Review: The future of cystatin engineering

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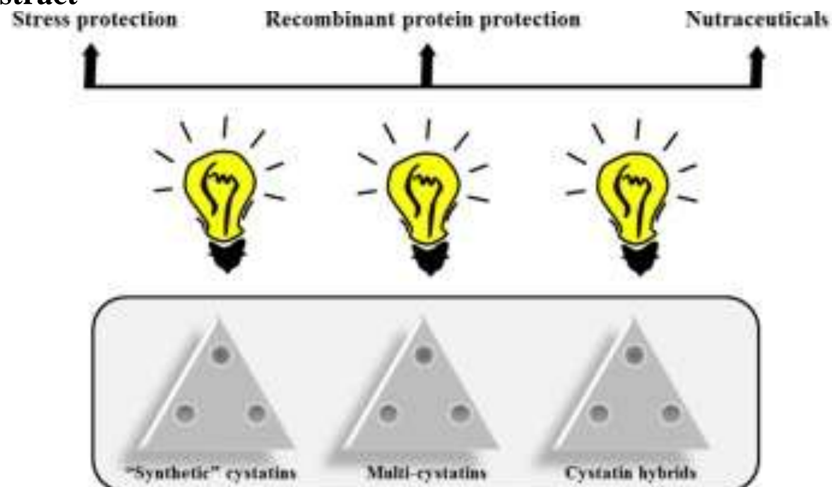
Highlights:

- Plant cystatins have a vital protective role in plant and human health
- Cystatin engineering is closely associated with plant genetic engineering
- Engineering involves amino acid substitution and multi-/hybrid cystatin formation
- Cystatins with rare or unique amino acids might help in cystatin engineering
- Engineering cystatins for the improvement of recombinant protein production is propitious

Graphical abstract



ABSTRACT

Plant cystatins are naturally occurring protease inhibitors that prevent proteolysis by papain-like cysteine proteases. Their protective action against environmental stresses has been relatively well characterised. Still, there is a need to greatly improve both potency and specificity based on the current rather poor performance of cystatins in biotechnological applications. Research in creating more potent and specific cystatins, including amino acid substitutions in either conserved cystatin motifs and/or at variable amino acid sites, is reviewed. Existing gaps for better understanding of cystatin-protease interactions are further explored. Current knowledge on multi-cystatins or hybrid protease inhibitors involving cystatins as an additional option for cystatin engineering is further outlined along with the nuances of how cystatins with rather unusual amino acid sequences might actually help in cystatin engineering. Finally, future opportunities for application of cystatins are highlighted which include applications in genetically modified transgenic plants for environmental stress protection and also as nutraceuticals, as part of more nutritious food. Further opportunities might also include the possible management of diseases and disorders, often associated with

lifestyle changes, and the most immediate and promising application which is inclusion into plant-based recombinant protein production platforms.

Keywords: Cystatin; plant; cystatin engineering; multi-cystatins; phytocystatin; protease inhibitor; proteolysis.

1. Introduction

Cystatins function as competitive inhibitors which coordinate the biological activity of C1 class (papain-like) cysteine proteases that are involved in proteolytic processes and are present in almost every form of life (for an overview see [1]). Cystatins bind to the active site of cysteine proteases as pseudo-substrates rendering target cysteine proteases incapable of cleaving peptide bonds. The Phytozome database (www.phytozome.net) currently contains over 300 such cystatin-like sequences from the Viridiplantae kingdom and 706 C1 cysteine protease sequences. This wealth of data not only allows for more detailed studies on the expression and functional analysis of cystatins and the cystatin-cysteine protease interactions, but also allows for the engineering of cystatins with improved potency and specificity against target proteases.

Cystatins have many beneficial properties for plants and humans. Cystatins function in the native host-plant defense system and they are expressed in response to wounding and pest infestation. In this regard, the potential of cystatins for pest control has been intensively explored in transgenic plants (for an overview, see [2]). Acting as anti-digestive compounds, cystatins cause protein deficiency, slowing pest development and reducing survival. The *Bacillus thuringiensis* (*Bt*) toxin is also currently widely applied for insect control with a number of insecticidal *Bt* Cry toxins, such as Cry1 and Cry2 for Lepidopteran pest control,

and Cry3 proteins for the control of pests from the Coleoptera order being used [3, 4]. *Bt* Cry toxins differ from most conventional insecticides. They are toxic only to a small range of related insects. This is due to the requirement of specific pH levels, enzymes, and midgut receptors to activate and bind a Cry toxin to midgut cells. Binding leads to pore formation in the insect's intestine and death. However, since cystatins are active at acidic pH levels, found in Coleopteran insects (for an overview, see [5]), cystatins could synergistically supplement *Bt* action in the effort to control Coleopteran insects and possibly delay build-up of *Bt* resistance in these insects.

Cystatins can also control fungal and viral pathogens acting against viruses by affecting their replication which requires cysteine proteinase activity [6]. Investigation of cystatin antifungal activity has already revealed a toxic effect on fungal growth [7]. However, the exact mechanism of control of fungal growth is still poorly understood.

Cystatins are further involved in the regulation of plant developmental processes ranging from seed germination [8] to natural and abiotic stress-induced senescence (for an overview see [9]). Expression of an exogenous cystatin in a genetically modified transgenic tobacco plant limits chilling and drought sensitivity [10] and in soybean, controls shoot branching and plant growth [11]. Exogenous cystatin expression also represses potato tuber sprout growth associated with loss of apical dominance and formation of an increased number of small buds at the skin surface [12]. In all instances, these phenotypic changes are very likely caused by preventing endogenous plant cysteine protease activity. A number of transgenic plant lines expressing exogenous cystatins were successfully produced and tested over the last 15 years for various protease targets. Nonetheless, serious doubts have been raised about the actual application potential of cystatins. This skepticism is based on findings that cystatin-mediated protection against pests is generally lower than that provided by potent conventional chemical pesticides. Lower potency and poor performance was based on the

