# C1A cysteine-proteases and their inhibitors in plants

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Plant cysteine-proteases (CysProt) represent a well-characterized type of proteolytic enzymes that fulfill tightly regulated physiological functions (senescence and seed germination among others) and defense roles. This article is focused on the group of papain-proteases C1A (family C1, clan CA) and their inhibitors, phytocystatins (PhyCys). In particular, the protease–inhibitor interaction and their mutual participation in specific pathways throughout the plant's life are reviewed. C1A CysProt and PhyCys have been molecularly characterized, and comparative sequence analyses have identified consensus functional motifs. A correlation can be established between the number of identified CysProt and PhyCys in angiosperms. Thus, evolutionary forces may have determined a control role of cystatins on both endogenous and pest-exogenous proteases in these species. Tagging the proteases and inhibitors with fluorescence proteins revealed common patterns of subcellular localization in the endoplasmic reticulum–Golgi network in transiently transformed onion epidermal cells. Further in vivo interactions were demonstrated by bimolecular fluorescent complementation, suggesting their participation in the same physiological processes.

## Introduction

Plant proteolysis is a metabolic pathway that involves broad metabolic networks, different subcellular compartments and types of proteases, mainly cysteine-, serine-, aspartic- and metallo-proteases (van der Hoorn 2008). Among the about 800 proteases encoded by plant genomes, more than 140 correspond to cysteine-proteases (CysProt) that can be grouped in 15 families in 5 clans (Rawlings et al. 2010). In particular, the papain-proteases C1A (family C1, clan CA), subdivided as cathepsin L-, B-, H- and F-like according to their gene structures and phylogenetic relationship (Martinez and Diaz 2008), are the most abundant. A high number of genes encoding C1A CysProt have been identified in diverse plant species. They share common features already analyzed but information about the roles of these genes is unknown or still fragmentary. The participation of individual C1A proteases in diverse processes such as senescence, abscission, programmed cell death and fruit ripening and in the mobilization of proteins accumulated in seeds and tubers has been published (Grudkowska and Zagdanska 2004, van der Hoorn 2008). Moreover, the expression of CysProt genes is enhanced under various environmental stresses, which trigger reorganization of metabolism, remodeling of cell protein components, degradation of damaged or unnecessary proteins and nutrient remobilization. C1A proteases are also involved in local and systemic defense in response to pathogen and pest attacks (Shindo and van der Hoorn 2008, McLellan et al. 2009).

Protease activity is regulated at the transcriptional and protein levels. Protease expression is controlled by

*Abbreviations* – BiFC, bimolecular fluorescent complementation; CysProt, cysteine-proteases; ER, endoplasmic reticulum; GFP, green fluorescent protein; PhyCys, phytocystatins; VPE, vacuolar processing enzymes.

transcription factors (Gubler et al. 1999, Sreenivasulu et al. 2008). Additionally, protein activity is regulated by binding to specific inhibitors and cofactors and through the activation of zymogens. C1A proteases are the main target of phytocystatins (PhyCys), plant proteinaceous inhibitors able to attenuate the protease activities by formation of complexes with the enzyme. From the functional point of view, PhyCys have a dual role: as defense proteins against heterologous proteases and as endogenous regulators of protein turn-over preventing the breakdown of essential proteins during metabolism (Benchabane et al. 2010).

CysProt and PhyCys have become important in the modern food and feed industry. On a commercial scale, CysProt, such as papain and bromelain, are used in food processing and others have been traditionally applied for brewing processes and in the milling and baking industries (Cabral et al. 1994). Additionally, PhyCys have a potential interest to prevent unintended proteolysis in plant protein biofactories (Benchabane et al. 2008).

This review focuses on our current understanding of the structure–function relationship between C1A CysProt and PhyCys through the protease–inhibitor coevolution and interaction, and their common participation in specific pathways throughout the plant's life.

