

From Flower to Seed Germination in *Cynara cardunculus*: A Role for Aspartic Proteinases

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ABSTRACT

Cardosins are plant aspartic proteinases (APs) isolated from the flowers of *Cynara cardunculus* L. (cardoon) and are responsible for their milk-clotting activity used to manufacture ewe's cheese. Cardosin A is mainly accumulated in protein storage vacuoles of the stigmatic papillae being suggested a possible role in pollen-pistil interaction. Cardosin B has been localized to the extracellular matrix of stylar transmitting tissue and a role in the remodelling or degradation of pistil extracellular matrix, during pollen tube growth, has been suggested. Also, cardosin B localization is closely correlated with programmed cell death (PCD) events in the nucellus of *C. cardunculus*, suggesting involvement in ovule and embryo sac development. Therefore, both cardosin A and B may fulfil important roles during sexual reproduction of the plant. In seeds APs may participate in protein hydrolysis, but, as they are present since early seed maturation they may take part both in zymogen activation and localised PCD in seed tissues, having a crucial role in the regulation of protein degradation and embryo nourishing. In the embryo cardosin A precursor form is accumulated in protein bodies and cell walls, a different localization from the described in cardoon flowers, suggesting a tissue-dependent pattern of accumulation of the protein. Furthermore, APs Plant Specific Insert seems to have a preponderant role in membrane reorganisation events and during water uptake and solute leakage, which supports the recently proposed bifunctional role of the AP precursor molecule. In this review, we intend to characterise cardosins developmental regulation in organs of *C. cardunculus* from the flower to post embryonic development and explore the putative roles assigned to these APs.

Keywords: cardosins, flower, PCD, PSI, seeds, sexual plant reproduction

Abbreviations: AP, aspartic proteinase; CP, cystein proteinase; ECM, extracellular matrix; ER, endoplasmic reticulum; LV, lytic vacuole; PB, protein body; PCD, programmed cell death; PSI, plant specific insert; PSV, protein storage vacuole

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ASPARTIC PROTEINASES

In common with aspartic proteinases (APs) from other origins, plant APs (Fig. 1) are active at acidic pH, are specifically inhibited by pepstatin and contain two aspartic acid residues at the active site (Rawlings and Barrett 1995). In most APs, the catalytic Asp residues are contained in a common Asp-Thr-Gly (DTG) motif in both lobes of the enzyme, but plant APs contain Asp-Ser-Gly (DSG) at one of the sites (Mutlu and Gal 1999).

Plant APs have so far been identified in numerous species, including gymnosperms (e.g. *Pinus*), monocotyledons (e.g. *Hordeum vulgare* - barley, *Zea mays* - maize and *Oryza sativa* - rice) and dicotyledons (e.g. *Arabidopsis*, *Brassica*, *Cynara cardunculus* - cardoon, *Solanum tuberosum* - potato, *Nicotiana tabacum* - tobacco, *Lycopersicon esculentum* - tomato). Although most of these enzymes were isolated

from seeds, there are also records of APs purified from other plant organs, namely leaves (potato, tomato), pollen grains (maize) and flowers (cardoon) (Mutlu and Gal 1999).

The presence of APs in floral organs is quite unusual in the plant kingdom, however, the massive accumulation of these enzymes in the flowers of several species from the genus *Cynara* appears to be a relatively common feature (Verissimo *et al.* 1998). In fact, APs have already been purified from the pistils of *C. cardunculus* (cardosins and cyprosins), *C. scolymus* (two APs related to cardosins), *C. humilis* (ChAP for *Cynara humilis* AP), and also from *Centaurea calcitrapa*, a close relative of the above cited species.

ASPARTIC PROTEINASES IN FLOWERS

Historically, flowers of *C. cardunculus* have been used in the Iberic Peninsula because of their milk-clotting activity

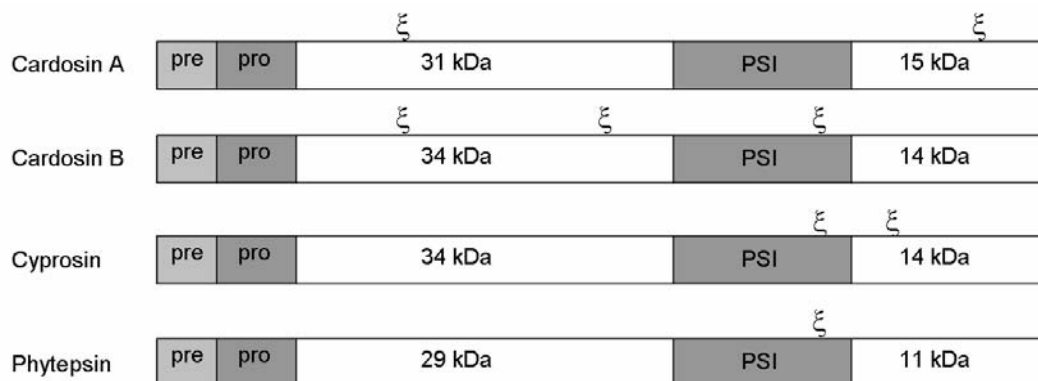


Fig. 1 Domain organization of cardosins A and B precursors as compared to other plant aspartic proteases. All include a signal peptide (pre), a prosegment in the N-end of the larger subunit (pro), and a plant specific insertion (PSI domain) separating the two chains of the mature form. Also shown are the N-glycosylation sites (ξ).

