Samples were separated by HPLC (Nucleosil 120 C18, 5 μ m, 250 $\times$ 4 mm, Tecknokroma) with an acetonitrile gradient of 10–85% for 30 min at 0.5 mL/min. Spectra were recorded at 254 and 280 nm. Commercial camptothecin (Sigma, SL, USA), OH-camptothecin (Santa Cruz, Te, USA) and quercetin (Sigma, SL, USA) were used as standards.

Ionic fragmentation of the ligand of Pru p 3 was performed by diluting the ligand in acetonitrile containing 0.1% formic acid (v/v), and the solution was applied directly to the MALDI target. The analysis was performed in a microTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an ESI source in positive ion mode. Typical instrument settings were as follows: capillary voltage, 4500 V; capillary exit, 130 V; dry gas temperature, 180 $^{\circ}$ C; dry gas flow, 4 L/min. Spectra were obtained in positive ion mode. The mass spectrometer was used to carry out two scans: a full-mass scan between 50

and 1000 m/z at a repetition rate of 2 Hz, and an MS–MS scan of the most abundant ions in the full-mass scan. Argon was used as the collision gas. Collision energy was ramped at between 5 and 25 eV. Mass calibration was performed using sodium formate clusters (10-mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid).

Inhibition assays of topoisomerase I activity

Since camptothecin is known to inhibit topoisomerase I, this activity was examined in the ligand of Pru p 3 using camptothecin assays, as reported elsewhere (Wright et al. 2015). We incubated 200 ng of plasmid pBSK with 1 U of human topoisomerase I (Inspiralis, UK) and increasing amounts of the ligand of Pru p 3 after heating (5 min 90 $^{\circ}$ C) and (S)-(+)-camptothecin (Sigma-Aldrich, Germany) for 30 min at 37 $^{\circ}$ C. The reaction was run on a 0.8% agarose gel at 5–10 V/cm. The gel was then stained with ethidium bromide for 30 min, washed briefly with water, and photographed in a UV transilluminator.

Modelling of structures of the ligand and the Pru p 3-ligand complex

The initial geometry of camptothecin was taken from the X-ray structure of its complex with human DNA topoisomerase I [PDB code 1T8I; (Wright et al. 2015)]. The initial geometry with the proper (2S, 3S, 4R) configuration of phytosphingosine was firstly built up with modelling software Chimera 1.10.2 (Pettersen et al. 2004). The ligand model was constructed by binding the two molecules through an amide moiety formed by lactone carbonyl from camptothecin and amine nitrogen from phytosphingosine. Hydroxyl was added to camptothecin C10, and the whole geometry was optimized. Hydrogens were then added and several geometry minimization cycles were run. This modelling was also performed with Chimera 1.10.2 (Pettersen et al. 2004).

Phytosphingosine alone was docked in the hydrophobic cavity of the crystal structure of Pru p 3 PDB code 2ALG; (Pasquato et al. 2006) by means of AutoDock Vina calculations (Trott and Olson 2010). Three docking results with the lowest affinity energies were used to prepare three initial geometries of the Pru p 3-ligand complex that then underwent 10-ns molecular dynamics (MD) simulations in water to find optimized structures. These calculations were performed with NAMD 1.10 (Phillips et al. 2005) according to a recently reported procedure (Garrido-Arandia et al. 2014). Force field parameters for the modelled ligand were assigned using SwissParam (ZoetteV et al. 2011). One of the three structures showed the best results for stability of ligand atoms (lowest RMSD), protein-ligand interaction

energy, and total energy of the complex upon completion of the three MD simulations. This optimum structure was the final model for the Pru p 3-ligand complex (see below).

Plant material

Tissues from the Super Crimson Gold peach cultivar were used to study gene expression during flower development and fruit set. Pollinated and unpollinated flowers at different time points (−2, −1, 0, 1, 7, 14, 21, 26, 28, 35, 51, 58, 65, 73, 80, 87, 94, 101 and 105 days post-anthesis [dpa]) from anthesis to mature fruit. The anthers, stigma-style, ovary, and pedicel from the flower and seed and peel from the fruit were excised separately, frozen immediately in liquid nitrogen, and stored at −80°C for RNA study.

