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MINI-REVIEW

The complex world of plant protease inhibitors: Insights into a Kunitz-type cysteine protease inhibitor of *Arabidopsis thaliana*

Sachin Rustgi[a,b], Edouard Boex-Fontvieille[c], Christiane Reinbothe[c], Diter von Wettstein[b,‡], and Steffen Reinbothe[c]

[a]Department of Plant and Environmental Sciences, Clemson University Pee Dee Research and Education Center, Florence, SC, USA; [b]Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA; [c]Laboratoire de Génétique Moléculaire des Plantes and Biologie Environnementale et Systémique (BEeSy), Université Grenoble Alpes, Grenoble, France

ABSTRACT

Plants have evolved an intricate regulatory network of proteases and corresponding protease inhibitors (PI), which operate in various biological pathways and serve diverse spatiotemporal functions during the sedentary life of a plant. Intricacy of the regulatory network can be anticipated from the observation that, depending on the developmental stage and environmental cue(s), either a single PI or multiple PIs regulate the activity of a given protease. On the other hand, the same PI often interacts with different targets at different places, necessitating another level of fine control to be added in planta. Here, it is reported on how the activity of a papain-like cysteine protease dubbed RD21 (RESPONSIVE TO DESICCATION 21) is differentially regulated by serpin and Kunitz PIs over plant development and how this mechanism contributes to defenses against herbivorous arthropods and microbial pests.

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Introduction

Proteases are involved in all aspects of plant life, ranging from seed germination to plant senescence. Proteases can do so by participating in key developmental processes such as fertilization, embryogenesis, seedling growth, plant maturation and the protection of adult plants from predators and abiotic stress.[1-3] Environmental cues like the amount, intensity and quality of incident light, water supply, nutrient availability, as well as biotic and abiotic stresses trigger the production or activation of proteases to induce changes in cell and plant morphology, metabolism and developmental state. In addition, proteases serve major housekeeping functions for example, the removal of damaged or misfolded proteins, transit peptides during intracellular targeting, or repressors blocking inducible signaling cascades and metabolic shunts under normal growth conditions. Based on their activity, proteases can be categorized into different classes, referred to as serine proteases, cystein proteases, aspartic acid proteases, metallo proteases and threonine proteases.[1]

Serine proteases are characterized by the presence of a single Ser residue in the catalytic His-Asp-Ser triad. Cysteine proteases contain catalytic Cys-His dyads that interact with a specific Asn residue in the active site. By contrast, the catalytic dyad of aspartic acid proteases is comprised of two aspartate residues, whereas metallo proteases need divalent metal cation such as Zn^{2+}, Mg^{2+} or Ca^{2+} for activity. Last but not least, threonine proteases possess an NH_2-terminal threonine as active site residue (see refs 1 and 4, for reviews).

Serine proteases constitute the largest family of plant proteases. Distinct serine protease activities have been identified in leaf tissues undergoing senescence and participate in the degradation of the photosynthetic apparatus and other chloroplast constituents.[5,6] The second largest family of plant proteases is constituted by aspartic acid proteases that are associated with nitrogen recycling in plants deprived of nutrients. Other, less abundant classes of plant proteases comprise cysteine and threonine proteases. Cysteine proteases are frequently found in lytic vacuoles, whereas threonine proteases are exclusively found in association with the 28S proteasome.[6] Since most plant proteases assume regulatory functions, it is of utmost importance to keep their activity under

CONTACT Sachin Rustgi srustgi@clemson.edu Department of Plant and Environmental Sciences, Clemson University Pee Dee Research and Education Center, 2200 Pocket Road, Florence, SC 29506, USA; Steffen Reinbothe sreinbot@ujf-grenoble.fr Laboratoire de Génétique Moléculaire des Plantes and Biologie Environnementale et Systémique (BEeSy), Université Grenoble Alpes 38041 Grenoble cedex 9, Grenoble, France.