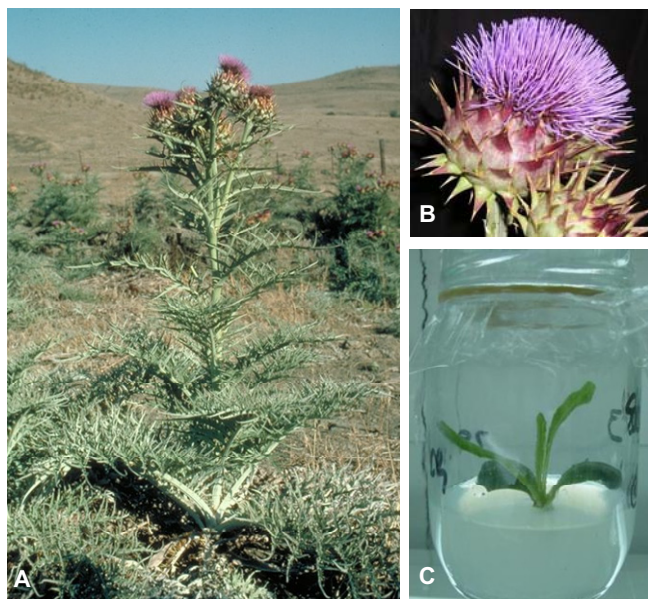


Fig. 2 *Cynara cardunculus* plant. Plant growing wild in its natural environment (A), detail of an inflorescence (B) and plant *in vitro* (C).

in the ewe's cheese manufacture for several centuries. However, the biochemistry underlying the milk-clotting capacity of cardoon pistils was only disclosed 15 years ago with the identification of two APs named cardosin A and cardosin B (Faro *et al.* 1992, 1998; Veríssimo *et al.* 1996; Ramalho-Santos *et al.* 1996, 1997, 1998).

C. cardunculus plants grow wild (Fig. 2A) in the south and west parts of the Mediterranean region, southern Portugal, Madeira and the Canary Islands. Cardoon plants are perennial herbs with erect stems and spiny leaves, clustered at the base of each segment. Flowering occurs in the second year of the plant life cycle and inflorescences are organized as compact capitula (Fig. 2B) consisting of numerous individual hermaphrodite flowers (Sampaio 1947; Tutin *et al.* 1976).

C. cardunculus flowers possess very long papillate stigmas (Fig. 3A) of the dry type. Four different structural regions can be distinguished in cardoon stigmas: the external papillate epidermis, the sub-epidermal region with several parenchyma layers, an area with support tissue surrounding the vascular bundles and, more internally, the transmitting tissue (Fig. 3B). The external papillae compose the uniseriate epidermis of this organ, are unicellular, densely arranged and matchstick-shaped, and throughout flower development show an increase in vacuolar volume. In the differentiated stigmatic papillae (Fig. 4A), two morphologically different vacuoles (electron-dense and electron-transparent) exist (Duarte *et al.* 2006). It is possible that these membrane-enclosed structures correspond to the co-existence of two distinct vacuolar populations in mature epidermal cells.

These putatively distinct vacuoles label differently for the presence of cardosin A, specifically detected in the electron-dense structures (Fig. 4B) where it accumulates throughout flower development (Ramalho-Santos *et al.* 1997; Duarte *et al.* 2006). The electron-dense compartments may therefore be protein-storage vacuoles (PSVs), while the electron-transparent regions are probably vacuoles of the lytic type (LVs) (Paris *et al.* 1996). However, this statement still remains speculative as the available Tonoplast Intrinsic Protein (TIP) antibodies (Johnson *et al.* 1989; Maeshima 1992) do not recognize *C. cardunculus* TIP isoforms. The co-existence of two functionally distinct vacuole populations in mature cardoon stigma cells is significant, since the presence of different types of vacuoles in the same cell has mainly been reported for immature tissues (Paris and Rogers 1996; Jauh *et al.* 1999). Data on this subject is very scarce regarding floral organs. More recently, Park *et al.* (Park *et al.* 2004) demonstrated the existence of both LVs and PSVs in mature mesophyll cells of three species: *Arabidopsis thaliana*, *Nicotiana tabacum* and *Phaseolus vulgaris*, suggesting that the co-existence of distinct vacuole populations in plant cells is more common than previously believed.

CARDOSINS' ROLE IN THE STIGMA-STYLE COMPLEX

The style of *C. cardunculus* flowers is of the solid type (Duarte *et al.* 2006). The centre of this structure is occupied by vascular bundles and by transmitting tissue (Fig. 3C). The transmitting tissue is the specially differentiated tract between stigma and ovary (Fig. 3D-E), through which the pollen tube elongates. Cardoon transmitting tissue cells typically show loose cell walls immersed in the extracellular matrix (ECM) that considerably enlarges during flower development (Duarte *et al.* 2006).