Pollen was recovered from the same flowers by shaking and stored immediately at −80°C. For testing pollen germination, grains were incubated in 15% glucose in darkness. Pru p 3 (1 µg/mL) and the Pru p 3-ligand complex (0.1 µg/mL) were added to the solution directly.

Real-time PCR in flower and fruit tissues

Total RNA was extracted from the tissues following the method published (Tordesillas et al. 2013). Gel electrophoresis (1% agarose) and spectrophotometry (260/280 nm) were used to determine RNA quality and concentration. The first-strand cDNA was synthesized from 1 µg of DNA-free total RNA treated with DNase I (Roche, Basle, Switzerland) using random primers and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA).

Relative real-time PCR quantification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. Primers 18S-F and 18S-R (5'-GGGCATTCGTAT TTCATAGTCAGAG-3' and 5'-GGTTGAGACTAGGAC GGTA TCTGA-3') were used to amplify a 146-bp fragment of the endogenous control 18S from *Prunus persica* (GenBank Accession No. L28749).

Primers Pru p 3-F and Pru p 3-R (5'-TGTCATGG TTGTGAGCGTG-3' and 5'-GTTGTTGACGTTCTAAT GCCG-3') were designed to amplify a 143-bp fragment of Pru p 3 gene from *P. persica* (GenBank Accession No. AY792996).

The thermal cycling conditions were as follows: 10 min at 95°C, 15 s at 95°C, and 45 s at 60°C (40 cycles). The optimal cDNA and primer concentrations were determined by serial dilution experiments. Finally, 10 ng of cDNA per well were added, and the final concentration of the primers was 1.5 µM. The amount of *Pru p 3*, *STR*, and *S4D* mRNA expression was normalized using the endogenous control 18S, and relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$),

as previously described (Tordesillas et al. 2013). Changes in gene expression were calculated with reference to anthesis (7 dpa). The products of reactions were separated in 2% agarose gel and stained with ethidium bromide. All PCR experiments were carried out in triplicate, in three independent experiments.

Immunofluorescence and confocal microscopy

To detect the presence of Pru p 3, the samples were fixed with 4% formaldehyde in PBS (pH 7.4) at 4°C overnight. After washing in PBS and sectioning with a razor blade, the tissues were permeabilized (20 freezing/thawing cycles of 5 min each). Then, the specimens were washed with PBS and blocked with 5% BSA in PBS for 1 h. Immunohistochemistry was performed in whole-mount tissue by incubating overnight at 4°C with an anti-Pru p 3 polyclonal antibody [applied 1:50 in PBS; (Palacin et al. 2012)] and a secondary antibody, Alexa 488-conjugated anti-rabbit antibody (applied 1:500 in PBS, Molecular Probes). The tissue sections were mounted with glycerol:PBS (1:1) on 10-well slides (FisherScientific Inc., Pittsburgh, USA) and observed with a Leica TCS-SP8 confocal microscope using the laser excitation lines of 488 nm for the localization of Pru p 3 and 405 nm to detect alkaloid autofluorescence. Measurements of fluorescence intensity in ten independent ROIs on Z stack maximal projections were used to compare the immuno-expression of Pru p 3 protein and the alkaloid.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 and Statgraphics Centurion XVI. Gene expression was compared using the Mann-Whitney test. Statistical significance was set at $p < 0.05$ for all analyses. At least three independent experiments were used for all assays.

Results

Pru p 3 carried a lipid compound

Pru p 3 was isolated from peach peel following two chromatographic steps: cationic exchange (cationic-retained) and reversed phase (HPLC purified), as previously described (Diaz-Perales et al. 2000). The presence of a lipid compound bound to Pru p 3 was confirmed by thin layer chromatography in methanol as the mobile phase and stained with vanillin (Fig. 1b). A lipid stain was observed in the cationic-retained fraction, whereas no spot was found in the HPLC-purified protein.

