Loop replacement design: a new way to improve potency of plant cystatins

Karl J. Kunert[1,*] and Priyen Pillay[2]

[1] Department of Plant and Soil Sciences Department, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa

[2] Future Production, Chemicals Cluster, Council for Scientific and Industrial Research, Pretoria, South Africa

*Correspondence to K. J. Kunert, Department Plant and Soil Sciences, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Hillcrest, Pretoria 0002, South Africa. Tel: +27(0)12 420 3770. E-mail: karl.kunert@up.ac.za

Abstract

Plant cystatins function as competitive inhibitors of cysteine proteases. Similar to other defence proteins, cystatins include hypervariable, positively selected amino acid sites presumably impacting their biological activity. Protein engineering approaches, such as point mutations, at these functionally relevant amino acid sites have already been found to be a powerful tool in improving the inhibitory properties of cystatins. Such engineered cystatins not only better protect against digestive proteases of herbivorous arthropods but also against cysteine proteases of several other plant pests as well as against cysteine proteases produced in plant during stress-induced senescence. Despite previous engineering successes, an urgent need still exists to further improve both plant cystatin potency and specificity. Tremblay and colleagues propose in this issue a new cystatin engineering strategy to substitute the function-related structural elements (SEs) of a cystatin by the corresponding elements of an alternative cystatin. This strategy, possibly combined with direct cystatin gene editing in a target plant, might provide an innovative way to control cysteine protease activity.

Keywords: cystatin loop replacement; gene editing; hybrid cystatin; plant cystatin; protein engineering

Abbreviations

LRD - loop replacement design

SE - structural element

SpCas9 - streptococcus pyogenes Cas9

Introduction

Plant cystatins function as competitive inhibitors of cysteine proteases. They act, for example, as inhibitors in the digestive tract of herbivorous arthropods causing amino acid shortage, growth delays and eventual death of the herbivorous enemy [[1, 2]]. Engineering of cystatins is therefore a powerful tool to improve the inhibitory properties of the inhibitor against digestive proteases of herbivorous arthropods [[3, 4]]. In protein engineering, a protein sequence is modified through substitution, insertion or deletion of nucleotides in the encoding gene, with the goal of obtaining a modified protein that is more suitable for a particular application or purpose than the unmodified protein. The recent methodological progress in, and also novel applications of, protein engineering and directed evolution in plant research has been recently reviewed [[5]]. Particularly, previous cystatin engineering approaches have included, as a feasible and powerful strategy to obtain more potent and specific cystatins, either the substitution of amino acids in individual cystatins or the creation of cystatin fusions. Plant cystatins have hypervariable, positively selected amino acid sites which impact their biological activity. Specifically, these sites have been targeted in the past to optimize cystatin action [[6, 7]]. In a first ground-breaking work, positively selected amino acid sites were identified in cystatins which allowed for successful modulation of their inhibitory profile [[8]]. These positively selected amino acid sites were specifically located at strategic positions on the protein. They included, for example, sites surrounding the conserved glycine residues in the N-terminal region. Other sites are located within the first and second inhibitory loops entering the active site of target enzymes, and adjacent to a conserved LARFAV motif in the α-helix. In particular, amino acid substitutions in the N-terminal trunk, or the inhibitory loops, of cystatins proved highly useful to enhance, for example, the inhibitory potency or changing the affinity profile of plant cystatins towards insect cysteine proteases [[9]].

Loop replacement as a new tool

Cystatin engineering by taking advantage of the potential of existing alternative cystatins as a source of function-related structural elements, as proposed by Tremblay and colleagues, is an interesting new and innovative approach [[10]]. They specifically applied a 'loop replacement design' (LRD) by which a function-related element of a protein is changed for the corresponding element of a related protein [[11]]. Such an approach allows conformational changes on a length scale and not only at a selected amino acid site. In their LRD approach, they followed the scheme by first applying protein modelling aimed to establish a cystatin model. Modelling also included replacing a loop of the cystatin model by a naturally occurring loop from an existing cystatin to create various cystatin hybrids. These hybrids were designed *in silico* by substituting the N-terminal trunk, first inhibitory loop and/or second inhibitory loop of a model tomato cystatin with corresponding element(s) of cystatins derived from different plant taxa. Cystatin–cysteine protease docking simulations were then carried out to evaluate the binding of the newly created hybrid cystatin to a target model cysteine protease. Finally, the newly designed hybrid cystatins were recombinantly expressed and then tested in assays for potency against protease containing extracts. Figure 1 provides a general overview of the test scheme followed. Overall, this excellent work by Tremblay and colleagues has clearly demonstrated the potential of naturally occurring cystatins as a reservoir for structural elements to generate potent broad-

spectrum cystatins. Work has further demonstrated the overall power of protein modelling to rationally design hybrid plant cystatins with higher potency and specificity. LRD-designed hybrid cystatins might ultimately not only be applicable to protect against digestive cysteine proteases in pests but also against cysteine proteases expressed in plants, for example, during stress-induced senescence. In particular, cysteine proteases, which are naturally recalcitrant to cystatin inhibition, might also be among the targets for such hybrid cystatins. Such proteases, which sustain leaf consumption and larval growth, include proteases that are specifically upregulated in the Colorado potato beetle to prevent cystatin action [[12]].