Cardosins account for the majority of the total soluble protein in mature stigmas and such an abundance of proteases in the flowers of a plant is in itself extraordinary. Plant APs identified so far are either vacuolar or secreted and cardosins A and B provide an example of this variety. Cardosin A is a vacuolar protein present in the mature stigmatic papillae (Figs. 4B and 5A) (Ramalho-Santos *et al.* 1997; Duarte *et al.* 2006), whereas cardosin B is mainly located in the stigma and style transmitting tissue (Figs. 5C, 5E) and is secreted to the cell walls and ECM (Fig. 6A) (Vieira *et al.* 2001).

Cardosin A is abundantly present in the stigma throughout floral development, mainly in the stigmatic papillae but also in the subepidermal parenchyma layers. In the epidermal papillae of the stigmas, cardosin A is conspicuous and specifically localized in the electron-dense protein storage-like vacuoles (Fig. 4B). Cardosin A is also present in the epidermis of the style but at much lower levels (Duarte *et al.* 2006). In contrast, cardosin B is confined to the inner region of the pistil; its presence in the transmitting tissue increases from the upper region of the stigma to the lower style (Vieira *et al.* 2001; Duarte *et al.* 2006) and, occurring also in the ventral face of the ovary wall and the nucellus,

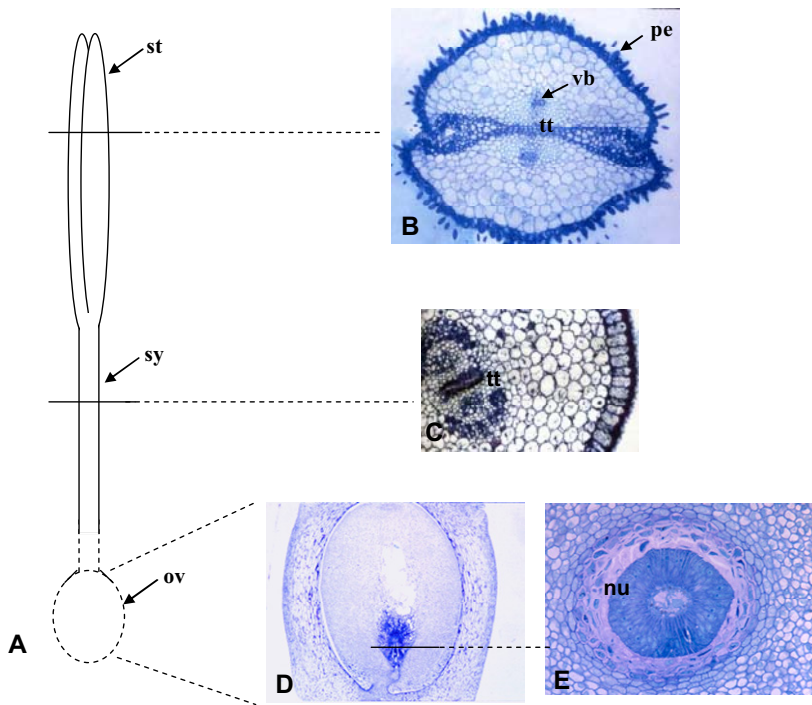


Fig. 3 Schematic diagram of a *Cynara cardunculus* pistil (A); the stigma has two stigmatic portions not completely united covered by a papillate epidermis (B), the inner core is occupied by the transmitting tissue continuum along the style (C) to the ovary; the ovary has a single ovule, here seen in longitudinal section (D), with large integuments surrounding the nucellus (nu) shown in cross section (E). The lines mark the plane in the flower from which the sections were taken. pe, papillate epidermis; st – stigma; sy – style; ov – ovary; tt, transmitting tissue; vb, vascular bundle.

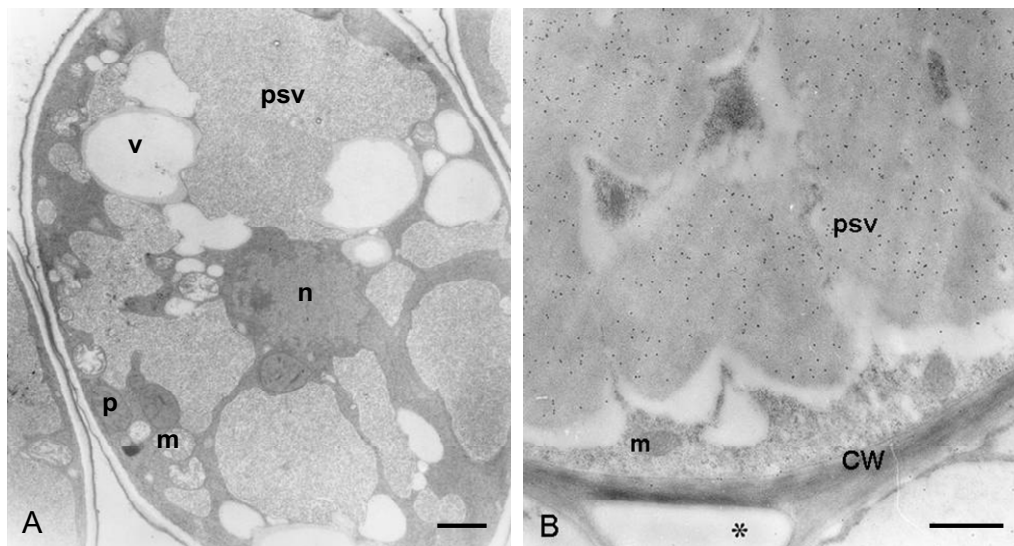


Fig. 4 Localization of cardosin A in stygmatic epidermal papillae of *C. cardunculus*. Cell ultrastructure of the stigma papillate epidermis (A). Immunolabelling for cardosin A appears specifically in protein storage vacuole (B). CW, cell wall; p, plastid; m, mitochondria; n, nucleus; v, vacuole; psv, protein storage vacuole; *, intercellular space. Bar: 1 µm.