The secondary structure of Pru p 3 from the cationic-retained fraction was studied using circular dichroism and

Fig. 1 **a** The retained fraction from cationic chromatography (5 µg; *Cationic retained*) and purified Pru p 3 (5 µg; *HPLC purified*) were separated by SDS-PAGE and stained with Coomassie R-250 (Coomassie) or incubated with polyclonal antibodies (anti-Pru p 3). **b** Both fractions were also separated in silica gel-coated plates (Merck) using thin layer chromatography (TLC) in methanol:water (7:3 v/v) in a saturated chromatography chamber for about 20 min. The plates were stained with vanillin (Sigma-Aldrich, Steinheim, Germany). **c** Both fractions were used to compare their secondary structure by far-

UV (190–260 nm) circular dichroism spectrum in water on a JASCO Spectropolarimeter J-810 (Jasco, Victoria, Canada) using 1-mm path-length quartz cells equilibrated at 20 °C. Spectra were recorded from 190 to 260 nm with 0.5 nm resolution at a scan speed of 50 nm/min. Data were fitted using the Dichroweb analytical tool. **d** Increasing amounts of the ligand of Pru p 3 was *dot-blotted* were incubated onto a nitrocellulose membrane. After blocking, it was incubated with a Pru p 3 solution (1 µg/mL) and the binding was revealed by specific antibodies

revealed an alteration in the far UV region, a feature that is typical of lipid binding (Fig. 1c). In contrast, HPLC-purified protein from RP-HPLC showed a circular dichroism spectrum typical of helices with no alteration in this region.

To characterize the interaction between Pru p 3 and this lipid compound, i.e. the ligand of peach LTP, the compound was isolated from the cationic-retained fraction by exclusion chromatography (70% methanol), followed by RP-HPLC in acetonitrile. The lipid fraction was

confirmed as the ligand of Pru p 3 by dot-blot binding assay. Increasing amounts of this fraction diluted in PBS were dotted onto a nitrocellulose membrane, and, after blocking, the membrane was incubated with Pru p 3 solution (1 µg/µl). The interaction was revealed by specific antibodies (Fig. 1d). These results showed tight binding affinity between Pru p 3 and its ligand, represented by a dissociation constant estimated at $K_d = 0.2 \mu\text{M}$ in a 1:1 ratio.

The ligand of Pru p 3 included a hydroxylated derivative of camptothecin bound to phytosphingosine

The identification of the ligand was analysed using standard compounds and by comparing retention times in HPLC (Fig. 2a). The ligand showed a peak overlapping to OH-camptothecin.

The presence of hydroxy-camptothecin and phytosphingosine were identified by ion fragmentation (Fig. 2b). Based on the pattern described for ceramides (Shin et al. 2014) and camptothecin (Zhao 2010), a fragmentation for the ligand of Pru p 3 was studied. The characteristic camptothecin fragment ions were detected as 364.81 m/z and 202.83 m/z. Phytosphingosine fragment ions were identified as 218.79 m/z and 230.01 m/z. The results also revealed peaks corresponding to both bound compounds, as 380.77 m/z and 424.82 m/z.

The chemical identification of the ligand of Pru p 3 was also confirmed by its capacity to emit blue light

when excited with UV radiation (254 nm; Fig. 3a), a feature also shown by camptothecin (Sirikantaramas et al. 2007). Moreover, camptothecin is well known for its ability to inhibit the catalytic activity of topoisomerase I, the enzyme responsible for relaxing supercoiling DNA (Wright et al. 2015). Activity assays performed with the ligand of Pru p 3 clearly showed inhibition of topoisomerase I after heating, whereas no inhibition was observed when the ligand was used directly (data not shown) (Fig. 3b). The inhibitory activity of topoisomerase I occurs through the closed E-ring of camptothecin (Fig. 4a). However, linking to phytosphingosine through the amide bond in the ligand of Pru p 3, forces the open form of the E-ring, thus rendering the ligand inactive in the inhibition of topoisomerase. Given that this α -hydroxy-lactone E-ring is susceptible to hydrolysis upon thermal treatment, the inhibition results are consistent with the chemical nature proposed for the ligand of Pru p 3.