pest's adaptation to the presence of an exogenous cystatin in transgenic plants and in turn expresses cystatin-insensitive proteases. Insects have developed mechanisms through coevolution, such as changes in gene expression in the gut, to circumvent any anti-digestive effect caused by their plant host [13]. This 'weakness' has raised the need to improve the potency of natural cystatins for any realistic inclusion into future biotechnological applications. Such a potent cystatin should ideally be very specific, e.g. specific against a pest's gut protease and less active against a plant cysteine protease. This would limit any pleiotropic effects in the plant (for an overview, see [2]). One strategy to obtain better specificity is the use of more specific promoter sequences. Such promoter might allow cystatin expression only when the plant is pest-infested. Expression of an exogenous cystatin under a wound-specific promoter, such as the 5' region of the wound-inducible wun1 gene from potato is a possibility, in particular, for controlling chewing insects [14]. Another possibility for a wound-inducible promoter is the recently characterized promoter region for the rice DNA binding with one finger (Dof) protein [15]. Dof proteins are plant-specific transcription factors, with a particular class of zinc-finger DNA-binding domain and four wound-response-like *cis*-acting elements (PI-II, EIRE, W box, and G box-like elements) which were recently identified in the *OsDof1* promoter region. Rationally designed and constructed synthetic promoter sequences, based on cis-motif engineering (for an overview see [16]), could become a powerful and efficient method for precise regulation of targeted cystatin expression. Recent identification and sequencing of members of cystatin families in various plant species with information deposited in databases like the Phytozome database (www.phytozome.net) will already allow identification of such wound-response-like *cis*-acting elements.

There is a need to improve the potency and specificity of natural cystatins for any realistic inclusion into biotechnological applications, and as such, we have taken a specific

focus within this review on cystatin engineering as an effective tool for creating new cystatins with higher potency and specificity. Cystatin engineering and the potential of such cystatins in biotechnological applications has not been extensively reviewed [2, 5, 17]. Therefore, in this review, we particularly detail what has been achieved thus far by cystatin engineering where various amino acids were substituted in the cystatin amino acid sequence, either in the conserved protein regions or at variable amino acid sites. A further motivation for writing this review also arose when we recently studied the significance of naturally occurring diversity within conserved cystatin motifs. These motifs contribute to the strength of interaction with cysteine proteases of different biological origins. During this investigation, we found that a papaya cystatin had much lower activity against banana weevil cysteine proteases [18]. Subsequently, we tried to improve its potency by amino acid exchange. When we applied site-directed mutagenesis, the interaction affinity of the papaya cystatin changed and the cystatin was as potent as the oryzacystatin-I (OC-I) from rice against both papain and cathepsin-L [19]. We further highlight in the review the current knowledge gaps and provide possible solutions to fill those gaps. We also summarize other recent approaches for the improvement of cystatin potency and specificity such as the developing of multi-cystatins or hybrid protease inhibitors with cystatin inclusion. Furthermore, we outline how unique and naturally occurring cystatins might help in altering cystatin design. Finally, we focus on future challenges for cystatin engineering and highlight possible short and long-term opportunities for such engineered cystatins.

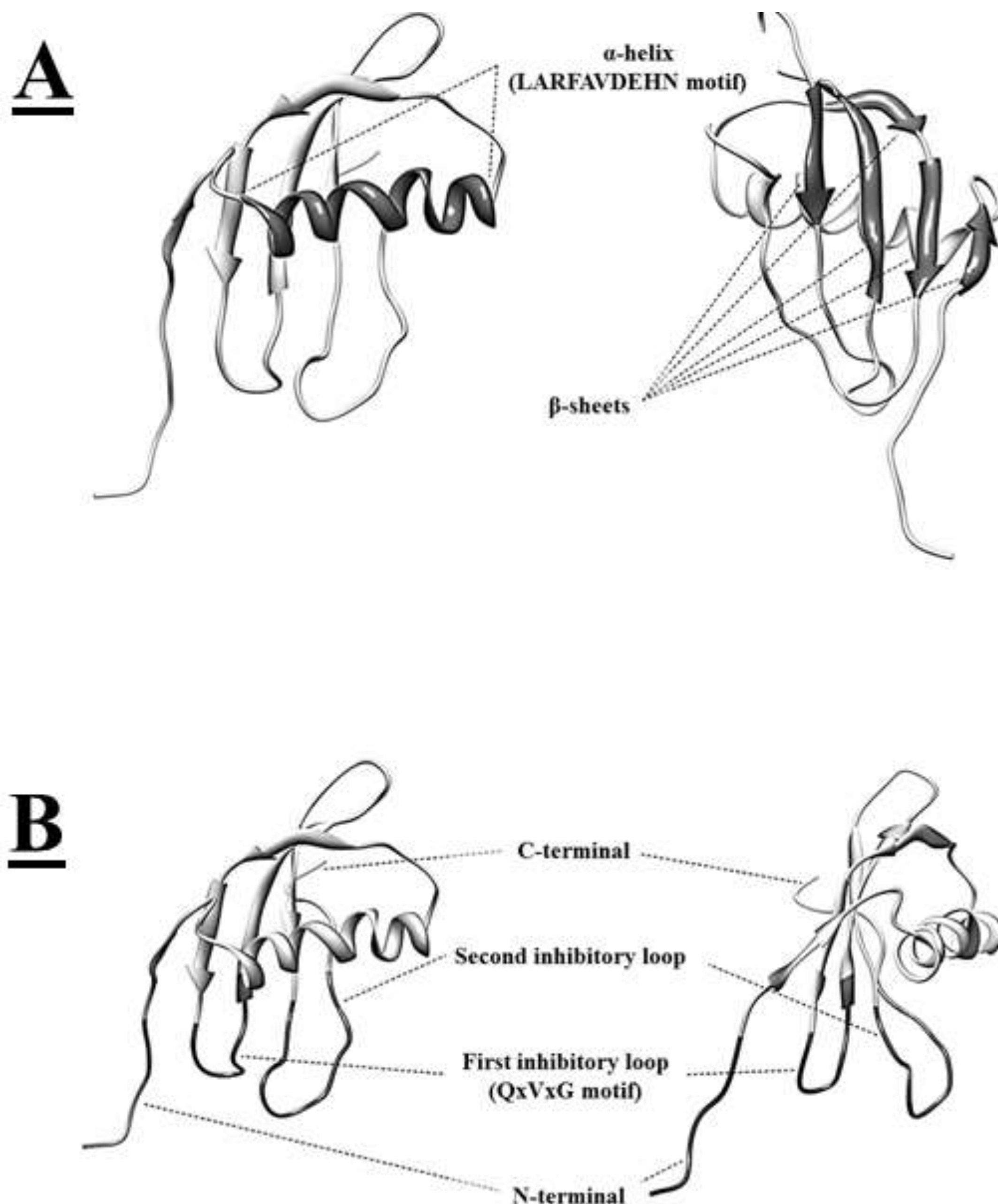
2. Engineering cystatins

Protein engineering is a powerful and efficient tool to alter a target protein, in a relatively short time period. This allows for the best combinations of amino acid sequences

