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Review article

Review: The future of cystatin engineering

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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.

Contents

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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 Ref. [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 706C1 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 Ref. [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 Ref. [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 Ref. [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 Ref. [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 Ref. [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