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‡Deceased April 13, 2017

tight control. For example, most plant proteases are synthesized as zymogens amenable to activation by autocatalytic processing or trans-activation.[7] To make the regulation highly stringent and specific, plants evolved in terms of protease inhibitors (PIs), another layer of control.[8] PIs often represent peptides with low molecular mass. In plants, proteases and PIs co-exist in a surprisingly large variety. Based on their target protease specificity, PIs are classified as aspartic acid protease inhibitors (pepstatins), serine protease inhibitors (serpins), cysteine protease inhibitors (cystatins) and metallo carboxy protease inhibitors.[9] As a function of structural and biochemical properties, such as composition of the active site, presence or absence of disulfide bridge, mechanism of action, three-dimensional structure, as well as stability under heat shock conditions or in the presence of detergents, PIs are also classified as Bowman-Birk serine protease inhibitors, cereal trypsin/α-amylase inhibitors, cysteine protease inhibitors, metallo carboxypeptidase inhibitors, mustard trypsin inhibitors, potato type I inhibitors, potato type II protease inhibitors, serpins, soybean trypsin (Kunitz) inhibitors and squash inhibitors.[10] More recently, Rawlings and co-workers grouped PIs into 76 families, based on sequence homologies of the PIs' inhibitory domains.[11] In this classification, PIs with single inhibitor domains are dubbed simple inhibitors and those with multiple inhibitor domains are called complex inhibitors. Among the various inhibitors, Kunitz PIs are most interesting and the focus of the present review.

Plant Kunitz protease inhibitors

Kunitz PIs are widespread in the plant realm. They are capable of binding serine, cysteine and aspartic acid proteases.[12] Kunitz PIs form six groups, differing in the number of Cys residues and disulfide bonds, as well as component polypeptide chains.[12] Most Kunitz inhibitors have four conserved Cys residues forming two intramolecular or intermolecular disulfide (S-S) bridges. The occurrence of a single S-S bridge, unique (singular) cysteine residues or even the absence of cysteine residues are not exceptional, however. Kunitz PIs reversibly interact with their target proteases, forming stable complexes and inhibiting their catalytic activities in a competitive or non-competitive manner.[12] In addition, Kunitz PIs are characterized by molecular masses of ≈20 kDa, a low overall cysteine content, the presence of α-helices, and a common structural fold consisting of a β-trefoil formed by 12 antiparallel β-strands with long interconnecting loops.[12] Detailed information on these and other protease inhibitors can be retrieved from the PLANT-PI database at http://www.plantpis.ba.itb.cnr.it/.

Water-soluble chlorophyll proteins (WSCPs) constitute a small Kunitz PI sub-family. WSCPs are unique amongst chlorophyll (Chl) binding proteins in that they are hydrophilic proteins that do not contain carotenoids.[13] WSCPs are present in only a limited number of plant species belonging to the *Amaranthaceae*, *Brassicaceae*, *Chenopodiaceae*, and *Polygonaceae*.[2,3] Based on their unique photochemical properties, WSCPs are grouped into photoconvertible (class I) and non-photoconvertible (class II) WSCPs. Class II WSCPs can be further subdivided into class IIA and class IIB, using their affinities for chlorophyll (Chl) *a* and Chl *b* and the resulting Chl *a*:Chl *b* ratio. Class IIA WSCPs have been so far extracted from various plant species, including cauliflower,[14] black mustard,[15] rapeseed,[16] Brussels sprout,[17] Japanese radish,[18,19] kale[20] and *Arabidopsis thaliana*.[2,3,21,22] On the other hand, class IIB WSCPs have been exclusively found in *Lepidium virginicum*.[14,15,23,24]

Crystal structure of the Lepidium virginicum WSCP

To the best of our knowledge, the only crystal structure resolved so far is that of the WSCP from *L. virginicum*.[25] In this structure, four WSCP monomers of 180 amino acids each form a tetramer enclosing four tightly packed Chl molecules. The presence of a central hydrophobic cavity shields the Chls from the aqueous environment and thereby prevents their light excitation and subsequent interaction with molecular oxygen that, by triplet-triplet interchange, would easily provoke the formation of singlet oxygen operating as cytotoxin and cell death factor.[26] The unique 3D structure also explains why the Chls are largely protected from photooxidation, in the absence of carotenoids. Other WSCPs differ from the *L. virginicum* WSCP by the number of bound Chls, accounting to 1–4 per protein tetramer.[13] Moreover, the Chl *a*:Chl *b* ratio can be highly distinct and range from 1.5-10.[13] The WSCP from *Arabidopsis thaliana* (AtWSCP), a frequently used model plant, differs in this respect from the WSCPs of *L. virginicum* and *Brassica oleracea*, and it remains largely monomeric upon Chl binding.[2,22] Nevertheless, the Lepidium and Arabidopsis WSCPs share a highly conserved structure and topology if 3D-modeling is being performed (Fig. 1; see also Figs. S1-3).