that appropriately modify the characteristics of the protein to be identified. Therefore, tailoring the cystatin amino acid sequence by applying protein engineering tools offers an exciting option to improve both cystatin potency and specificity. In contrast, searching for naturally occurring cystatins with more potency and specificity is a much more laborious task despite the current extensive genome sequencing initiatives for many plant species and the discovery and characterization of cystatin families [20, 21]. Protein engineering requires a detailed knowledge of the structure and function of the protein to make any desired changes. This information is already available for cystatins [22]. The rational design for protein engineering is based on 3-D models for protease-protease inhibitor complexes, which are needed for the identification of target amino acids to be changed. A human stefin B-papain complex has served as a structural model and has allowed the identification of cystatin target sites that might be changed [5]. The well-developed technique of site-directed mutagenesis has then been a relatively inexpensive and simple tool for amino acid change. Any relevant target sites for site-directed mutagenesis were further identified by high throughput screening with a molecular phage display. In phage display, bacteriophages 'display' the investigated protein on their surface and displaying phages are then screened against a target protein [23]. Due to the possibility of synthetically producing proteins with the size of cystatins, testing of these synthesized mutant cystatins, is possible. This offers the option to evaluate relevant target sites in the cystatin's structure for their involvement in potency and specificity.

Cystatin engineering has currently focused on substituting amino acids. Substitution has been mainly done in the three cystatin structural elements. Cystatins are stabilized through a helix-sheet architecture formed between a central α-helix and a five-stranded antiparallel β-sheet core [24] (Figures 1A-C). They interact with target cysteine proteases [22] in order to prevent access of the active site of proteases to protein substrates. Recent engineering approaches have also included exploring amino acids at hyper-variable sites in

Fig. 1. Molecular cystatin model based on OC-I to illustrate positions of α-helix and the 5 β-sheets, characteristic of plant cystatins. (A) Side and (B) front views of the rice OC-I model with conserved motifs typically found in the structure of plant cystatins and (C) cartoon of the generalized secondary structural elements with β-sheets (numbered from 1 to 5), the spiral representing the single α-helix and positions were loops occur are indicated with a paper clip mark and labelled 1 to 4. Indicated regions contain amino acid residues involved in the interaction with target protease consisting of N-terminal with GG amino acid string, first inhibitory loop with the conserved QxVxG motif and the second inhibitory loop with the PW amino acid string.



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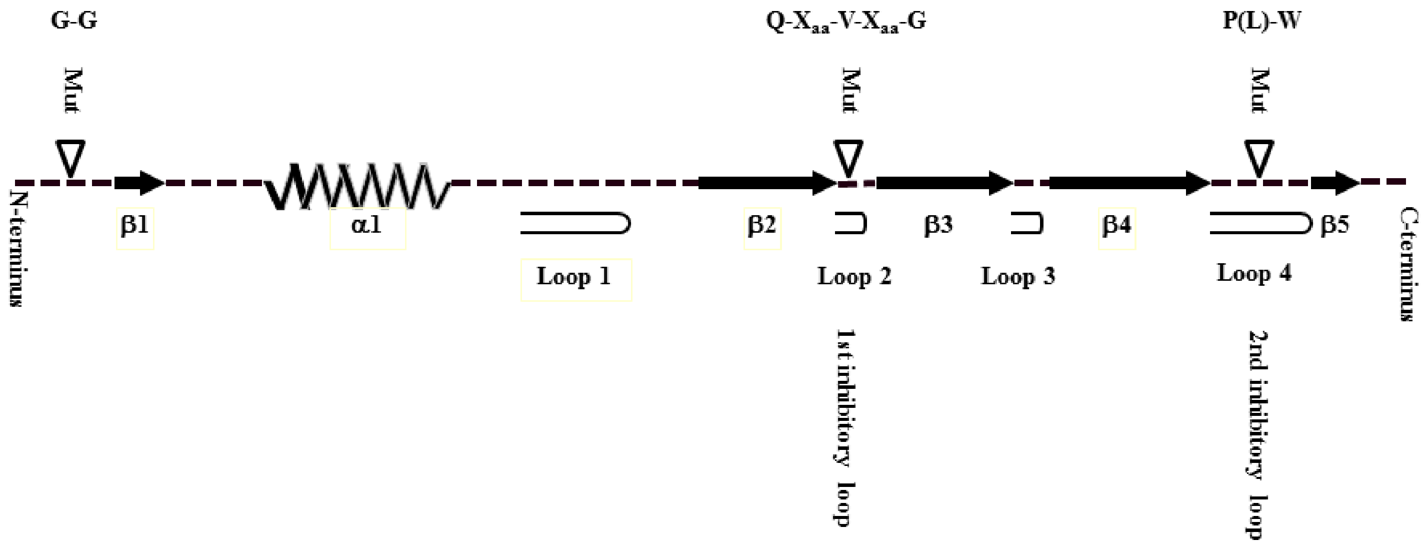