Fig. 2 **a** The ligand of Pru p 3 was separated by phase reverse-HPLC. Commercial OH-Camptothecin/, camptothecin and quercetin were used as standards. **b** ESI-micrOTOF-Q II. Deconvolution chromatogram corresponding to the camptothecin obtained from the ligand fraction when it was dissolved in methanol-formic acid

Fig. 3 **a** Commercial quercetin (1 μ g Sigma), the ligand of Pru p 3 (1 μ g; Ligand), commercial camptothecin (1 μ g Sigma; CPT) and hydroxy-camptothecin (1 μ g Santa Cruz; OH-CPT) were loaded onto a silica gel plates and UV light (354 nm) was applied. **b** Inhibition of topoisomerase I activity. Plasmid pBSK (200 ng) was incubated with topoisomerase I (1 u; Inspiralis, UK) for 30 min at 37°C in the

presence of camptothecin (1 μ g, 0.5 μ g; Sigma, UK) or of the ligand (1 μ g, 0.5 μ g), heated for 5 min at 90°C. After the mix was separated on a 0.8% agarose gel at 5–10 V/cm, the gel was stained with ethidium bromide (10 mg/mL) and destained briefly with water. The gel was illuminated with a UV transilluminator

Modelled structure of the Pru p 3-ligand complex

Phytosphingosine is an 18-carbon chain with a polar head consisting of three hydroxyl groups and one amine group at the first four carbons followed by a 14-carbon saturated hydrophobic tail (Fig. 4a). Camptothecin is composed of a pyrroloquinoline moiety (rings A, B, and C in Fig. 4a), a conjugated pyridine ring (D), and a α -hydroxy-lactone ring (E) that is susceptible to hydrolysis and is open in the amide bound to phytosphingosine. In our model structure of the Pru p 3-ligand complex (Fig. 4b), which was obtained as explained in Methods, the phytosphingosine tail is inserted into the hydrophobic tunnel of the protein, whereas its polar head lies at the entrance. The camptothecin part of the ligand is located unburied in the large cleft outside the cavity, with rings A–C exposed to the aqueous solvent and the 10-hydroxy group protruding from the structure (Fig. 4c).

LTPs are known to bind a wide variety of hydrophobic ligands with polar groups, even in two opposite orientations inside the tunnel (Pacios et al. 2012). An X-ray structure is available for wheat LTP in a complex with two molecules of a phospholipid (lyso-myristoyl-phosphatidylcholine, LMPC) at opposite orientations with respect to the tunnel axis (PDB code 1BWO; (Charvolin et al. 1999)). Interestingly, the superposition of this wheat LTP–LMPC complex and our modeled Pru p 3-ligand complex places the ligand of Pru p 3 and an LMPC molecule in orientation B (Pacios

et al. 2012) at a remarkably similar position (Fig. 4d). Given that this spatial coincidence arises from superposition of proteins only, the finding lends strong support to our model structure.

The Pru p 3 gene is expressed mainly in the style of pollinated flowers

Expression of the *Pru p 3* gene revealed maximum levels in the flower, particularly high in the stigma-style in contrast to that observed in the ovary and pedicel, where the expression was very low (about 1000-fold less than in the stigma-style; Fig. 5a). In the style of non-pollinated flowers, *Pru p 3* expression decreased after the flower opened and continued to decrease after that stage. However, in pollinated flowers, *Pru p 3* expression increased from 0 dpa until 21 dpa.