seems to establish an inner route from the upper stigma to the embryo sac in the ovule.

Based on the observations compiled so far and on previously published results (Ramalho-Santos *et al.* 1996; Verissimo *et al.* 1996; Ramalho-Santos *et al.* 1997; Faro *et al.* 1998; Ramalho-Santos *et al.* 1998; Faro *et al.* 1999; Vieira *et al.* 2001), several hypotheses concerning the biological functions of cardosins in flowers arise. Cardosin A accumulates massively in the stigmatic papillae of *C. cardunculus* flowers, therefore it is reasonable to hypothesize that in cardoon, cardosin A may play a role in pollen recognition events. In about half of all plant families, including those to which *Arabidopsis* and *C. cardunculus* belong, the stigma surface is dry (Swanson *et al.* 2004). In plants with dry stigmas, pollen self-incompatibility is typically sporophytic (Lord and Russell 2002), with pollen grain recognition occurring at the stigmatic level. Pollen adhesion in plants with dry stigmas has been investigated with different assays, leading to apparently contradictory results (Lord and Russell 2002) that can be resolved when the distinct stages of pollen adhesion are taken into account: “pollen capture” – the earliest stage and exine-dependent; “cross-linking” –

second stage of adhesion involves the formation of a foot of pollen coat at the point of contact with the stigma; “tethering” – the final adhesive stage, when the pollen tube penetrates the stigma cuticle “tethering” the emptying grain to a papilla (Swanson *et al.* 2004). Protease digestion assays argue for the role of stigma surface proteins in pollen cross-linking and, through careful dissection of *Brassica* stigma and pollen coat proteins, at least one pollen-stigma cross-linking pair, SLR1 and SLR1-BP (PCP-A2), has been identified (Swanson *et al.* 2004). In cardoon, no homologous protein to SLR1 has been identified so far. However, the presence of cardosin A in the stigma’s papillate epidermis, in the vacuole and also in the cell wall, together with the identification of a putative receptor for this protein isolated from pollen extracts – a 100 kDa protein (Faro *et al.* 1999), is indicative of a possible physiological role of cardosin A in pollen-pistil interactions. Faro and co-workers (1999) demonstrated that cardosin A contains an Arg-Gly-Asp (RGD) motif, a well known integrin-binding sequence, through which interaction with the 100 kDa putative pollen receptor occurs. On the other hand, the accumulation of cardosin A in mature flowers of *C. cardunculus* may indicate a role of

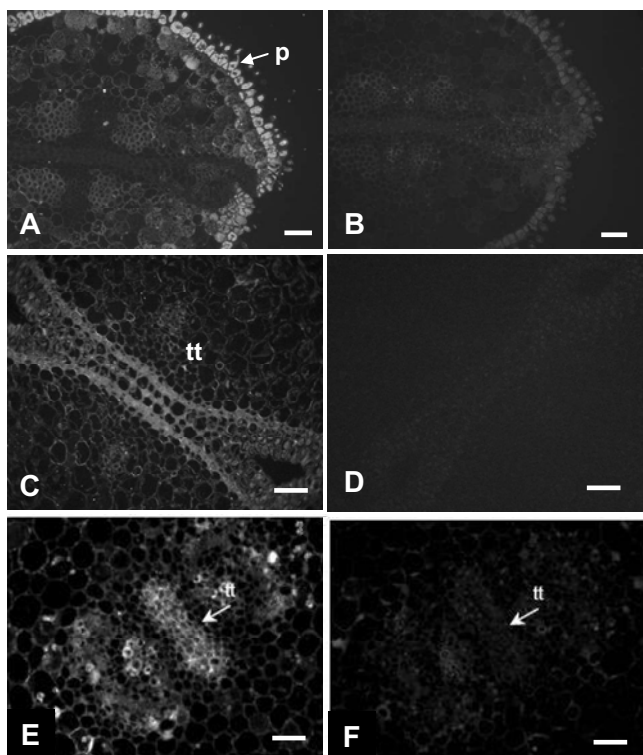


Fig. 5 Immunofluorescence localization of cardosins A and B in stigma and style of *Cynara cardunculus*. In stigma cross sections cardosin A is abundantly present in the papillate epidermis (p) and subepidermal parenchyma layers, being absent from the transmitting tissue (A). Cardosin B was detected in the transmitting tissue (tt) both in the stigma (C) and style (E) sections. (B), (D) and (F), control sections. Bar: 25 µm.