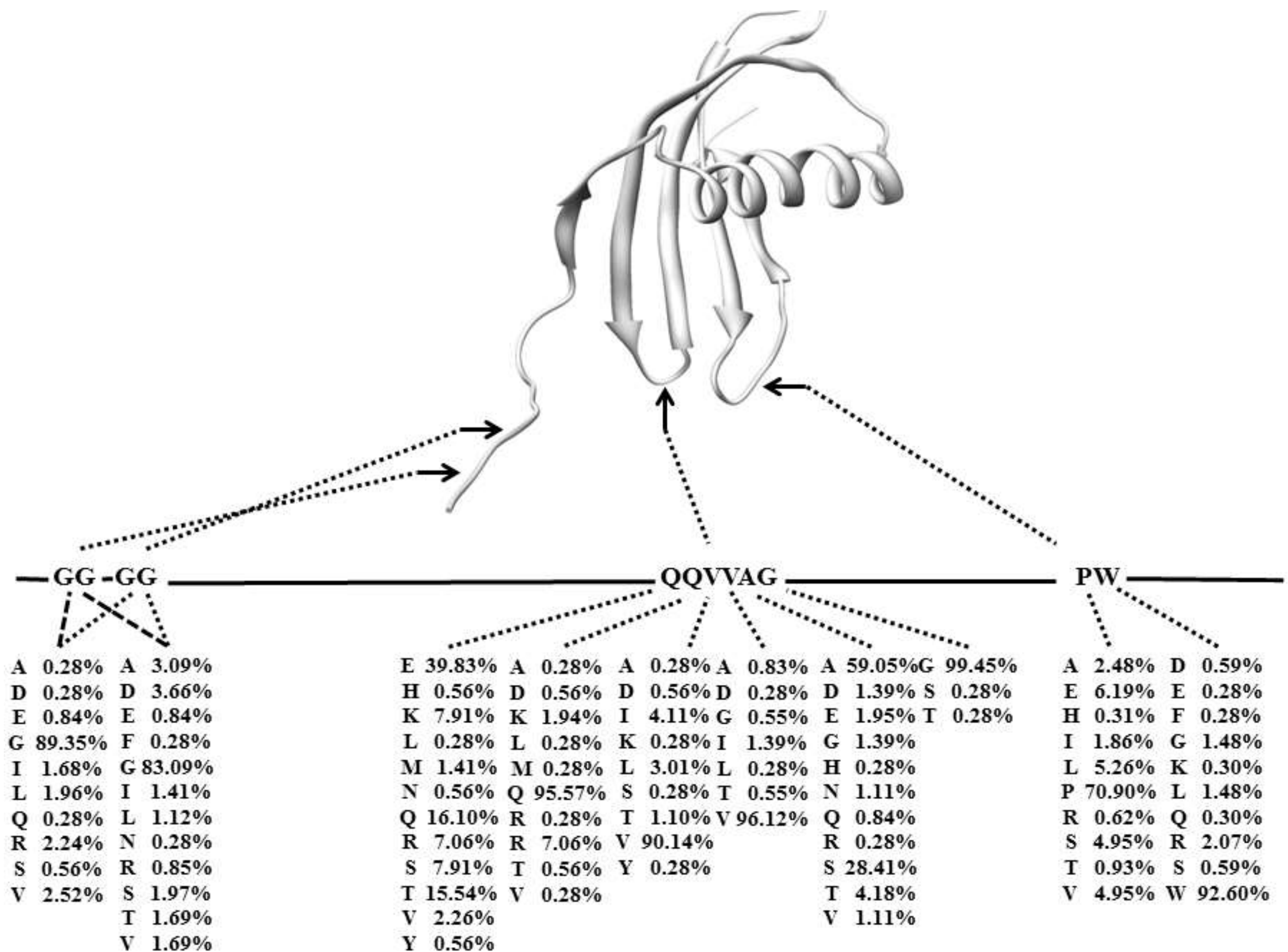
the cystatin. The best-characterized naturally expressed plant cystatin is rice OC-I, which is expressed in the rice endosperm, was first described 30 years ago (for an overview see [5]). Rice OC-I has 102 amino acid residues, but no disulfide bonds, and rice OC-I was the most potent cystatin when tested against papain and compared to a range of soybean cystatins [20]. In the following sections, we briefly review the achievements obtained thus far in cystatin engineering.

2.1 Conserved cystatin motifs

Improvement of cystatin potency and specificity has mainly focused on substituting amino acids in conserved cystatin motifs. These motifs are within two inhibitory hairpin loops that penetrate the active site cleft of the target cysteine protease [5] (Figures 1B and C). An additional N-terminal mobile element does not penetrate the active site, but contributes to binding. A further conserved 'LARFAVDEHN' motif, with still unknown function, exists in

the first central α-helix (Figure 1A). The N- and C-termini further contribute to cystatin specificity [5].

Fig. 2. Comparison of conserved positions (underlined; GG, QVVAG, PW in rice OC-I) of 332 phytocystatin amino acid sequences, from different plant species, scored and illustrated on the amino acid sequence of rice OC-I.



We recently obtained over 300 plant cystatin sequences from the Phytozome database and a multiple sequence alignment was performed. From this alignment, the frequency of each possible amino acid was determined for each of the conserved motifs in the protein

sequence (Figure 2). Frequently occurring amino acid variations in cystatins were changed to infrequent variants, or *vice versa*. In the experiments, purified natural and mutated cystatins were used. Purified cystatins can easily be produced in a bacterial expression system with the *E. coli* strain, BL21. In the process, a cystatin sequence is cloned into a plasmid allowing expression of a gene fusion, for example, a Glutathione S-transferase (GST)-cystatin fusion [20, 25]. The fusion protein produced is then purified with affinity chromatography (Glutathione Sepharose when a GST-cystatin fusion is expressed). The tag is finally cleaved with a protease (for example Factor Xa) to release the purified cystatin. Most of the amino acid exchanges reported in conserved motifs and predominantly carried out with rice OC-I against papain as a target cysteine protease, resulted in reduced cystatin activity. Changes in the N-terminus included the highly conserved G-10 [26], substituting Q and V (V→D) residues in the central Q-X_{aa}-V-X_{aa}-G motif of the first inhibitory hairpin loop as well as substituting the conserved P (L)-W motif in the second binding loop of the C-terminus [27]. The tryptophan (W) in the C-terminal P (L)-W motif is by far the most conserved amino acid in cystatins. A recent screen of 332 cystatin sequences found that 92% contained this tryptophan (Figure 2). Partial deletions and removing the tryptophan in the C-terminal P (L)-W motif decreases cystatin activity [27]. Furthermore, two barley cystatins without this tryptophan had very little activity against cysteine proteases [21]. In addition, exchanging this tryptophan in the C-terminal P (L)-W motif in rice OC-I, such as substituting the tryptophan with glycine (G), caused a loss of cystatin activity [19]. Although exchange or deletion of this amino acid decreased the cystatin potency, the amino acid is probably not essential for the overall cystatin's inhibitory capability [21].