## Coevolution of plant C1A CysProt and PhyCys

To date, more than 600 amino acid sequences for plant C1A CysProt and 150 for PhyCys have been included in the specific MEROPS database for proteases and their inhibitors. Figure 1 summarizes the main results obtained by Martinez and Diaz (2008) on the evolution of both gene families in several plant species. C1A proteases are present in all plant species analyzed, the algae *Ostreococcus tauri* and *Chlamydomonas reinhardtii*, the moss *Physcomitrella patens*, the spikemoss *Selaginella moellendorffii* and the angiosperms *Oryza sativa*, *Arabidopsis thaliana* and *Populus trichocarpa*. They have been originally described as cathepsins in mammals and traditionally classified based on their homology to them (Beers et al. 2004). The classification following this criterion, grouped C1A proteases as homologous to the human B-, F-, H- and L-cathepsins. Several algal papain-like proteases do not share enough similarities to mammalian cathepsins to be considered as their plant counterparts. In Fig. 1, the number of C1A proteases belonging to each group is shown. B-like C1A are present in all clades, and its number does not vary during plant evolution. F-like proteases are also maintained from algae, although they are not present in the *Chlorophyceae*, and their numbers

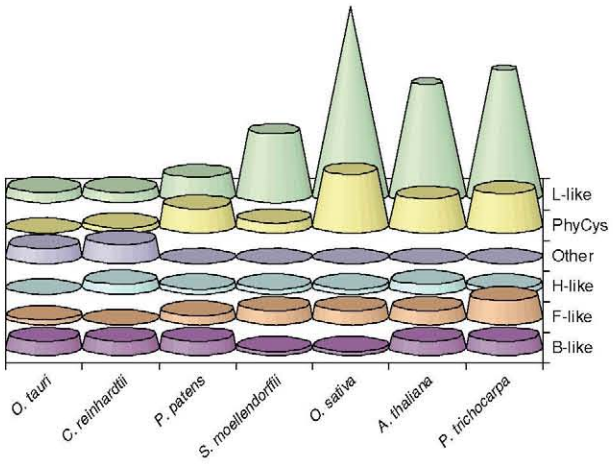


**Fig. 1.** Number of C1A CysProt grouped in cathepsin L-, H-, F- and B-like and their inhibitors PhyCys, in some plant clades.

slightly increased in angiosperms. H-like proteases are present in *Chlorophyceae* and are conserved from moss to angiosperms. L-like proteins show the most striking pattern. They first appeared in algae, slightly increased in number in the moss and they present a sharp increase from the spikemoss to the angiosperms. Finally, C1A proteases not related to mammalian proteases (indicated as other) were only identified in algae.

Coevolution of C1A CysProt and their inhibitors, PhyCys, should have been crucial to acquire their actual roles in the plant. Cystatins are also present in all species analyzed with the exception of the prasinophyceae algae *O. tauri*. Globally, a higher number of cystatins in angiosperms than in the non-seed land plants is accompanied by a sharp increase in the number of papain-like CysProt. Evolutionary forces leading to the control of these proteases in the plant or to control exogenous proteases from pests could have determined the increase in the cystatin number in angiosperm species.

## Features of plant C1A CysProt

### Molecular characteristics

Members of the papain-like subfamily C1A are the most widely studied among plant CysProt. All C1A proteins present disulphide bridges to determine their functional structure, contain a catalytic triad formed by three conserved amino acid residues Cys, His and Asn and have a Gln residue involved in maintaining an active enzyme conformation. C1A CysProt from plants are synthesized as inactive or little active precursors to prevent inappropriate proteolysis. Preproteins comprise an N-terminal pro-peptide of 130–150 amino acids,

and the mature protein is about 220–270 residues long. To become active, the C1A proteases need to be processed either by self-processing or with the aid of processing enzymes. Activation takes places by limited intra- or inter-molecular proteolysis cleaving off the inhibitory pro-peptide (Wiederanders et al. 2003). The pro-sequences play important roles as modulators of the protease activity to guarantee that the mature enzyme is formed in the right place and/or at the right time (Demidyuk et al. 2010). The pro-peptides contain the consensus motif GxNxFxD, which seems to be essential for the correct processing of the protease precursors and the non-contiguous ERFNIN signature (Ex3Rx3Fx3Nx3I/Vx3N) found in cathepsin L- and H-like or the ERFNAQ variant in cathepsin F-like, both of unknown function (Grudkowska and Zagdanska 2004, Martinez and Diaz 2008). In contrast, cathepsin B-like proteases lack this motif (Wiederanders 2003, Martinez and Diaz 2008). Additionally, some cathepsin L-like members also contain a C-terminal extension sequence, which includes a Pro-rich region and a granulin domain with a high homology to animal proteases of the epithelin/granulin family (Yamada et al. 2001). This granulin-like domain may participate in the regulation of protease solubility and in its activation (Beers et al. 2004). These plant granulins are considered as a subclass of the papain family.