this AP in the senescence of this organ (Ramalho-Santos *et al.* 1997; Simões and Faro 2004), something previously proposed for cyprosins, APs also present in cardoon flowers (Cordeiro *et al.* 1994), and for cenprosin, the AP from *Centaurea calcitrapa* (Domingos *et al.* 2000). In fact, plant APs are known to be associated with developmentally regulated PCD phenomena such as organ senescence (Panavas *et al.* 1999; Beers *et al.* 2000) and tracheary element differentiation (Runeberg-Roos and Saarma 1998). Association of cysteine and serine proteases with TE formation namely the final process of TE PCD is well documented (Pesquet *et al.* 2005) but few reports describe the association of aspartic proteases with TE formation. Barley AP phytepsin presen-

ting homology with mammal cathepsin D and Yeast vacuolar proteinase A, both implicated in PCD, was shown to accumulate in metaxylem vessels (Runeberg-Roos and Saarma 1998). Regarding cardosin B, our results clearly show that this AP is specifically localized along the stylar transmitting tissue throughout flower maturation. Results obtained by Vieira and co-workers (2001) showed specific localization of cardosin B in the cell wall and ECM of the style transmitting tissue (Figs. 5C, 6A). These data suggest that cardosin B may be associated or be responsible for the loosening process of the transmitting tissue's ECM in order to facilitate pollen tube growth.

CARDOSINS INVOLVEMENT IN PROGRAMMED CELL DEATH

The ovule of *C. cardunculus* is anatropous (Fig. 7A), with thick integuments that surround the embryo sac and the nucellus.

The cardoon embryo sac is of the *Polygonum* type. In its proximal end the filiform apparatus is evident as well as the strongly cytoplasm polarised synergids and egg cell. In the middle region the central cell is present, occupied mostly by the large vacuole and where, near the polar nuclei, several cell ingrowths appear. At the central cell distal end, electron dense structures corresponding to nucellar remnants are visible. At the embryo sac distal region there are at least three antipodal cells that persist after fusion of the polar nuclei (Figueiredo *et al.* 2006).

Two specialised nucellar layers can be distinguished at the distal end of the embryo sac, prior to pollination: the podium and the hypostase. The podium cells are located adjacent to the embryo sac presenting a rich cytoplasm and a large nucleus with condensed chromatin at the periphery. Surrounding the podium, the hypostase cells emerge, with thick cell walls, electron-dense cytoplasm with amorphous condensed material and strong vesiculation. At the micropylar end only nucellar remnants are present, composing the epistase (Figueiredo *et al.* 2006). All these morphological features are typical of cells undergoing programmed cell death (PCD) (Bouman 1984). As the cardoon embryo sac differentiates TUNEL PCD labelling is present at the nucellar podium and hypostase (Fig. 8), concomitant with an absence of nuclear fragmentation in ovules prior to nucellar differentiation and embryo sac maturation (Figueiredo *et al.* 2006).

In *C. cardunculus*, the internal layer of the inner integument differentiates into the endothelium that encircles the embryo sac. The endothelium cells with a rich, dynamic cytoplasm are radially oriented and become bilayered as ovule

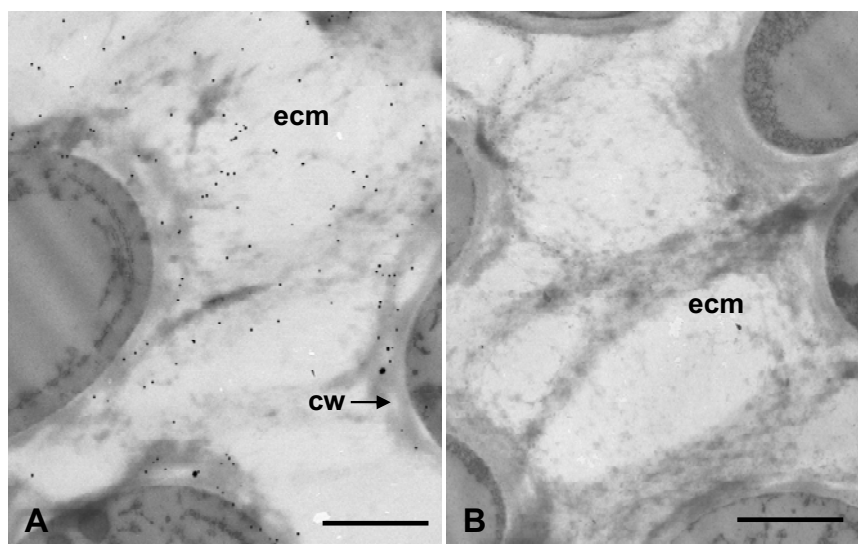


Fig. 6 Immunogold-electron microscopy localization of cardosin B in the stylar transmitting tissue. Labeling is present in the extracellular matrix (ecm) and cell walls (cw) of the transmitting tissue cells (A) and some labelling can be seen in the cytoplasm. No appreciable labelling is present in sections incubated with the preimmune rabbit serum (B) as control sections. Bar: 1 µm.