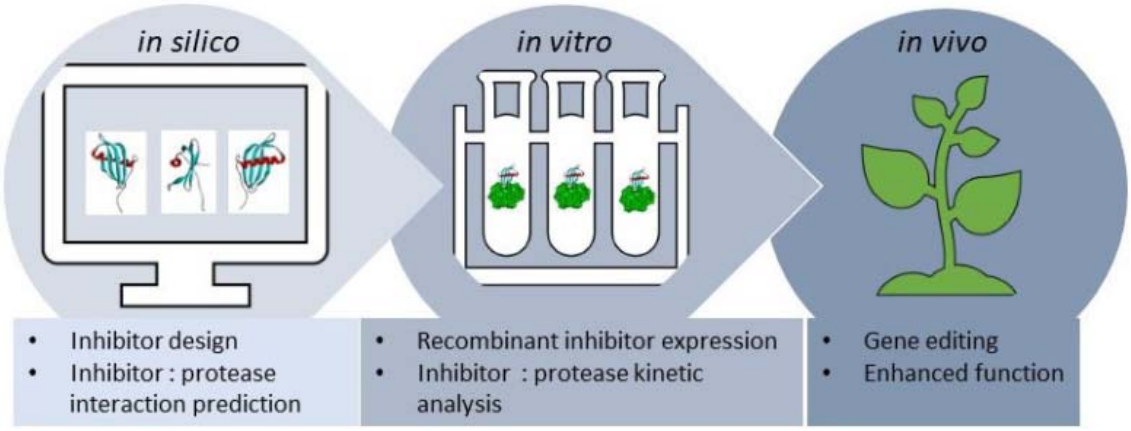

Fig. 1. Scheme of *in silico* cystatin hybrid design and cystatin–cysteine protease docking simulations followed by *in vitro* assays testing cystatin hybrid for potency against proteases and protease-containing extracts and final *in vivo* expression of newly designed hybrid cystatin in plants after cystatin editing to obtain improved potency.

Where to go

The question is, however, where to go next and what potential does this new LRD strategy actually have? Tremblay and colleagues propose several useful immediate follow-up studies for further experimental proof of the strategy. Such experiments include comparison of the protective effect of potent cystatin hybrids with potent natural cystatins or single-variant cystatins and also to test replacement of further 'structural elements'. As Tremblay and colleagues outline, an unsolved question at this point is, however, the actual relevance of the new approach in practice. In this regard, the functional variability among members of the plant cystatin family and any possible negative effects of any high inhibitory efficiency, already measured for some hybrid cystatins, have to be carefully considered. In addition, serious doubts have been previously raised about the actual agricultural application potential of any engineered cystatin. This scepticism is based on findings that cystatin-mediated protection against pests is generally lower than that provided by potent conventional chemical pesticides. In the past, any cystatin engineering approach was further strongly driven by the prospect to use such engineered cystatins in genetically engineered plants, and plant genetic engineering was an attractive option for plant improvement [[4]]. In recent years, however, any further major research in cystatin engineering unfortunately suffered from the declining interest in genetically engineered plants in many parts of the world due to non-scientific public and political resistance against such plants and also possible biosafety concerns.

Research results of Tremblay and colleagues will, without doubt, form an interesting future basis for gene editing to directly engineer a selected cystatin in the genome of a target plant. The ability to efficiently edit plant genomes at scale currently alters our approach to trait development. Gene editing has the advantage that an engineered cystatin transgene requiring sufficient expression does not have to be first integrated into the plant genome. Gene editing already works in both monocot (e.g. maize, wheat and rice) and dicot (e.g. tomato, potato and soybean) crop species [[13]]. A further interesting approach is cystatin editing directly in a 'bio-factory' plant like tobacco to ultimately establish a master bio-factory plant where cysteine proteases no longer compromise foreign protein production. In general, plant genome editing has currently been mostly achieved by transferring DNA encoding the Streptococcus pyogenes Cas9 (SpCas9) protein and an engineered guide RNA into plant cells by Agrobacterium-mediated transformation followed by regeneration of an edited plant through a tissue culture process [[14]]. The gene-editing revolution has been, however, slow to be realized in plants due to the still inefficient delivery methods of DNA allowing for constitutive expression of the CRISPR/Cas9 machinery for targeted DNA modifications. New technological approaches, such as *de novo* induction of gene-edited meristems and the use of RNA viruses, to create gene edits through infection, are therefore currently being tested [[15]]. There are also additional challenges due to lack of editing efficiency and off-target mutations in the edited products [[15, 16]]. Furthermore, precision breeding of plants through gene editing is still banned in the EU following a 2018 ruling by the European Court of Justice. This technique is subject to the 2001 EU directive banning genetically modified organisms (GMOs). In a break with the European Union's stance, the United Kingdom, however, plans to ease requirements for field research. Gene-edited crops are no longer required to submit risk assessments [[17]]. But the UK government still does not allow selling such products in supermarkets.