These results were confirmed by immunofluorescence assays in whole-mount tissue (Fig. 6). Pru p 3 was located specifically in the pollinated flower stigma from 7 to 21 dpa, with a maximum expression at 21 dpa, which fell and then finally disappeared at 28 dpa.

The Pru p 3-ligand complex inhibited pollen germination

To gain a deeper understanding of the role of the Pru p 3-ligand complex in pollination, pollen grains were

Fig. 4 **a** Structural formula of the modeled ligand of Pru p 3 composed of 10-hydroxy-camptothecin (red) and phytosphingosine (blue). **b** Model structure of the Pru p 3 (ribbon)-ligand (sticks with C, green; N, blue; and O, red) complex. **c** Molecular surface of the Pru p 3-ligand complex structure shown in b. **d** Superposition of proteins in wheat LTP (light orange ribbon)-LMPC (sticks with carbons in yellow) complex (crystal structure, PDB id 1BWO) and Pru p 3 (green ribbon)-ligand (sticks with carbons in green) complex (model structure, present study)

germinated in 15% glucose for 4 h in the absence of Pru p 3 (Control) and in the presence of 1 µg/mL of Pru p 3 (Pru p 3) or Pru p 3 (1 µg/mL) together with its ligand (0.1 µg/mL) (Pru p 3+ligand). Germination was essentially inhibited in the presence of the Pru p 3-ligand complex (Fig. 5b).

The Pru p 3 gene is expressed mainly in the trichomes of peel

In the case of fruit development (Fig. 5c), expression of the *Pru p 3* gene was concentrated in the peel, starting at 70 dpa and reaching its maximum at 80 dpa. From that time on, mRNA levels decreased to 105 dpa.

Thus, Pru p 3 protein was also located mainly in the peel of ripe fruits, particularly on trichomes (Fig. 7). Interestingly, the alkaloid signal was also localized on peach peel trichomes; this finding is consistent with the kinetics of expression of *Pru p 3* obtained in our study.

Discussion

The present study is the first to characterize the ligand of the peach LTP Pru p 3 as a derivative of camptothecin bound to phytosphingosine. Ligand binding to LTPs is accompanied by slight changes in the unstructured C-terminal tail of the protein, which acts as a lid on the hydrophobic cavity, although the α -helical fold is preserved (Salcedo et al. 2007; Salminen et al. 2016). Consistent with the behaviour described for LTPs upon ligand binding, the presence of the ligand produced a distortion in the circular dichroism spectrum of Pru p 3 in the vacuum UV region (190–200 nm).

In the mass spectrometry analysis, the ligand of Pru p 3 suggested the presence of two different chemical species, each of which was identified as a monohydroxylated derivative of camptothecin and phytosphingosine. The presence of camptothecin was also confirmed by its autofluorescence properties and its ability to inhibit topoisomerase I activity, two well-known features of this alkaloid. However, given their chemical structures, both molecules barely occupy the hydrophobic central cavity of Pru p 3. We therefore suggest that the two species forming the ligand and are linked through an amide bond that involves the

Fig. 5 a Expression profile of *Pru p 3* gene in style, ovary, and pedicel of peach flower collected from -2 to 35 days post-anthesis (dpa), in pollinated and unpollinated flower. All expression values are normalized to the 18S gene. Relative expression is calibrated on the corresponding gene expression at ovary '0' time. The *bars* show the standard error. **b** Effect of the ligand on the pollen germination pro-

cess. Pollen grains were grown in 15% glucose for 4 h, in the absence of Pru p 3 (Control), in the presence of Pru p 3 (1 $\mu\text{g/mL}$; + Pru p 3), and in the presence of Pru p 3 and its ligand (0.1 $\mu\text{g/mL}$; + Pru p 3 and ligand). The percentage of germinated grains (%) was quantified in both samples. **c** Expression profile of *Pru p 3* gene in peach peel and seed fruit collected from 51 to 105 dpa

Fig. 6 Immunolocalization of Pru p 3 in styles from flowers collected at different dpa. v, unpollinated flowers; +, pollinated flowers. Pru p 3 was detected using a specific monoclonal antibody, which showed fluorescence signal in green (Pru p 3 revealed by Alexa 488)

E-ring of camptothecin, which is known to be responsible for the inhibitory activity of topoisomerase I in its closed form (Wright et al. 2015). Since sphingoid molecules are also known to bind disparate moieties as substituents of amino hydrogens, the amide bond seems a reasonable link between alkaloid and sphingoid components of the ligand.