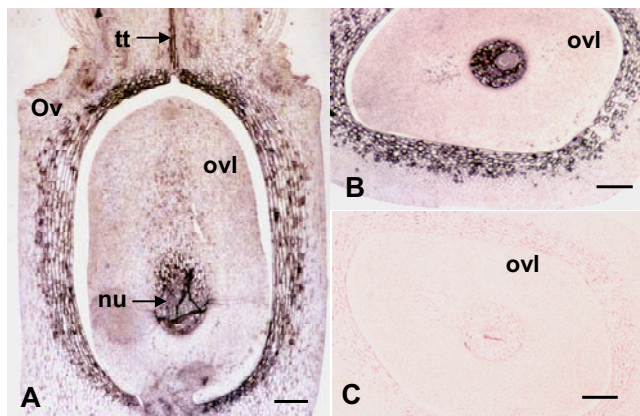


Fig. 7 Immunocytochemistry localisation of cardosin B in sections of the ovary (Ov) of *Cynara cardunculus*. Sections were labelled with anti-cardosin B and visualised by staining for alkaline phosphatase activity. Cardosin B appears along the transmitting tissue (tt) and the ventral side of the ovary wall, through the micropile and also at the nucellar region of the ovule, as seen in longitudinal (A) and cross (B) sections. (C) Control without primary antibody. nu, nucellus; ovl, ovule; tt, transmitting tissue. Bar: 500 µm.

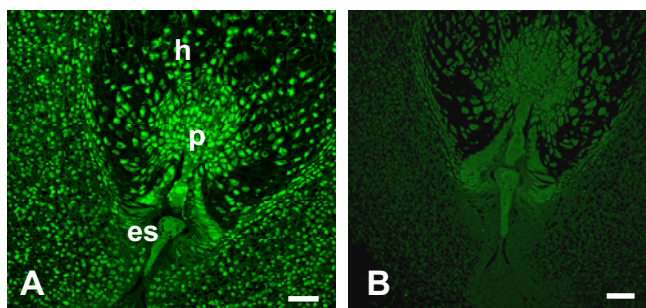


Fig. 8 PCD localisation in the ovules of *Cynara cardunculus*. TUNEL reactions with fluorescein (Roche) were performed in sections and observed in a confocal microscope. (A) Ovule with a mature embryo sac (es). The nucellar layers, hypostase (h) and podium (p), shown are at different stages of PCD. (B) Negative control without terminal deoxynucleotidyl transferase. Bars: 100 µm.

maturation proceeds (Figueiredo *et al.* 2006). One possible function of the endothelium is the regulation of nutrient flow from the nucellus towards the embryo sac and later to the endosperm. Other function putatively assigned to this region could be the protection of the embryo sac from PCD-associated proteases present within the degenerating tissues that surround the endothelium. *Solanum americanum* RNAi transgenic lines with silenced expression of proteinases inhibitor proteins and subsequent premature endothelium PCD illustrate the importance of this tissue by the lack of endosperm development and embryogenesis (Sin *et al.* 2006a, 2006b).

Cardosin B largely coincides with the pollen tube path to the egg cell, from the lower stigma, through the style, ventral side of the ovary wall (Fig. 7) and the micropyle (Vieira *et al.* 2001; Figueiredo *et al.* 2006) supporting a role in softening and loosening of the cell walls to facilitate pollen tube progression. On the other hand, after fertilization, extracellular proteases are expected to modify or degrade peptides or proteins secreted by the female gametophyte as guidance signals for the pollen tube, in order to avoid, for example, polyspermy (Dresselhaus 2006).

In the nucellus cardosin B expression (Fig. 7B) is highly restricted to the hypostase cells undergoing PCD (Fig. 8) where it localises in the cell wall and ECM of TUNEL-positive cells showing strong vesiculation, condensed cytoplasmic material and thick cell walls. Even after pollination, when the hypostase cells have already lost their nuclei, cardosin B is still restricted to the cell walls and the

ECM. It is noteworthy that this protein is completely absent in the nucellus before nucellar degeneration initiation (Figueiredo *et al.* 2006). Cardosin B present in the hypostase cells undergoing PCD may be associated with proteolytic activity of cell death related proteins that function as a supply of nutrients to the growing embryo sac as proposed for nucellin (Chen and Foolad 1997). These hypostase cells show strong vesiculation, a feature correlated both with efficient transport of nutrients and growth regulators of *Arabidopsis* suspensor cells to and from the embryo proper and shown to be a part of the suspensor death program (Bozhkov *et al.* 2005). Alternatively, cardosin B may just be involved in hypostase cell wall loosening and reorganisation to facilitate the traffic of nutritive substances to nourish the embryo sac, a role also indicated for nucellain (Linnestad *et al.* 1998; Wu and Cheung 2000). APs may also activate other proteins or release signal molecules that have a more direct participation in pistil cell death processes (Wu and Cheung 2000).

Cardosin B involvement in PCD events is a recent finding but there are several evidences linking plant APs to PCD in several organs and/or situations: developing flowers, senescence and stress responses (Beers *et al.* 2000). There are several studies, carried out during embryogenesis, which show the involvement of APs in PCD in the aleurone layer (Chen and Foolad 1997). In contrast, few studies were undertaken during seed germination and post-embryonic development. However, recent results put forward a possible role for cardosin A in PCD events occurring during radicle emergence and seedling formation (Pereira *et al.* 2007).