Plant serpins

Serpins constitute another family of plant PIs. The Arabidopsis genome contains 41 potential PI genes, belonging to 8 of the 67 known protease inhibitor families in the MEROPS peptidase database. Serpins are grouped in family I4. Their name was originally derived from the fact that they are active as serine PIs,[27] although some

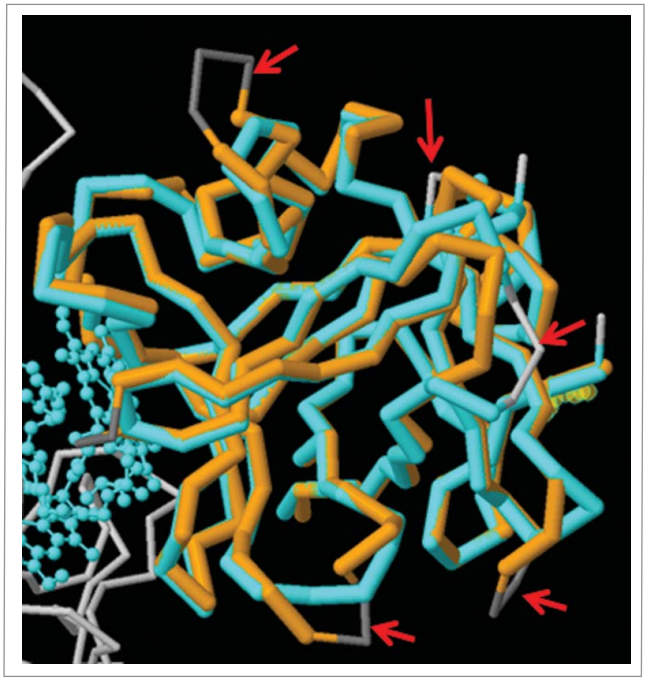

Figure 1. Three-dimensional structure of WSCPs from *Lepidium virginicum* and *Arabidopsis thaloiana*. 'Strucalign' was used to superimpose the AtWSCP model (monomer; ochre) on to the model of LvWSCP-tetramer (docked with chlorophyll molecules; light blue). Regions of disparity in the two models are marked with red arrows.

serpins inhibit cysteine proteases, whilst others have bifunctional activity or are non-inhibitory. All serpins displaying PI activity contain flexible bait loops and form covalent complexes with their target proteases. In this respect, serpins differ from other PIs, such as those in the Kunitz PI and potato PI families, which display 'standard' or 'Laskowski' mechanisms and from non-covalent, reversible complexes with their targets. The unique reaction mechanism of serpins explains why these PÏs produce dead-end complexes with their target proteases.

Multiple PIs can regulate the activity of a single protease during development

Despite the divergence in target protease specificity and mode of action, most PIs bind their target proteases in a substrate-like manner. Since related proteases show a high degree of homology in their active sites, substrate-like binding often leads to inhibitor promiscuity. With the availability of the human genome annotation, a ratio of approximately 1:5 was noted for proteases and PIs.[8] A similar ration was found for land plants, although the number of sequenced plant genomes is still too limited for definite clues. Anyhow, it appears that a plant protease is likely to be targeted by up to 5 PIs, belonging to one or more families. A particularly interesting example is provided by the cysteine protease RESPONSIVE TO DESICCATION 21 (RD21), an enzyme that was thus far implicated in desiccation responses and microbial defense.[28-31] RD21 is synthesized as a preproprotein that, like many other cysteine endo-proteases, has an NH_2-terminal propeptide with auto-inhibitory activity and a COOH-terminal granulin-domain containing propeptide with unknown function.[2,31] The NH_2-terminal propeptide is cleaved off by a yet unknown mechanism either requiring an autocatalytic processing under low pH or activity of a processing enzyme.[31]