Amino acids in conserved cystatin motifs were also successfully substitituted. When several amino acids of a barley cystatin (Hv-CPI) were mutagenized, the majority of variants retained antifungal and cysteine-proteinase inhibitory activity [28]. However, one mutant

with a C→G exchange, with C adjacent to G in the consensus cystatin-reactive site Q-V-V-A-G, was more active against papain and cathepsin B. An engineered rice OC-I-variant also had greater nematicidal activity when amino acid D-86 was deleted from the original rice OC-I sequence [29]. Deleting this amino acid very likely removed an interference caused by this amino acid in the cystatin-cysteine protease interaction. Soyacystatin N variants with higher activity against papain were also identified when variants carried mutations in the first and second hairpin loop [30]. Variants originating from screening a phage display library derived from a nematode cystatin and mutagenized in the first inhibitory hairpin loop were more potent against digestive proteases of the bean weevil, *Acanthoscelides obtectus* [31]. We also recently improved cystatin potency against papain when a rather weak-acting papaya cystatin was mutagenized in the Q-X_{aa}-V-X_{aa}-G motif to obtain a cystatin more similar to rice OC-I [19].

2.2 Hyper-variable sites

Interest in cystatin engineering also arose through the discovery of variable amino acid sites in cystatins. Variability and positive selection allows for functional diversification as a response to a selective pressure caused, for example, by stress. Such hyper-variable cystatin sites under selective pressure have been recently identified and characterized in more detail [32, 33]. These sites in rice OC-I are located within the N-terminus on each side of the conserved G residues (V-8, L-9, N-17), within the first inhibitory hairpin loop at Q-52 and V-54 and in the second inhibitory hairpin loop at P-83 and Q-91. Interestingly, such hyper-variable and positively selected amino acid sites have also been discovered in digestive cysteine proteases of herbivorous Coleopteran insects. This is probably an adaptation in response to plant cystatins [34].

The potential of cystatin engineering at such hyper-variable sites for improved inhibitory potency and specificity was clearly demonstrated with the eighth inhibitory unit of the tomato multi-cystatin, *Sl*CYS8. Single mutations at hyper-variable sites strongly influenced inhibition against cysteine proteases [33]. When a collection of 29 *Sl*CYS8 variants were assessed against insect and plant cysteine proteases, some of these multi-cystatin *Sl*CYS8 variants strongly inhibited digestive larval cysteine proteases of the Colorado potato beetle (*Leptinotarsa decemlineata*). They were only weak inhibitors in the two-spotted stink bug (*Perillus bioculatus*), a natural predator of the Colorado potato beetle. Furthermore, *Sl*CYS8 variants had various activities against endogenous potato leaf cysteine proteases. An interesting prospect may be to evaluate if additional site-directed mutagenesis of amino acids already substituted might further improve specificity of *Sl*CYS8 variants with existing variable activities against endogenous potato leaf cysteine proteases. Mutation of such hyper-variable amino acids by site-directed mutagenesis has also resulted in improved inhibition against cysteine protease activity [19]. The amino acid preceding the conserved first inhibitory hairpin loop of cystatins has been found to be a hyper-variable site [32, 33]. Better functionality against papain and cathepsin L was indeed achieved by mutating glutamine (Q) preceding the conserved first inhibitory hairpin loop of rice OC-I to glutamic acid (E), an amino acid found in several other cystatins (40%; Figure 2).

Together, these findings provide strong evidence that engineering at hyper-variable sites is an effective tool for improving cystatin potency and specificity. Such an engineering approach may ultimately assist in overcoming compensatory processes in insect guts. They may further limit interference with protease function in plants and also help to limit proteolytic processes in plants associated with both biotic and abiotic stresses. The specific function of these hyper-variable regions should be further evaluated in the future. In particular, the substitution of amino acids at these hyper-variable sites in combinations with

amino acid substitutions in conserved regions should be carried out. This may elucidate how such changes can affect both the overall cystatin potency and specificity.

3. Filling in the gaps

Several gaps in our understanding of the cystatin/protease interaction still remain. In our opinion, the approach of changing only a single amino acid in a conserved cystatin region might be too simplistic to obtain greatly improved potency, or specificity for that matter. Testing the consequence of the substitution of more than one amino acid in one, or even several, conserved regions should also be explored. This has, to our knowledge, not been done so far in more detail. In addition, testing mutated cystatins against a much wider range of cysteine proteases, and identifying in naturally occurring cystatins rare, or even unique, amino acids in conserved regions, should be an important immediate task. This might result in finding improved cystatin potency and specificity. An excellent example for unique amino acids in the Q-X_{aa}-V-X_{aa}-G motif is the unique alanine in the first position of the motif in a papaya (*Carica papaya*) cystatin [35]. Unfortunately, whether or not such rare, or even unique, amino acids like the unique alanine in conserved regions provide more potency, or specificity, against certain target proteases has not been very well investigated.

A new and interesting strategy, with regards to cystatin engineering, is DNA shuffling [36]. The approach can be applied to recombine two cystatins to produce a new hybrid cystatin with improved inhibitory activity. In short, the method consists of generating a shuffling library of random DNA fragments by fragmentation of genes with DNase I with a set of genes having similar DNA sequences. Repeated cycles of DNA annealing followed by amplification with added primers re-assembles fragments again into a full-length gene sequence where fragments prime on each other based on sequence homology. Recombination

occurs when fragments from one gene anneal to fragments from the other gene which results in a template switch. DNA shuffling has been recently successfully applied by Valadares *et al.* [36] with a sugarcane cystatin (CaneCPI-1) and rice OC-I with both sequences having a 56% DNA similarity. A clone with higher inhibitory activity towards cathepsin B was obtained with two unanticipated point mutations as well as an N-terminal deletion. The result also provided strong evidence that mutations do not always directly affect the inhibitory site. Such mutations can also act from some distance away from the actual mutation site. Mutations in the N-terminus might also result in better protein flexibility due to disruption of the hydrophobic cystatin core. Such increased flexibility will reduce steric hindrance with better direct binding of the two inhibitory hairpin loops to the catalytic site [36]. Future research is necessary though, to extend these findings to demonstrate the overall potential of the DNA shuffling technology with other plant cystatins and the cysteine protease strategy.