## Compartmentalization

Compartmentalization of C1A CysProt within cells provides a further level of regulation whereby plant cells contain several different types of organelles each with a specialized function. The inactive pro-enzymes are synthesized in the lumen of the endoplasmic reticulum (ER). Then, via the *trans*-Golgi network most C1A CyProt are finally transported to the vacuoles and lysosomes or externally secreted. Some of the cathepsin L-like C1A proteases also display a carboxy-terminal KDEL/HDEL motif, an ER retention signal for soluble proteins that regulate the delivery of proteins to other compartments (Schmid et al. 2001, Okamoto et al. 2003, Than et al. 2004, Helm et al. 2008). In particular, some KDEL-tailed peptidases are stored in ricinosomes from where are released upon acidification of the cytoplasm because of the breakage of the vacuole (Schmid et al. 1999, 2001). Other C1A members without the KDEL motif present similar enzyme subcellular location (Martinez et al. 2009). The relatively acidic pH of vacuoles and lysosomes provides the optimal conditions for protease processing, these organelles being not only the site of enzyme action but also the site of their activation. The protease precursor activation is a complex process involving appropriated pHs, other protease action, inhibitors and others compounds strongly dependent on the cellular or extra-cellular environment (Wiederanders 2003).

## Physiological roles

C1A CysProt are involved with a variety of proteolytic physiological functions in plants, such as senescence, seed reserves mobilization and plant defense.

Senescence is a strictly controlled process throughout plant development that can be modulated by endogenous and exogenous factors such as plant growth regulators (abscisic acid, cytokinin, ethylene, salicylic acid), sucrose starvation, dark, cold, heat, drought, salt, wound, pathogen infection (bacteria, fungi, virus) and insect attack (Parrot et al. 2010). It is characterized by the dismantling of the tissue's organization and a high-efficient mobilization and relocation of nutrients from old leaves, petals and other organs to sink tissues (tubers, cereal seeds, fruits) to sustain further growth and development. The timing of senescence affects the length of the active photosynthetic period and, therefore, crop yield. Thus, its control is of interest in breeding programs. A number of CysProt have been described to be involved in senescence in different tissues and species: petals of *Dianthus caryophyllus* and *Alstroemeria* (Sugawara et al. 2002, Wagstaff et al. 2002), wheat and *Arabidopsis* leaves (Martinez et al. 2008, Esteban-Garcia et al. 2010), and peel pitting of orange (Fan et al. 2009), among others. Antisense silencing of CysProt Cyp15a (classified in group F of C1A peptidases, according to MEROPS database) resulted in delayed senescence phenotypes in *Medicago truncatula* (Sheokand et al. 2005). Transgenic tobacco plants expressing the rice cystatin OC-I grew more slowly than controls and showed changes in leaf protein content with an increased abundance, among others, of two ribulose-1,5-biphosphate carboxylase/oxidase (Rubisco) activase isoforms, together with delayed senescence (Prins et al. 2008). Thus, according to these authors, Rubisco is likely a major target of CysProt during senescence and mechanisms of interaction through the vacuolar degradation of Rubisco-containing vesicles were put forward. All these data confirm the importance of C1A CysProt in leaf senescence and from them it can be concluded that a considerable more detailed picture of protease action in the senescence process has to be done.