RD21 is targeted by two different PIs over plant development: a serine protease inhibitor, dubbed AtSerpin1, and a Kunitz type protease inhibitor, namely, AtWSCP[2,32]. These two PIs are structurally highly distinct and thus exert different modes of action on RD21 (Fig. 2). Largely, Kunitz, Kazal, Bowman-Birk protease inhibitors follow a 'standard-mechanism' or 'Laskowski-mechanism' of protease inhibition and are generally much smaller in size than serpins.[33] As discussed above, Kunitz PIs form non-covalent and reversible complexes with their target proteases, in contract to serpins that

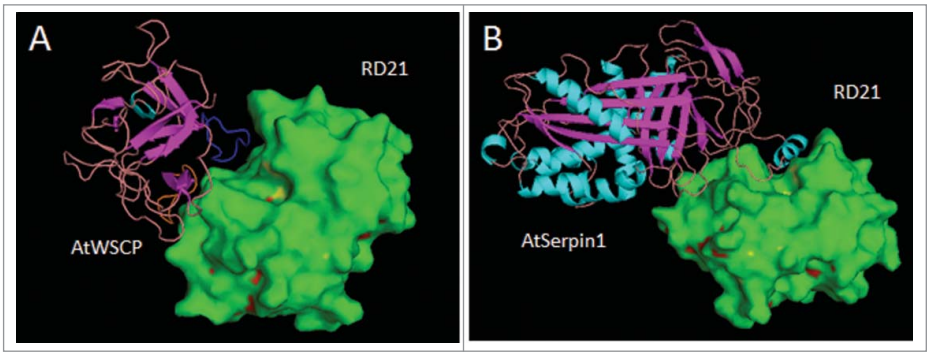

Figure 2. Interaction models of AtWSCP (A) and AtSerpin1 (B) with RD21 predicted using ClusPro 2.0. Structures of AtWSCP (A) and AtSerpin1 (B) are shown by ribbon diagrams, with the β-strands and α-helices highlighted in cyan and magenta, respectively. Loops in AtWSCP are shown in deep salmon, except for the 2nd and 5th loops, which were respectively shown in orange and blue. The structure of RD21 in either case is provided as surface model, with yellow = β-sheet, green = loop and red = α-helix.

form covalent, irreversible adducts.[33] AtWSCP displays a unique mechanism within the Kunitz PI family that resembles the one employed by cystatins.[2] In cystatins, some amino acid residues at the reactive center first bind in a substrate-like manner to residues of the active site of the target protease and a few subsiding amino acids turn away and out of the active site pocket, triggering other, surface-based interactions using exo-sites.[8,34] Unlike to what is observed in cystatins, the NH_2-terminal part of AtWSCP is not involved in the interaction with the target cysteine protease RD21. Instead, the second loop of AtWSCP binds to RD21 at an exo-site, which increases the surface area of the interaction.[2]

The inhibitory mechanisms of AtSerpin1 is fundamentally different and shall briefly be compared and contrasted with that of AtWSCP. The reactive center loop (RCL) of AtSerpin1 interacts with amino acids of the active site of the targeted protease RD21, whereas in case of AtWSCP the fifth loop serves such function, along with the second loop providing further stabilization to the formed complex (Fig. 2). AtWSCP exhibits a secondary and tertiary structures similar to that of other Kunitz PIs, consisting of 10 antiparallel β-strands connected by long loops that establish a β-trefoil or an antiparallel β–barrel. However, subtle structural differences exist between AtWSCP and other Kunitz PIs that concern the amino acid composition in the fourth loop comprising the RCL and the second loop containing the LHCII (light harvesting complex II) domain involved in Chl binding. The amino acid sequences of the 4^{th} and 5^{th} loops indeed show no conservation amongst other Kunitz PIs.[2,35] In addition, unlike other Kunitz PIs, AtWSCP possesses only two conserved cysteine residues that possibly form a disulfide bridge between the 2^{nd} and the 5^{th} loops.[2,35] Collectively, all of these structural features contribute to the function and target specificity of AtWSCP. In previous studies, an Arg/Lys residue at the P1 position in the RCL of Kunitz PIs was shown to be essential for their interaction with different serine proteases.[36,37] Interestingly, a unique Gly residue in the 4^{th} loop of AtWSCP replaces the Arg/Lys residue found in other Kunitz PIs.[2,35] Moreover, other specific residues at the 5^{th} and 2^{nd} loops were found to be interacting with the residues at the active site and an exo-site, respectively, of the target cysteine protease RD21 (Fig. 2) and, as mentioned earlier, a disulfide bridge likely connects the two loops and thereby contributes to the stability of the complex.[235]