ASPARTIC PROTEINASES IN SEEDS

Cardoon seeds are characterized by a thick and impermeable seed coat and a reduced endosperm (one or two cell layers). The embryo occupies the majority of the seed with two large cotyledons. Cardoon cotyledon cells are highly vacuolated with one or more protein bodies (PB) that can vary in shape and texture and the cytoplasm appears compressed against the vacuolar tonoplasts, and distributed along the cell as a net (unpublished). Protein bodies are ER-derived compartments specialized in the storage of reserve proteins, composed of a dense matrix and occasionally a core (Muntz *et al.* 2001). As germination proceeds, PBs enlarge due to the coalescence of the multiple initial PBs in the cell. Often, a big PB, occupying most of the cell can be observed and, eventually, is enclosed in large central vacuoles. PBs matrix, initially dense, becomes reticulated and, by the time of radicle emergence, large amounts of ribosomes/polyribosomes and endoplasmic reticulum (ER), frequently along the cortical cytoplasm adjacent to the plasma membrane, are observed indicative that protein metabolism is at the maximum rate due to the high metabolic activity of the seed (unpublished). The loss of density observed can be related to the physiological events taking place as the reticulated shape is a consequence of storage proteins proteolysis/degradation (Elpidina *et al.* 1990).

The presence of APs in seeds has been approached through different ways (Elpidina *et al.* 1990; Martilla *et al.* 1995; Bethke *et al.* 1996; Hiraiwa *et al.* 1997; Bleux *et al.* 1998; Capocchi *et al.* 2000; Gruis *et al.* 2004) and several roles have been assigned, only in a hypothetical way. However APs have been detected inside protein bodies, and according to Bethke and co-workers (1996), APs work together with cystein proteinases (CPs) in storage protein mobilization and/or zymogen activation. Despite that, these authors state that APs activity is lower than CPs in protein bodies, results confirmed later by Capocchi and co-workers (2000). APs precursors seem to accumulate in protein bodies during seed maturation (Muntz *et al.* 2001) where they show no proteolytic activity, either because they remain inactive or their substrate is not accessible. On the onset of germination, structural modifications take place and protein hydrolysis begins. Recent work (Pereira *et al.* 2007) showed

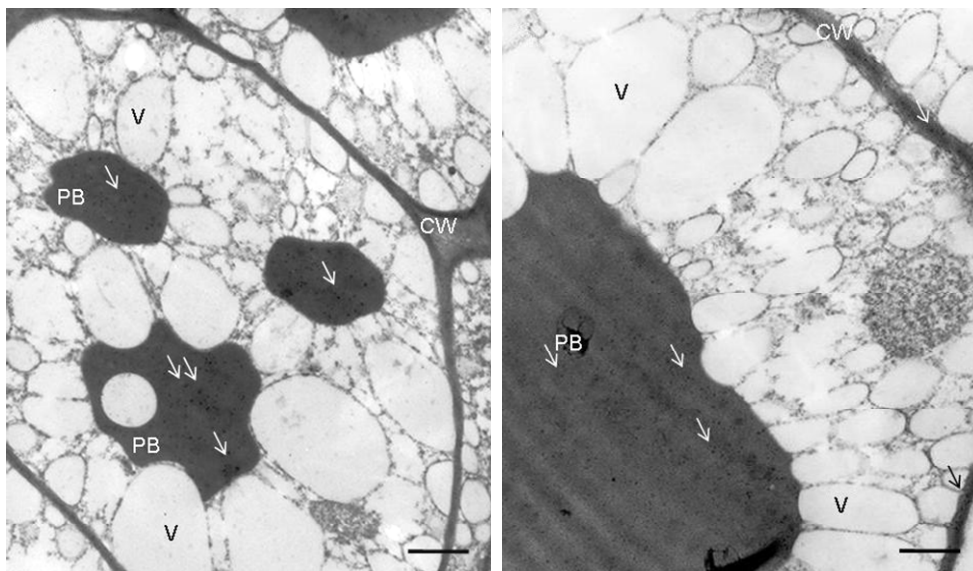


Fig. 9 Cardosin A immunogold labelling in cotyledon ultra-thin sections of *Cynara cardunculus* germinating seeds. Labelling is detected (arrows) in the protein bodies (PB) and, in lower amounts, in the cell wall (CW). V, vacuole. Bars: 4 μ m.

the presence of a cardoon AP – cardosin A – in seed tissues revealing an interesting dynamics during post-embryonic seed development events. In cotyledons Cardosin A precursor form was localised inside protein bodies and in cell walls (**Fig. 9**). Transcripts and protein analysis point to the presence of cardosin A in cotyledonal tissues, suggesting an involvement of this AP in the embryo basal metabolism. Martilla and co-workers (1995) have already suggested the same role for a barley AP and later, Bleux and co-workers (1998) supported this idea, stating that APs in dry seeds are responsible for the day-to-day protein hydrolysis. Also interesting is the observation (Pereira *et al.* 2007) that in the first stages of germination the two different cardosin A forms – precursor and mature – were present at the same time, but in different organelles. Since no cardosin A mRNA accumulation was detected it was argued that its mature form is probably reminiscent from mother tissues. This is rather interesting and worth to consider, because the cardosins processing steps are consecutive and therefore consistent with a time-lapse accumulation of its different forms. Martilla and co-workers (1995) also observed the precursor in PBs and the mature form of a barley AP inside lytic vacuoles, but in different time-points. As a whole, these results point out to a fine developmental regulation of APs in seeds and prompt an analysis of their possible role in the context of the physiology of seed germination and post-germination.