Regarding the seed germination, it is known that seeds store starch, proteins and lipids that are used during the germination until photosynthesis is fully established. The contribution of C1A CysProt to storage-protein mobilization has been shown in monocots and

dicotyledonous species. Most seed germination research has been done in cereals, particularly in barley, where the majority of analyzed events have been focused at the transcriptional level. In fact, a complex network of genes encoding multiple transcriptional factors has been described as genes involved in the control of hydrolase expression at the beginning of germination (Sreenivasulu et al. 2008). In contrast, there are few data related to post-germination processes where proteases and amylases hydrolyze and mobilize storage compounds. Cereal seed germination starts when water is uptake and ends when the radicle tip protrudes through the seed coat. Proteases and amylases are either stored in aleurone layers and embryo during seed maturation or are newly synthesized during early germination. These enzymes hydrolyze accumulated reserves that can be then absorbed by the scutellum to allow the development of the seedling. Zhang and Jones (1995) identified the presence of 27 CysProt among the 42 proteases implicated in the protein degradation during barley seed germination. Recently, a complete transcriptome analysis of barley grain germination in two tissue fractions (starchy endosperm/aleurone and embryo/scutellum) showed the induction of a high number of CysProt genes during germination, most of them being mediated by gibberellins (Sreenivasulu et al. 2008). Additionally, histochemical assays performed in germinating seeds of wheat have revealed the detection in the embryo of several triticains and gliadains, C1A CysProt, which are secreted into the endosperm to digest storage compounds. Some of these proteases are regulated by intrinsic cystatins (Kiyosaki et al. 2007, 2009). Moreover, several cathepsin L-like CysProt from barley, differentially expressed in seed tissues, have been characterized and their implication on the mobilization of hordeins, the main storage proteins from barley, has been shown (Davy et al. 1998, 2000, Martinez et al. 2009). Similarly, the orthologous genes encoding C1A proteases from wheat participate in bulk protein degradation of wheat endosperm during seed germination and subsequent seedling growth (Shi and Xu 2009). Mobilization of stored compounds in the seeds of dicotyledonous plants is also mainly mediated by proteases and amylases, although their spatial and temporal patterns of expression and activities differ considerably from monocots. In seeds of legumes and rape, globulins are the most abundant storage proteins in the grain and are first mobilized in the embryonic axis during germination. Only after protein reserves are depleted in the axis do the bulk of globulins become mobilized in the cotyledons after germination. Vacuolar processing enzymes (VPEs) belonging to the C1A CysProt seem to be important in the endoproteolytic cleavage of storage-protein mobilization in embryonic axis and cotyledons in different legume species (Tiedemann et al. 2001). Recently, Wang et al. (2009) have shown that seed type of VPE are essential enzymes in the maturation of rice glutelins, which constitute up to 80% of total endosperm proteins in rice.

CysProt C1A also play crucial roles in plant pathogen/pest interactions (Shindo and van der Hoorn 2008, van der Hoorn 2008, McLellan et al. 2009). Several reports indicate that plants use CysProt to protect themselves against different pests. Papain is a component of papaya latex involved in the defense of the papaya tree against different lepidopteran caterpillars (Konno et al. 2004). Mir1 is a CysProt from maize induced in response to the herbivorous armyworm *Spodoptera frugiperda* (Pechan et al. 2000). Once ingested, the proteolytic activity of Mir1 damages the insect's peritrophic matrix, impairing nutrient utilization (Pechan et al. 2002). On the other hand, their cystatins counterparts are also induced by herbivores and have a clear effect against plant pests after plant feeding (Schluter et al. 2010). Following cystatin inhibition, proteases secreted by the target pests to digest host plant tissues can no longer cleave peptide bonds, which results in a detrimental disruption of dietary protein assimilation leading to growth delays and mortality among target pest populations (Benchabane et al. 2010). Protease–inhibitor interactions in plant–pest systems are the result of a long coevolutive process involving both proteases and inhibitors from the plant and the pest.

Likewise, C1A CysProt are used in defensive processes against plant pathogens. Cathepsin B genes from *Arabidopsis* are involved in the hypersensitive response and are required for full basal resistance against the virulent bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (McLellan et al. 2009). Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens, the fungal *Cladosporium fulvum* and the oomycete *Phytophthora infestans*, target the tomato defense C1A CysProt Rcr3 (Song et al. 2009). These effectors are cystatin-like CysProt inhibitors, which support the important role that the interaction between C1A CysProt and PhyCys plays in plant defense.