Structural modeling to underscore the AtWSCP-RD21 interaction

In silico modeling of AtWSCP (At1g72290) performed by Boex-Fontvieille et al.[2] suggested that the 3D-structure of AtWSCP most closely resembled that of soybean Kunitz trypsin inhibitor and tamarind Kunitz inhibitor (TKI). All three proteins share the presence of an α-turn and 10 anti-parallel β-strands that form a barrel-like structure.[36,37] Similarly, the molecular modeling of RD21 (At1g47128) revealed a typical papain-like structure, with two almost equally sized lobes dubbed R (right) and L (left), divided by an active site cleft.[38] Studies on oryzacystatin-I and papain-like proteases as well as on TKI and its interactions with factor Xa and trypsin[34,36,37] suggest the second loop (Ala37-Leu46, orange) that spans β-strands 2 and 3, and encompasses the LHCII signature, and the fifth loop (Lys84-Ser95, blue) which connects β-strands 5 and 6, to establish the reactive-site loop (RSL) (Fig. 2; cf. ref. 2). In this interaction model, Try88 and Pro89 in the RSL of AtWSCP are predicted to intrude into the active site region of RD21 containing Cys161 and His297 and thereby to block its proteolytic activity (cf. ref. 2). Moreover, one amino acid residue, Lys92 in the RSL, and two amino acid residues, Leu41 and Pro42, in the LHCII signature sequence are predicted to form hydrogen bonds with amino acid residues Asp154 and Lys227, respectively, in RD21[2]. Together, these hydrogen bonds are expected to stabilize the observed AtWSCP-RD21 complex. On the other hand, the presence and close physical proximity of the LHCII signature of AtWSCP to the catalytic triad of RD21 seems to explain the observed light-triggered, chlorophyllide-dependent dissociation of the AtWSCP-RD21 complex in vitro and in planta.[2]

Biological significance of protease-PI interactions

There is an increasing amount of work that highlights the great biological significance of protease-PI interactions in nature. Fig. 3 highlights at least some of these interaction for animals and plants. Obviously, proteases and their respective PIs operate like twins in a 'Yin and Yang' fashion and antagonistically control almost all stages and levels of life. In a sense, they could be seen as Siamese twins that live together in the same body (organism) but have their own mind such that their interaction must be tightly controlled, assuring species survival in a continuously changing environment.

Kunitz PIs and serpins are two examples of serine protease inhibitors that are of ubiquitous occurrence in nature. Kunitz PIs contain a ≈60 amino acid signature motif stabilized by three disulfide bonds that can be present in a singular term or repeated several times and/or combined with other PI modules. Kunitz PIs are widespread in nature and have been reported to occur in microbes, animals and plants (see ref. 39, for review). In vertebrates, Kunitz PIs mostly operate in inflammatory processes, whereas in invertebrates they are involve in a