3.1 Cystatin sequence design

An important challenge of the future is to design cystatins that only target specific proteases without acting against non-target cysteine proteases. However, the selection of the right model proteases will be a difficult task due the complexity and diversity of protease systems existing in plants and insects [20, 37]. A clear strategy is altering the hydrophobic contacts of cystatin amino acid residues which are involved in protease binding and interact with the corresponding protease amino acid residues in the binding pockets. For designing such cystatins that only target specific proteases, a rational design approach can be applied. However, this requires knowledge on the function of the individual amino acids involved. Phage display and high throughput screening of amino acid variants is a further suitable experimental method [33]. A library of cystatins specifically mutated in the amino acids that form hydrophobic contacts is first produced by site-directed mutagenesis. Screening is then

followed by assessing potency of mutant cystatins against a variety of recombinant-produced purified target proteases with fluorescent protease substrates. Improved knowledge of preferential selection of cysteine proteases toward cystatin amino acids will, in our opinion, greatly assist in designing new cystatins with enhanced inhibitory potency. However, cathepsins B and/or H do not easily accommodate large aromatic residues in their S_3 and S_2 pockets. This is in contrast to cathepsin L, which has a preference for larger aromatic residues in its S_2 pocket [38].

Searching for the frequency and occurrence of evolutionary conserved amino acids at a specified tertiary position, such as the first cystatin inhibitory hairpin loop, will also provide a strong indication of such preferred amino acids. In addition, this type of data provides information about uniquely occurring amino acids that can be tolerated in the targeted enzymes-binding pockets. Applying a rational design then allows determining the relative importance of amino acids at specific tertiary positions, without detrimentally altering the structural backbone of the cystatin. Although over 50-80% of all amino residues can be changed without significantly altering the protein structure, conformational changes due to amino acid changes may impact protein function. This is particularly important when changes are connected to the binding sites [39]. Disruption of hydrophobic interactions, due to an amino acid exchange through the introduction of charged residues into buried sites or changes that break beta-sheets, can have severe impacts on protein function. Changing a single amino acid residue can alter the complementary shape, interacting charge and polarity of the binding face. It also influences the neighboring amino acids, which are dependent on the local changes in amino acid side chains and the resulting functional charges. An excellent example of how an amino acid can change the cystatin structure and function was reported by Urwin *et al.* [29]. Deleting the aspartic acid in rice OC-I at position 86 increased cystatin activity against nematode cysteine proteases, presumably by altering the relative orientation

of some side chains of adjacent amino acids and altering intermolecular contacts. This could include the proline-tryptophan (P-W) motif in the second cystatin binding loop.

An interesting future aspect to look at is if data from identified cystatin sequences will substitute for other approaches as to how to engineer cystatins. Despite a similar mechanism of action and structural homology among them, cystatins naturally exhibit distinct differences in enzyme affinities [20, 40]. Unfortunately, the majority of sequence data currently available is still either from a model plant, such as *Arabidopsis*, or from plants where commercial interest has strongly driven the genomic and transcriptomic exploration in these plants. Currently, this severely limits the exact knowledge of distinct differences that may exist among the many uncharacterized plant families and species.

3.2 Cystatins from landraces, orphan crops and indigenous plant species

Locally adapted landraces, which have evolved over time by adaptation to the natural environment, as well as orphan crops and indigenous plant species, remain greatly unexplored on a genomic and transcriptomic level. Limited to no research has been conducted on cystatins from such plants. These plants are all a potential treasure trove for undiscovered and potentially rare, or even unique, cystatins. In contrast, major crop plants have been bred for specific desired traits which has led to a loss in genetic diversity. An excellent example for the potential of such orphan crops is the previous isolation of an antifungal cystatin from taro (*Colocasia esculenta*). Taro is a perennial, tropical plant native to South East Asia used as a vegetable [41]. This cystatin contains an amino acid variance in the second binding loop of the C-terminus, which contributes to the binding interaction between the cystatin and proteases. Only 8 of the 323 (2.48%) recently screened cystatins varied in this region (Figure 2). Another excellent example is a cystatin recently identified in turmeric (*Curcuma longa*). Tumeric, a herbaceous perennial plant of the ginger family, is

native to India [42] and, although the turmeric cystatin sequence is similar to that of rice OC-I, it also contains an SNSL (Ser-Asn-Ser-Leu) amino acid motif. This motif has been associated with inhibitory activity against legumain-like proteases. The SNSL amino acid motif is a carboxy-terminal extension of some long cystatins. Such cystatins are bifunctional and capable of inhibiting both papain-like and legumain-like activities, comparable to mammalian cystatins [43].

We have also recently started to explore cystatin sequences from marama bean (*Tylosema esculentum*) to uncover more potent and also more specific cystatins. Marama bean is a perennial legume of Southern Africa growing in the Kalahari Desert. The bean is possibly an excellent source for identifying naturally occurring plant protease inhibitors with pharmaceutical value. A marama serine-type protease inhibitor, recently identified, specifically blocks elastase activity involved in inflammatory disorders and also skin diseases [44]. We are currently also searching a marama whole genome database for cystatins that might have similar anti-inflammatory characteristics. So far, such characteristics have, to our knowledge, not been studied in greater detail for plant cystatins similar to the marama serine-type inhibitor or human cystatin C [45].