## Features of PhyCys

Plant cystatins are proteinaceous inhibitors that can act against CysProt of the papain C1A and C13 families and are considered an independent subfamily within the whole cystatin family of inhibitors (Martinez and Diaz 2008, Rawlings et al. 2010). Most PhyCys are proteins of small molecular size of about 12–16 kDa, without disulphide bridges and lack of putative glycosylation

sites. However, there are PhyCys of higher molecular weight, of approximately 23 kDa, characterized by the presence of a carboxy-terminal extension essential for the inhibition of a second family of CysProt, the C13 legumain peptidases (Margis-Pinheiro et al. 2008, Martinez et al. 2007, 2009, Martinez and Diaz 2008). Additionally, PhyCys of 85–87 kDa, designed as multicystatins because they contain several cystatin domains, have been also reported (Madureira et al. 2006, Nissen et al. 2009). Cystatin inhibition of CysProt is due to a tight and reversible interaction. It involves the N-terminal part of the protein that contain a glycine residue, and two hairpin loops in which a conserved QxVxG motif and a Trp residue are located (Stubbs et al. 1990). Besides, PhyCys have a plant-specific signature ([LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N) located in a $\alpha$-helix (Martinez and Diaz 2008). Most PhyCys have a signal peptide suggesting a non-cytosolic protein location. Madureira et al. (2006) and Nissen et al. (2009) detected multicystatins from solanaceous species such as potato and tomato in vacuoles and in other cytoplasm organelles. Similarly, oryzacystatins from rice have been found in the cytosol, chloroplasts, vacuoles and extra-cellular area (Prins et al. 2008, Tiang et al. 2009). Moreover, the subcellular location of the 13 barley cystatins fused to a green fluorescent protein has allowed establishing their differential presence in cytoplasm (mainly in the ER and Golgi complexes) and nuclei (Martinez et al. 2009).

Most reviews and articles on PhyCys describe these inhibitors as proteins involved in two main roles, as defense proteins and as plant regulators of endogenous protein turn-over (Martinez et al. 2009, Benchabane et al. 2010). The defense function is probably the most analyzed, and it has been inferred from their capability to inhibit the activity of digestive proteases from insects and acari in vitro, in experiments using artificial diets and in multiple bioassays on plants stably transformed with PhyCys genes (Atkinson et al. 2004, Alvarez-Alfageme et al. 2007, Carrillo et al. 2011a). It has also been described antimite activities and antipathogenic effects against fungi and viruses carried out by these inhibitors (Gutierrez-Campos et al. 1999, Martinez et al. 2003, Carrillo et al. 2011b). However, the main and less known function of PhyCys is the regulation of endogenous CysProt to prevent the breakdown of essential proteins involved in multiple physiological processes. PhyCys participate in different stages of plant growth and development, senescence, programmed cell death, as well as in the accumulation and degradation of reserves of seeds and tubers (Solomon et al. 1999, Martinez et al. 2009, Weeda et al. 2009).

## C1A proteases and PhyCys, partners in common function roles

Protein interactions are crucial for many cellular processes. Diverse approaches have been used toward understanding which proteins interact and how they interact. Physical in vitro interactions may be determined by resolution of crystal protein complexes. The Protein Data Bank (PDB, Berman et al. 2000) contains, relative to single protein structures, only a few complexes because of the difficulty of isolation and solving complex structures. From 1968, date in which the three-dimensional structure of papain was determined by X-ray diffraction at 2.8 Å resolution (Drenth et al. 1986) the crystal structure of several plant C1A CysProt has been resolved (e.g. castor oil CysEP, Than et al. 2004, barley EPB2, Bethune et al. 2006). For cystatins, the nuclear magnetic resonance (NMR) structure of oryzacystatin-1 (OC-1) from rice was the first available (Nagata et al. 2000), and recently, a crystal structure from potato multicystatin 2 (PMC2) was resolved (Nissen et al. 2009). Until now, a few structures of human cystatins in complex with papain-like peptidases have been determined, such as the human stefin B-papain, stefin A-cathepsin H and stefin B-cathepsin B complexes (Stubbs et al. 1990, Jenko et al. 2003, Renko et al. 2010). From plants, the structure of the tarocystatin–papain complex has been recently resolved (Chu et al. 2011) showing that human and plant cystatins have a similar binding mode to inhibit CysProt activity. As crystallization of protein complexes is sometimes very difficult, in silico