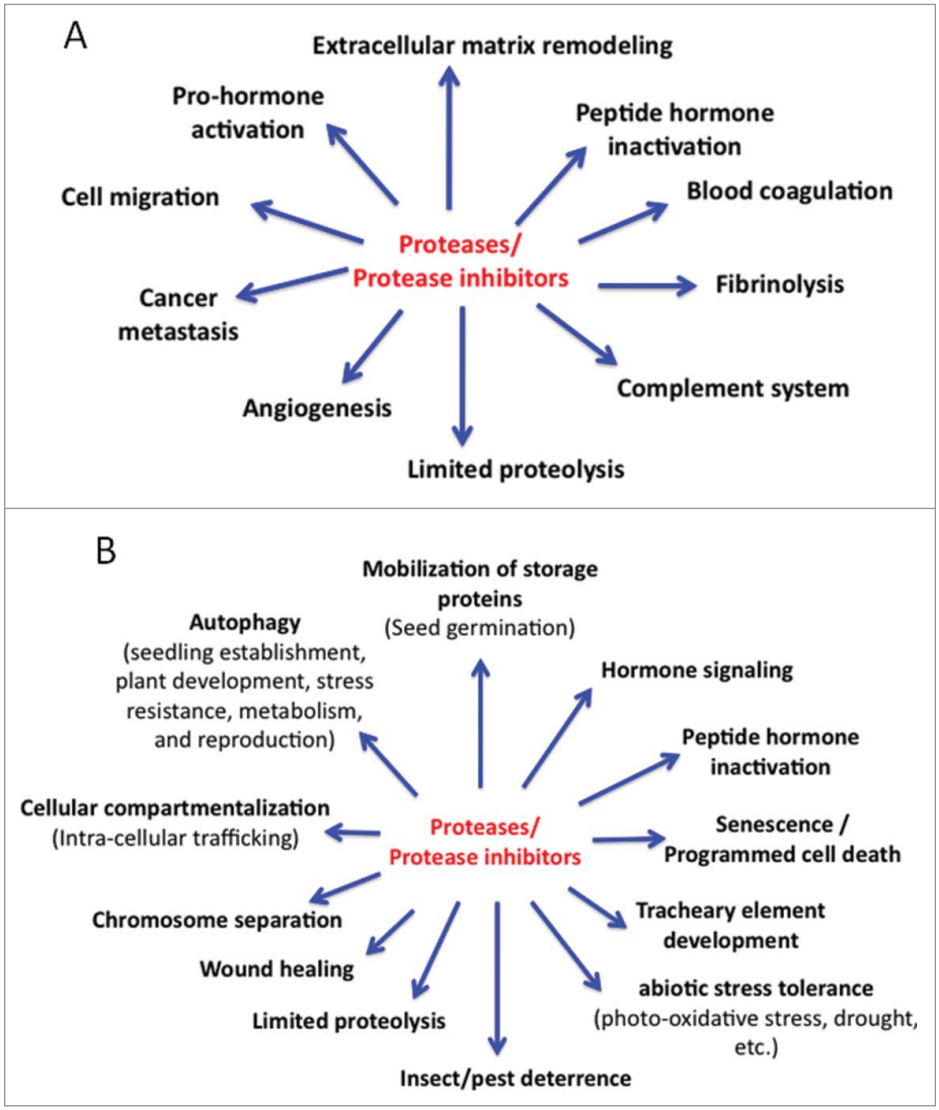

Figure 3. Biological significance of protease and protease inhibitor interactions in animals (A) and plants (B).

vast range of biological processes, covering predation and defense, such as found for scorpions and cone snails where they operate as both neurotoxic and protease inhibitory activity or typical Kunitz type toxins. Other examples comprise Kunitz PIs that protect parasitic helminthes from host digestive proteases. As a last example, Kunitz-type PIs were found to function as inducers of IgE-mediated allergic reactions in nematodes and anti-coagulant factors in blood sucking arthropods and were additionally implicated in defenses against microbial pests.

Serpins as a second, large family of serine proteinase inhibitors perform a similarly wide variety of biological functions (see ref. 40, for review). In the fruit fly *Drosophila*, serpins control development and reproduction. In arthropods, they regulate insect innate immunity via inhibition of serine proteinase cascades that initiate immune responses such as melanization and antimicrobial peptide production. In addition, several serpins with anti-pathogen activity serve as ad-hoc defense compounds that are expressed in response to infection. Beyond their function in host-pathogen interactions, serpins are ingredients of several venoms in parasitoid wasps and saliva of blood-feeding ticks and mosquitoes. Because of their distinct features as immune-suppressors and anti-coagulants, some serpins are of interest for vaccine development.

Both, Kunitz PIs and serpins have been identified in marine venomous animals, such as sea anemones, as well as terrestrial venomous animals, such as scorpions, spiders, Anurans, and Hymenopterans (see ref. 41, for review). Venomous arthropods such as Brown spiders use their venom comprising, besides Kunitz PIs and serpins, phospholipase D, metalloproteases (astacins) and insecticidal peptides (knottins) for predation and defense. Additional components that might be involved in these processes include hyaluronidases, allergen-like

toxins and histamine-releasing factors. Spider bites in humans provoke several injuries including spreading dermonecrosis, hematological abnormalities and impaired renal function, collectively referred to as loxoscelism, such that understanding PI function may contribute to new therapies.