PSI AS A MEMBRANE DESTABILIZING DOMAIN

Egas and co-workers (2000) propose PSI as a putative membrane destabilizing domain, interacting with vesicles and inducing the leakage of their contents. Later on, Simões and Faro (2004), based on studies with cardosin A, suggested that AP precursors are bi-functional molecules containing a membrane destabilizing domain (PSI) in addition to their protease domain. Recently, Pereira and co-workers (2007) added more evidences to this debate, showing, in cardoon seeds, the detection of cardosin A precursor in cell walls. They argue that it may be implicated in the high membrane reorganization events occurring in actively growing tissues, as radicle emergence and elongation, participating in water uptake and solute leakage as well as in lipid turnover during membrane biogenesis. An important role is here assigned to procardosin A PSI in the membrane structural changes. In another approach, Brodelius and co-workers (2005) proposed a role for PSI in the biogenesis of membranes in tissues under high growth rates, where a high turnover of lipids is expected. Years earlier, Martilla and co-workers (1995)

have already detected this AP in the same tissues (protective root tissues and aleurone layers). The presence of AP precursor forms in the cell walls may be related to the proposed role of PSI in membrane lipid conversion in actively growing tissues.

TRAFFICKING PATHWAYS IN SEEDS

Trafficking pathways in seeds have recently been approached, raising the interest among the scientific community, given the peculiarities observed. Protein sorting may be, in many cases, tissue specific, behaving differently and depending on the tissue they are expressed in (Robinson *et al.* 2005). A good example, among others, is cardosin A. In cardoon flower tissues (Ramalho-Santos *et al.* 1997), and even in heterologous systems (Duarte 2005) like *Nicotiana tabacum* and *Arabidopsis thaliana* leaves, this aspartic proteinase accumulates in vacuoles (storage and lytic) while in seed tissues it is found inside protein bodies and in the cell wall (Pereira *et al.* 2007). This observation, allied to the fact that the proteinase is found under different forms in these different locations (precursor and mature) supports the idea of different sorting events in storage tissues. Storage proteins are synthesised in the endoplasmic reticulum, where the first maturation steps occur. From here, the route taken to the storage vacuole or to the protein bodies may differ, considering their passage through the Golgi apparatus, or directly to their final destination. According to Hillmer and co-workers (2001) there are three ways for storage proteins to leave the endoplasmic reticulum: the first implies protein aggregates autophagy; the second one involves release of precursor accumulating vesicles; and the last implies transport through the Golgi. Vitale and Galilli (2001) propose a variation for this model still considering a route through and bypassing Golgi. What makes storage proteins follow one or another route is still essentially unknown. Apparently, the ones destined to the protein bodies forms storage protein aggregates at endoplasmic reticulum level and are exported in ER derived vesicles. On the other hand, proteins targeted to the protein storage vacuole enter the Golgi and are distributed along the stacks, in a gradient dependent manner as described by Hillmer and co-workers (2001). Despite all the differences and all the controversies, almost all authors agree that storage proteins that enter the Golgi apparatus leave in cis/median derived dense vesicles and not in clathrin coated vesicles, as they normally do not reach the trans-Golgi region (Hohl *et al.* 1996; Hilmer *et al.* 2001; Molina *et al.* 2006). Furthermore, the sorting and travel of storage proteins may not depend only on the recognition of a single

sorting signal by a specific receptor, but proteins aggregation may be sufficient to trigger the sorting process (Shimada *et al.* 2003; Castelli and Vitale 2005).

CONCLUSIONS

Given the evidence presented it is plausible that cardosins perform significant roles in the sexual reproduction of *C. cardunculus*. The fact that cardosins are localized differentially in *C. cardunculus* flowers – cardosin A throughout the stigma and cardosin B at the lower stigmatic region, style and ovary – supports the idea that cardosins A and B have distinct biological functions. Regarding their trafficking pathways, in the flowers cardosin A accumulates in the vacuoles and cardosin B is secreted. This fact may be related to a tissue-specific expression, as in developing seeds cardosin B is not detected and cardosin A is found in protein bodies and in the cell wall. It is known that proteins frequently adapt their trafficking pathways and/or their final destination according to cell needs, in a tissue dependent manner. In seeds, APs may have other roles besides the traditionally ones assigned and PSI could have a preponderant role in this matter. The involvement of cardosin A in lipid membrane destabilisation during water/solute exchanges and in actively growing structures reinforces the bifunctional role of this protein.

Most of the proposed biological functions of plant APs are still speculative and further work is required to either confirm already arisen hypotheses or unveil new *in vivo* functions for APs in plants.

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