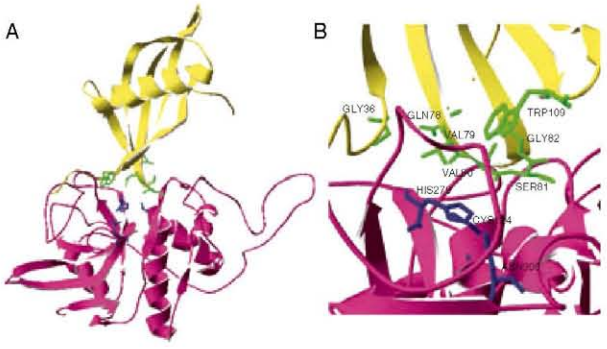


**Fig. 2.** Molecular modeling of C1A cysteine protease–cystatin interaction. The complex formed by barley cathepsin B HvPap-19 (pink) and barley cystatin HvCPI-6 (yellow) were modeled superimposing their homology-based three-dimensional models onto the three-dimensional structure of the stefin A-cathepsin B complex (PDB accession number: 1K9M) using the fit routines of the SWISS-PDB viewer program (Guex and Peitsch 1997). The catalytic triad of the CysProt is depicted in blue and the residues involved in the protease inhibitory activity of PhyCys are colored in green. (A) Complete model. (B) Close-up of the predicted region of interaction between the cystatin and the protease.