4. Engineering multi-cystatins or cystatin-containing hybrid protease inhibitors

In addition to directly substituting amino acids in the cystatin amino acid sequence, an interesting approach to follow is to engineer multi-cystatins or hybrid protease inhibitors containing a cystatin (for an overview, see [5]). Possible constructs may include combinations consisting of naturally occurring cystatins and engineered cystatins. Naturally occurring multi-cystatins have already been identified in several plant species including: potato, tomato, sunflower and cowpea (for an overview, see [5]). Multi-cystatins are single

polypeptides consisting of different cystatins in tandem. Natural multi-cystatins very likely originate from multiple gene duplications in plants [5] with the stoichiometric ratio for the polypeptide comparable to the overall ratio of the individual cystatins [46]. Such multi-cystatins not only improve, but also specifically broaden, the overall efficiency of cystatins against various target proteases. They can be constructed either by ligating engineered DNA-encoding sequences directly, or separated by a peptide linker [47]. The general potential of such multi-proteins has been demonstrated in several studies with various expression strategies. These include stable fusion protein production, cleavable poly-proteins or poly-cistronic DNA constructs with the co-translational release of proteins [40]. More simple two component multi-cystatins are cystatin combinations to obtain simultaneous resistance against an insect and a phyto-pathogen [48]. Cystatins have also been combined with a pesticidal protein to obtain a synergistic delay in insect development. An excellent example exists within *Nicotiana benthamiana* plants where expressing multiple defense genes, such as sporamin, a taro cystatin and chitinase, was achieved. Such plants have broad-spectrum resistance against insects, pathogens and abiotic stresses not achievable with single transgene expression [49]. Another excellent example of a functional engineered multi-protease inhibitor is the coupling of a cysteine and a serine protease inhibitor. Coupling was achieved via a translational fusion of the two proteins to produce a polyprotein with functional properties derived from each of the original proteins. By linking them with a peptide, the translational fusion is refractory to cleavage *in planta* and the fusion has successfully controlled nematodes [50]. Potatoes expressing this translational fusion were more resistant to proteolytic degradation when compared to resistance provided by individual components of the fusion. Another compelling engineering approach was the creation of a novel type of protease inhibitor with multi-inhibitory activities. These activities were obtained by replacing the cystatin domains with serine protease inhibitors in a sunflower multi-cystatin [51]. Two

of these chimeric inhibitors acquired trypsin inhibitory activity in addition to activity against papain. However, this technology gives different results depending on whether it is a C- or N-terminal fusion. Fusing amino acids of a potato carboxypeptidase A inhibitor to the rice OC-I C-terminus decreased OC-I potency against papain [52]. In contrast, a hybrid protease inhibitor targeted against aspartate and cysteine proteases and generated by fusing a cathepsin D inhibitor to the N-terminus of corn cystatin II inhibited both cystatin-sensitive and cathepsin D-like enzymes in the Colorado potato beetle [53].

Overall, the application of engineered multi-cystatins or hybrid protease inhibitors containing cystatins to obtain a broader, multi-purpose, inhibitory spectrum against proteolytic processes certainly has the potential to target cysteine proteases and also protease combinations. However, finding the right balance of potency and specificity is still a challenge. This application also requires the identification of specific promoters for the induction of expression of an inhibitor partner in multi-cystatins or protease inhibitor hybrids to differentially regulate targeted protease activity.

5. Engineered cystatins - future potential and applications

Despite previous success in cystatin engineering as well as in large-scale cystatin identification with genomic and transcriptomic approaches, the question, as to whether there is any future in cystatin engineering, still remains. In the past, any cystatin engineering approach was strongly driven by the prospect to ultimately apply engineered cystatins in genetically modified transgenic plants. Much of the cystatin engineering activity therefore, occurred at the end of the last century and the beginning of the new century. At that time, plant genetic engineering was an attractive option for plant improvement around the world. In recent years, any further major research in cystatin engineering unfortunately suffered from

both an increasing concern about the safety of growing transgenic plants in many countries and also the limited adoption of protease inhibitors in crop protection due to poor protease inhibitor efficiency when compared to traditional chemical control methods or even the *Bt* technology. Thus, current progress in selecting, or designing, more potent and specific cystatins has been stunted. However, we are confident that future public acceptance of transgenic plants in many more countries, as well as the discovery of new application fields for cystatins, will provide the basis for more intense future cystatin research. Besides synergistically acting with *Bt* cry toxins, completely new application fields might also emerge such as designing plant cystatin-based novel non-antibody scaffold proteins, termed Adhirons [54]. Adhirons are highly specific binding reagents which have the potential to partly replace the application of antibodies in scientific research, diagnostics and therapy. Cystatins generally fulfil the requirements for an excellent scaffold protein. Cystatins are small, monomeric, with high solubility and stability. They also lack disulfide bonds and glycosylation sites that are both required by antibodies for stability. Cystatins also allow cysteine introduction for site-specific coupling of biotin and fluorescent labels as well as of polyethylene glycol to enhance stability. In contrast to antibodies, cystatins are also easy to express in a microbial system.

In the following sections, we would like to highlight further possible applications where future engineered cystatins might have a significant role with a prospect of short-, medium- or long-term success.

5.1 Genetically modified plants

A clear target, with great potential, is developing genetically modified plants expressing a single or even multiple engineered cystatins for pest control. This is particularly important when targeting a specific key proteolytic process in an organism, but not

interfering with non-target organisms. Inclusion of cystatin transgenic plants into integrated pest management approaches and synergistically acting with other compounds, such as the *Bt* toxin or chemicals, is also an interesting option. This can decrease pesticide application and also support any strategy based on the application of a biological agent for pest control. In addition, the reliance on one major strategy for insect resistance e.g. *Bt* toxin, has raised serious concerns about the development of resistant insects [55]. Therefore, searching for new additional insecticidal proteins for plant protection, which also includes protease inhibitors, has been a recent activity to avoid the build-up of, or delaying resistance development. In addition, such transgenic plants expressing a more potent and specific exogenous cystatin, might also be less susceptible to post-harvest decay due to fungal and bacterial attack. Furthermore, by targeting particularly endogenous plant cysteine proteases with cystatins might also result in plants with reduced un-desired shoot elongation and more shoot branching for better shading [11]. Targeting endogenous plant cysteine proteases could also help in developing potato varieties with longer storage capabilities [12] and improve the nutritional value of many plants by obtaining a higher amount of edible seed proteins [11].