Conclusions

Cystatin engineering using cystatin segments of naturally occurring cystatins as a reservoir for engineering is a new and innovative strategy to greatly extend cystatin engineering. To combine this engineering technique with the powerful tool of gene editing might offer, in the future, a major breakthrough to not only control digestive cysteine proteases in an herbivorous arthropod but ultimately also in all pests as well as within the plant itself.

Acknowledgements

KK was funded by NRF incentive funding.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

KK and PP both conceived, designed and wrote the Commentary.

References

1. Schlüter U, Benchabane M, Munger A, Kiggundu A, Vorster J, Goulet M-C, et al. Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective. *J Exp Bot*. 2010; 61: 4169– 83.
2. Martinez M, Santamaria ME, Díaz-Mendoza M, Arnaiz A, Carrillo L, Ortego F, et al. Phytocystatins: defense proteins against phytophagous insects and acari. *Int J Mol Sci*. 2016; 17: 1747.
3. Tremblay J, Goulet M-C, Michaud D. Recombinant cystatins in plants. *Biochimie*. 2019; 166: 184– 93.
4. van Wyk SG, Kunert KJ, Cullis CA, Piullay P, Makgopa ME, Schlüter U, et al. The future of cystatin engineering. *Plant Sci*. 2016; 246: 119– 27.
5. Engqvist MKM, Rabe KS. Applications of protein engineering and directed evolution in plant research. *Plant Physiol*. 2019; 179: 907– 17.
6. Goulet M-C, Dallaire C, Vaillancourt L-P, Khalf M, Badri AM, Preradov A, et al. Tailoring the specificity of a plant cystatin toward herbivorous insect digestive cysteine proteases by single mutations at positively selected amino acid sites. *Plant Physiol*. 2008; 146: 1010– 9.
7. Vorster J, Rasoolizadeh A, Goulet MC, Cloutier C, Sainsbury F, Michaud D. Positive selection of digestive Cys proteases in herbivorous Coleoptera. *Insect Biochem Mol Biol*. 2015; 65: 10– 9.
8. Kiggundu A, Goulet M-C, Goulet C, Dubuc JF, Rivard D, Benchabane M, et al. Modulating the proteinase inhibitory profile of a plant cystatin by single mutations at positively selected amino acid sites. *Plant J*. 2006; 48: 403– 13.
9. Rasoolizadeh A, Goulet M-C, Sainsbury F, Cloutier C, Michaud D. Single substitutions to closely related amino acids contribute to the functional diversification of an insect-inducible, positively selected plant cystatin. *FEBS J*. 2016; 283: 1323– 35.
10. Tremblay J, Goulet MC, Vorster J, Goulet C, Michaud D. Harnessing the functional diversity of plant cystatins to design inhibitor variants highly active against herbivorous arthropod digestive proteases. *FEBS J*. 2022; 289: 1827– 41.
11. Clark LA, Boriack-Sjodin PA, Day E, Eldredge J, Fitch C, Jarpe M, et al. An antibody loop replacement design feasibility study and a loop-swapped dimer structure. *Prot Eng Des Select*. 2009; 22: 93– 101.
12. Zhu-Salzman K, Zeng R. Insect response to plant defensive protease inhibitors. *Annu Rev Entomol*. 2015; 60: 233– 52.
13. Nasti RA, Voytas DF. Attaining the promise of plant gene editing at scale. *Proc Nat Acad Sci USA*. 2021; 118:e2004846117.
14. Zhu H, Li C, Gao C. Applications of CRISPR–Cas in agriculture and plant biotechnology. *Nat Rev Mol Cell Biol*. 2020; 21: 661– 77.
15. Nadakuduti SS, Enciso-Rodríguez F. Advances in genome editing With CRISPR systems and transformation technologies for plant DNA manipulation. *Front Plant Sci*. 2021; 11:637159.
16. Jin S, Zong Y, Gao Q, Zhu Z, Wang Y, Qin P, et al. Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science*. 2019; 364: 292– 5.
17. Ledford H. New rules will make UK gene-edited crop research easier. *Nature*. 2021. https://doi.org/10.1038/d41586-021-01572-0