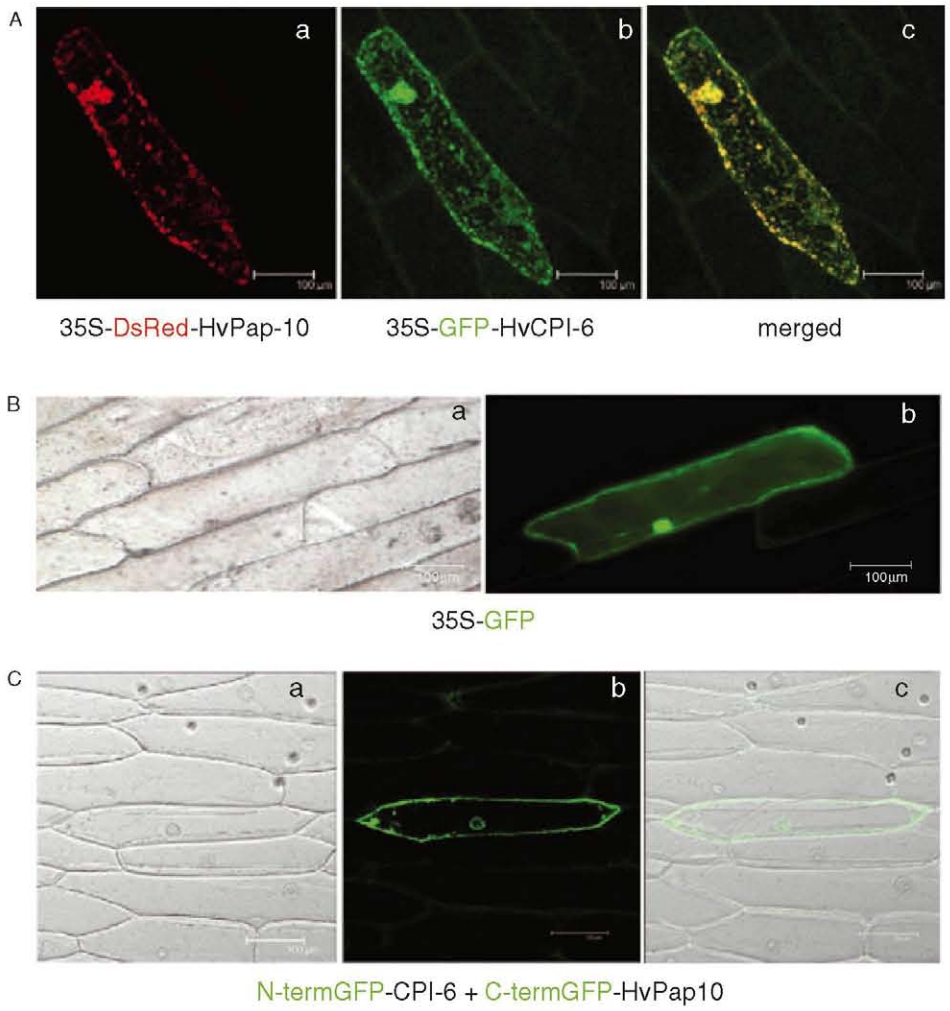


**Fig. 3.** Colocalization and protein–protein interaction of a C1A CysProt and a PhysCys from barley. (A) Subcellular colocalization of the barley cathepsin L-like, HvPap-10 fused to DsRed fluorescent protein (panel a) and the barley cystatin HvCPI-6 fused to the GFP-green fluorescent protein (panel b). Merged figures are in panel c. (B) Subcellular location of the GFP protein used as a control. Observation under Nomarski bright field is shown in panel a. (C) Subcellular localization of reconstituted GFP in transiently cobombarded onion epidermal cells with HvCPI-6 fused to the N-terminal GFP fragment (N-term) and HvPap-10 fused to the C-terminal GFP fragment (C-term). Nomarski bright field is shown in panel a. Merged figures are in panel c. Samples were observed in a confocal ultraspectral microscope. Scales are indicated in micron meter.

docking techniques become important tools to solving and understanding protein interactions. So, homology modeling is a promising new approach for predicting protein complex structures. In a recent study, homology-based docking approach was applied for CysProt and their inhibitor structures (Bishop and Kroon 2011). The conserved interaction between CysProt and inhibitors and the availability of crystal structures within the family are important factors that make the method feasible and the models of high accuracy. An example of homology modeling for protein complexes, between the cathepsin B (HvPap-19) and the cystatin 6 (HvCPI-6) from barley, is shown in Fig. 2. On the other hand, the interaction among cystatins and proteases can be indirectly tested by inhibitory protease activity assays using a substrate able to be degraded by the protease. In these assays, recombinant proteins purified from bacterial or yeast cultures are commonly used. The inhibition constant values obtained from these analyses are a measure of the specificity and capacity of inhibition of each cystatin on each protease and, therefore, indicate a physical interaction between both proteins.

In addition to in vitro assays, interactions between CysProt and their inhibitors may be tested by experiments using plant tissues. Only two studies have reported plant protease–cystatin complexes purified by immunoaffinity

or hydrophobic chromatography from leaves of maize and spinach (Yamada et al. 1998, Tajima et al. 2011), which indicate the difficulties of this approach to characterize the proteases and inhibitors that interact in the plant tissues. An alternative approach is to test the subcellular collocation followed by the interaction of both proteins in plant tissues using a molecular reporter such as fluorescence proteins. To demonstrate the interaction between barley PhyCys and CysProt in vivo, Martinez et al. (2009) used the Bimolecular Fluorescence Complementation (BiFC) assay to detect protein–protein interaction in the plant cell. Proteases and cystatins were translationally fused to the C- or N-terminal fragments of the GFP (green fluorescent protein) and cobombarded into onion epidermal layers. When a combination of a cystatin and a protease interacts, the GFP is reconstituted and fluorescence is recovered. Microscope observations corroborated in plant cells the in vitro inhibitory interactions between the PhyCys and C1A CysProt. An example of subcellular collocation and the GFP reconstitution that indicates protease–inhibitor interaction of barley proteins is shown in Fig. 3.

Besides the molecular interaction, a functional relationship between C1A proteases and cystatins has been supported by a reduced number of reports. The coincident in vivo localization of CysProt and PhyCys in the same subcellular compartments in the heterologous system of onion epidermal cells, further demonstrated by fluorescence complementation experiments, suggests their participation in common processes. The availability of specific antibodies will permit to extend these studies to other tissues at sequential developmental times. The confirmation of these lines of evidence requires the systematic gathering of additional data under different physiological conditions. Pairwise or multiple combinations of putative interacting CysProt and PhyCys, based on constants of inhibition and spatial–temporal expression patterns, will identify the partners involved in a particular process and their regulation throughout development in a tissue-dependent manner.

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