Both Kunitz PIs and serpins control the plant cysteine protease RESPONSIVE TO DESICCATION 21 (RD21) and thereby establish an efficient means to deter both microbial pathogens and arthropodous crustacean such as pillbugs and woodlice and fine-tune these activities over plant development.[2,3,35] To avoid any uncontrolled RD21 action, nature in fact has evolved different strategies to fine-tune the expression and activation of RD21 over plant development. The first mechanism is to keep RD21 silent until needed. This is achieved in seeds by virtue of maintaining RD21's primary structure as proprotein comprising the NH_2-terminal auto-inhibitory domain.[21,31] Only during seed germination when pH drops below 5, intramolecular conformational changes are supposed to take place that lead to the removal of RD21's propeptide and its activation.

A second mechanism to control RD21 activity is provided by fine-tuning the expression of the corresponding PIs, AtWSCP and AtSerpin1. Whereas AtWSCP is expressed during skotomorphogenesis and accumulates in the apical hook, but not the cotyledons, AtSerpin1 shown an inverse expression pattern and is highly abundant in the cotyledons and undetectable in the apical hook.[2,32,33,35,42] Meta-expression profiling of *AtWSCP*, *RD21* and *AtSeprin1* using the eFP Browser revealed 1.37 to 8.03 fold increase in *AtWSCP* expression during the first 4–12 h of plant etiolation (skotomorphogenesis). Similar, though less pronounced, was the shift in *RD21* expression (from 0.01- to 0.35-fold) during this developmental stage, suggesting some temporal overlap in *AtWSCP* and *RD21* expression. *AtWSCP* gene expression is negatively light-regulated and under hormonal control by jasmonic acid and ethylene.[3,43] By contrast, *AtSerpin1* expression was constitutive and insensitive to phytohormones (refs. 3 & 43, and unpublished results).

A third mechanism to regulate RD21 activity is suggested by in silico localization data. Whereas AtWSCP and RD21 accumulation overlapped spatially and was similarly detectable in the Golgi apparatus, the endoplasmic reticulum, the extracellular space and the vacuole, AtSerpin1 localized exclusively to the cytosol and, to some extent, to chloroplasts.[2,35] These differences were supported by findings showing that AtWSCP traffics to vacuoles under certain conditions to inhibit papain-type cysteine proteases including RD21.[21,42] Moreover, cytological studies carried out by Boex-Fontvieille et al.[2] revealed a localization of AtWSCP in cell wall and/or apoplastic spaces. Interestingly, also AtSeprin1 was found in the apoplast (and the endoplasmic reticulum).[44] Thus far, RD21 from Arabidopsis has not been localized in intercellular spaces, although the defense protease and RD21 homolog of tomato, C14, is an apoplastic protein. In other studies, RD21 was reported to be an abundant vacuolar protein.[30] Because the vacuole rapidly collapses and releases its content into the apoplast during the hypersensitive response,[30] a set-point control mechanism was proposed that could limit the activity of RD21 during defenses of microbial foes.[42] Apart from the above data, all three genes, *AtWSCP*, *AtSerpin1* and *RD21*, map to the long arm of Arabidopsis chromosome 1 and thus could be part of the same transcriptional domain (Fig. S4).[45] However, the observed differences in *AtWSCP*, *AtSerpin1* and *RD21* expression noted in the in silico transcript profiling studies are suggestive of additional control mechanisms that need to be explored in the future work.

In summary, our findings and those of other groups highlight that the activity of RD21 is controlled by two distinct PIs over plant development, a Kunitz PI dubbed AtWSCP that operates during skotomorphogenesis[2,3,35,45] and flower development[46] and a serine protease inhibitor dubbed AtSerpin1 that acts in leaves during stress responses, defense against microbial pathogens and presumably also during senescence.[35] Unique regulatory mechanisms assure that the two PIs (AtWSCP and AtSerpin) do not compete with each other for RD21. The AtWSCP-based mechanism of RD21 sequestration is part of a larger response that permits deterring herbivorous arthropod crustacean that often pray on seeds and young-born seedlings and attempt to consume them.[47] Due to the highly specific co-expression of RD21 and AtWSCP in the apical hook of etiolated seedlings, an efficient protection mechanism is provided, making the new-born sprouts "untasty" and thereby permitting apical hook opening during greening without damage by herbivorous arthropods. It is noteworthy that Arabidopsis avoids using serpins for this task because these PIs are quite abundant in arthropods[40] and would be easily neutralized by the devourers.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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