This hypothesis is supported by the data obtained in the mass spectrometry spectra in which the camptothecin ions with the amide bond are identified by the peaks

Fig. 7 Immunolocalization of Pru p 3 in trichomes from mature fruits (105 dpa) detected using specific monoclonal antibody. The fluorescence signal is shown in green (Alexa 488) and alkaloids in blue in a; only blue light is observed in b; and the face contrast in c. $\times 40$

with m/z values of 380.77 and 424.82. The phytosphingosine segment is able to occupy the hydrophobic cavity of Pru p 3 because of the hydrophobic nature of amino acid side chains inside the cavity, which stabilizes binding of the hydrocarbonated nonpolar tail while leaving the polar groups close to the amide bound with camptothecin exposed to the solvent. Thus, given that the E-ring is open when the two segments are bound, the ligand of Pru p 3 lacks the pro-apoptotic activity characteristic of the alkaloid, and the LTP then merely transports an inactive compound causing no plant cell damage. However, if the ligand is released in the apoplast, hydrolysis of the E-ring breaks the bound to phytosphingosine, and camptothecin is

released in the closed form of the ring, thus regaining its activity and inhibiting cell division and pollen tube growth. The link can be also broken by extreme conditions such as heat and acid treatment. Therefore, the analysis with high voltage lasers in mass spectrometers could be responsible for splitting both molecules, thus explaining the presence of the two peaks observed.

The fact that the phytosphingosine tail forces the E-ring of camptothecin to remain open could be interpreted as a detoxification mechanism. In many cases, secondary metabolites and their pathway intermediates are phytotoxic. Plants have evolved several defence mechanisms to avoid self-toxicity from secondary metabolites (Sirikantaramas et al. 2007). A basic strategy to avoid self-toxicity is tight regulation of biosynthesis in terms of temporal/spatial gene expression and localization. In this sense, the highest expression of *Pru p 3* and the *Pru p 3*-ligand activity in pollinated stigma-styles led us to propose that both features might inhibit double pollination. Moreover, the capacity to inhibit pollen germination, together with timing of expression, also suggests a role in preventing the pollination if one considers that maximum expression has been shown to match fertilization time in peach flowers (Herrero and Arbeloa 1989).

Similarly, both the timing of *Pru p 3* expression and the presence of the *Pru p 3*-ligand complex in fruit peel (mainly in trichomes), might also inhibit predator feeding in fruit. The maximum expression of *Pru p 3* coincides with the end of embryo maturation and, thus, with the end of lignin deposition (Dardick et al. 2010) and accumulation of storage substances (amino acids and sorbitol) (Lombardo et al. 2011). Protection associated with expression of defence proteins such as LTPs is therefore necessary at that time. LTPs are expressed mainly in the cells of the exocarp in unicellular trichomes secreting alkaloids, as evidenced by immunolocalization of *Pru p 3* and ultraviolet fluorescence. Hence, *Pru p 3* might prevent ingestion of fruit until the embryo is fully developed.

In summary, *Pru p 3* can carry a monohydroxylated derivative of camptothecin bound to phytosphingosine. This LTP is expressed at two key times of flower and fruit development in peach, namely, during pollination and during embryo development. In both stages, the ligand of *Pru p 3* can inhibit cell division, although with two different objectives: first, to avoid pollination, and second, to prevent the plant from herbivores.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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