However, a number of important issues have to be solved before any use of engineered cystatins in a transgenic crop plant can be envisaged. These certainly include promoting the interest in transgenic plants particularly in countries which still exhibit a low public acceptance of such plants. Ultimately proving biosafety with no risks to human and/ or animal health and to the environment will still be important despite the fact that natural cystatins should not pose a risk to humans. This is due to the absence of any target cysteine proteases in the human gut and will therefore not act as an anti-feedant in mammals, in comparison to other types of protease inhibitors [56]. Testing for biosafety will be particularly true for any newly designed engineered cystatin. Also any improved cystatin properties, mainly obtained at the bench with *in vitro* systems with isolated and purified

target proteases, have to ultimately be translated to useful products. For a commercial company, an engineered cystatin offers the obvious benefit of obtaining intellectual property rights on such “synthetic” cystatin not found in nature with superior properties. Specifically, commercial companies might be interested in such “synthetic cystatins to extend, or improve the control of chewing pests in transgenic crop or vegetable plants. To our knowledge, and by also searching the USPTO (US Patent and Trademark Office) database, no such engineered “synthetic” cystatin, by manually changing nucleotides in the cystatin DNA sequence to obtain an amino acid substitution, has been currently protected for application as a crop protectant. Interest in any engineered cystatin technology might further come from seed processing companies. Such companies store large quantities of seeds, which are prone to insect attack. Target insects which are responsible for large post-harvest losses would include, for example the red flour beetle or the large grain borer, that are worldwide chewing beetles of stored grain products.

5.2 Nutraceuticals

Finally, engineered cystatins might also act in the longer term as powerful nutraceutical compounds in a plant-derived ‘designer’ food product to better combat pathogen infection in the human digestive system. There is so far no evidence that cysteine proteases absent in the human gut system have, in comparison to serine proteases, any role in human food degradation. Plant cystatins would therefore have useful characteristics for inclusion as a nutraceutical due to their antimicrobial and antiviral properties. They can only interact with microbial or viral expressed cysteine proteases in the human gut, and not with cysteine proteases in the human digestive system [56]. There are also signs that the industry is preparing itself for a major investment into such ‘designer’ food with benefits to humans.

Such food, which ultimately could dwarf the demand for drugs, might also help to manage diseases and disorders often associated with a changed life-style.

Plant cystatins could also play various other beneficial roles in human health [45]. Disturbances in human cystatin C expression and localization are implicated in several pathological processes. These include inflammatory skin diseases and neurodegenerative disorders and different forms of cancer. In particular, association of cystatin C with amyloid-beta formation in Alzheimer's disease has been demonstrated. Low levels of cystatin increases neuronal vulnerability and impairs neuronal ability to prevent neurodegeneration [57]. Although in cancer the inhibitory effect of cystatins beneficially counteracts tumour-associated proteolytic activity, cystatins can also impair the functions of cathepsins in the anti-tumour immune response [58]. Since human cystatin C has, based on NMR spectroscopy, the same cystatin fold as the plant cystatin rice OC-I [24], an interaction with cysteine proteases expressed during this pathological processes can be expected. However, no data currently shows, nor suggests, that 'designer foods' containing recombinant cystatins might indeed help to prevent tumour growth and malignancy. A particular target might be any tumour growth in the digestive system, such as colon cancer, which is currently the third most common type of cancer. However, how plant cystatins might play a beneficial role and methods for effective delivery of such cystatin-based drugs, have yet to be investigated in greater detail. In this regard, an interesting option would be to administer cystatins via plant cells, for example from carrots, which express a recombinant cystatin. Since plant cells have cellulose cell walls rendering them resistant to enzyme degradation when passing through the digestive tract, such plant cells could act as a vehicle. An active cystatin could be thereby administered orally, rather than through intravenous therapy, and, once released and absorbed, delivered to the bloodstream (http://www.protalix.com; accessed on 29 January 2016).

Another interesting nutraceutical application of cystatins might be as a natural sweetener. Monellin, a plant cystatin derived from the fruit of the West African shrub *Dioscoreophyllum cumminsii*, has the unique characteristic of eliciting a sweet taste. It is 800 to 2000 times sweeter than sucrose [59]. The water-soluble cystatin can therefore be extremely useful as a non-carbohydrate sweetener for food and drink used by diabetics who must control their sugar intake. Unfortunately, extraction of monellin from a plant source is still too expensive. Alternative production, such as chemical synthesis and/or expression in microbial or yeast systems, needs to be extensively investigated to confirm the commercial potential.

5.3 Recombinant protein production

There is an increasing interest to use plants, such as tobacco, as a platform for recombinant protein production. In this platform, tobacco leaves are agro-infiltrated and a recombinant protein is transiently expressed (for an overview see [60]). The method is applied for vaccine production such as the production of the Ebola virus vaccine http://mappbio.com/z-mapp/; accessed on 29 January 2016). Proteases, however, can severely affect recombinant protein production [60]. There is already evidence that a transgenic tobacco plant expressing the exogenous rice OC-I facilitates better accumulation of a transiently expressed recombinant protein [60]. Co-expression of an engineered cystatin to protect a recombinant protein transiently expressed in a plant is therefore a feasible strategy. It would have, in our opinion, the greatest potential for immediate use of an engineered cystatin. We recently also identified several cysteine proteases, as possible targets for exogenous engineered cystatins, induced by the agro-infiltration process (Pillay, unpublished). Such cystatins with optimal activity and specificity might particularly protect recombinant proteins against induced protease activities without affecting other vital cellular

proteases. Since plants expressing an exogenous engineered cystatin would not be consumed by humans or animals, or even exposed to the environment, biosafety concerns can be minimized.

6. Conclusions

Previous cystatin engineering approaches have clearly demonstrated that either amino acid substitution in individual cystatins or the creation of cystatin fusions is a feasible and powerful strategy to obtain more potent and specific cystatins. Cystatin engineering will, without a doubt, also open up new possibilities beyond the application of cystatin expression in genetically modified plants for better protection against biotic and abiotic stresses. Such transgenic plant technology will also find momentum again with increasing public acceptance of transgenic plants in many more countries. The future demand for recombinant proteins with pharmaceutical values as well as more nutritious and bioactive food to manage diseases and disorders will also require more potent and specific cystatins as a valuable component. A more immediate application is the inclusion of engineered cystatins in plant-based platforms for recombinant protein production. Such production is not directly connected to the consumption of plants by humans, thereby rendering it more akin to pharmaceutical production in genetically modified bacteria, thereby reducing the expected public concern or debate.

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