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Research article

Differential in vitro and in vivo effect of barley cysteine and serine protease inhibitors on phytopathogenic microorganisms

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ABSTRACT

Protease inhibitors from plants have been involved in defence mechanisms against pests and pathogens. Phytocystatins and trypsin/α-amylase inhibitors are two of the best characterized protease inhibitor families in plants. In barley, thirteen cystatins (HvCPI-1 to 13) and the BTI-CMe trypsin inhibitor have been previously studied. Their capacity to inhibit pest digestive proteases, and the negative in vivo effect caused by plants expressing these inhibitors on pests support the defence function of these proteins. Barley cystatins are also able to inhibit in vitro fungal growth. However, the antifungal effect of these inhibitors in vivo had not been previously tested. Moreover, their in vitro and in vivo effect on plant pathogenous bacteria is still unknown. In order to obtain new insights on this feature, in vitro assays were made against different bacterial and fungal pathogens of plants using the trypsin inhibitor BTI-CMe and the thirteen barley cystatins. Most barley cystatins and the BTI-CMe inhibitor were able to inhibit mycelial growth but no bacterial growth. Transgenic *Arabidopsis* plants independently expressing the BTI-CMe inhibitor and the cystatin HvCPI-6 were tested against the same bacterial and fungal pathogens. Neither the HvCPI-6 expressing transgenic plants nor the BTI-CMe ones were more resistant to plant pathogen fungi and bacteria than control *Arabidopsis* plants. The differences observed between the in vitro and in planta assays against phytopathogenic fungi are discussed.

1. Introduction

Plant defence against pathogens is a complex process that involves the activation or repression of different signalling pathways leading to the overexpression of target genes with defence properties. One of the main groups of proteins induced after plant pathogen exposition corresponds to the protease inhibitors. These proteins are mainly located in seeds or tubers and are induced in vegetative organs as leaves or roots. Two functions have been related to these proteins: i) regulation of endogenous plant proteases; and ii) inhibition of exogenous proteases from arthropod pests and phytopathogenous microorganisms [1].

Formerly, protease inhibitors were grouped according to the kind of protease inhibited. Then, they were classified as cysteine, serine, aspartic, and metalloprotease inhibitors [2]. However, several homologous inhibitors are able to inhibit different kind of proteases and they are now classified in function of their sequence similarities and tridimensional structures [3]. Two of the most abundant plant protease inhibitors are the cystatins, family I25, that are cysteine protease inhibitors, and the cereal trypsin/α-amylase inhibitors, family I6 [4,5].

Plant cystatins (PhyCys) are plant proteinaceous inhibitors of cysteine proteases of the papain C1A family integrated in an independent subfamily on the cystatin phylogenetic tree [6,7]. The cystatin inhibitory mechanism is produced by a tight and reversible interaction with their target enzymes. It involves a tripartite wedge formed by the partially flexible N-terminus containing a glycine residue and two hairpin loops carrying a conserved QxVxG motif and a tryptophan residue, respectively. Most PhyCys are small proteins with a molecular mass in the 12–16 kDa range, but there are some with a molecular weight of 23 kDa. These PhyCys have a carboxy-terminal extension which has been involved in the inhibition of a second family of cysteine proteases, the C13 legumain peptidases [7,8]. From a functional viewpoint, PhyCys have been implicated in regulation of the protein turn-over and as

Abbreviations: PhyCys, plant cystatins; ORF, open reading frame; EC_{50}, effective concentration for 50% inhibition; BTI, barley trypsin inhibitor; CPI, cystatin protease inhibitor.

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defence proteins [4]. The defence role has been inferred from: i) the ability of PhyCys to inhibit digestive proteases from herbivorous arthropods in vitro, in artificial diets as well as by bioassays on transgenic plants over-expressing PhyCys genes [9–11]; ii) their transcript induction in response to mechanical wounding or methyl-jasmonate [12,13], and; iii) their deleterious effects against phytopathogenic fungi and viruses [14–16].

The implications of PhyCys in defence against fungal plant pathogens are supported by a high number of PhyCys genes with antifungal in vitro activity [16–20]. However, the mechanism of inhibition is still not clear. According with a previous report, the inhibition of *Botrytis cinerea* growth by the barley cystatin HvCPI-1 is not associated with its cysteine protease inhibitory properties and correlates with the absence of intra- and extra-cysteine protease activity in this fungus [15]. Alternatively, it was reported an inhibitory effect of the tarocystatin on *Sclerotium rofsii* cysteine proteases [21]. At this point, it is unknown how cystatins inhibit fungal growth. Furthermore, neither are evidences on the effect of PhyCys on the growth of phytopathogenic fungi in vivo nor in the in vitro and in vivo growth of plant pathogenous bacteria.

On the other hand, the plant family of the cereal trypsin/α-amylase inhibitors is formed by proteins that accumulate in the seed [5]. Their members can be classified as trypsin inhibitors, α-amylase inhibitors, and dual trypsin/α-amylase inhibitors [22]. Three different roles have been attributed to the family I6 inhibitors: i) regulators of seed germination; ii) storage proteins, and iii) defence proteins. Their role as defence proteins is supported by their specificity against amylases and trypsins from insect pests [23,24].

The defence function would be also related to fight against phytopathogenic microorganisms. The implication of trypsin and chymotrypsin inhibitors on fungal and bacterial growth inhibition has been previously reported [25–28]. Among the family I6 inhibitors, the 14 kDa protein from maize seed was able to inhibit spore germination and mycelial growth of nine different plant pathogen fungi [29].

In barley, the complete family of cystatins has been previously characterized. Thirteen cystatins have been described and their evolutionary relations with their target proteases analyzed [7,30]. These cystatins have shown different gene structure, variations in the mRNA expression patterns and subcelullar location, and important changes in the deduced amino acid sequences affecting their inhibitory properties [17,31,32]. Regarding to defence, the barley cystatins HvCPI-1 to 7 have been tested against the phytopathogenic fungi *Fusarium oxysporum* and *B. cinerea*. Likewise, *Arabidopsis* and maize plants have been transformed with the HvCPI-6 cystatin and their partial resistance against acari and aphids characterized [11,33].

The most characterized I6 trypsin inhibitor in barley is the *Itr1* gene encoding the protein BTI-CMe, which has been putatively involved in plant defence. This gene is specifically expressed in the barley endosperm and the purified protein BTI-CMe has been shown to be active in vitro against insect trypsin proteases [23]. Likewise, transgenic rice, wheat and tobacco plants expressing this protein were tested against the performance of several herbivorous pests showing a negative impact on their performance [34–36].

In this study we analyze the in vitro antifungal capability of the thirteen barley cystatins (HvCPI-1 to HvCPI-13) and the barley BTI-CMe inhibitor against three important phytopathogenic fungi, *Magnaporthe grisea*, *Plectosphaerella cucumerina* and *F. oxysporum*, and two plant pathogen bacteria, *Dickeya dadantii* and *Pseudomonas syringae*. Likewise, we construct *Arabidopsis* plants expressing the trypsin inhibitor BTI-CMe. These plants and transgenic *Arabidopsis* plants expressing the HvCPI-6 cystatin were tested to know the resistance to the same fungi and bacteria.

2. Results

2.1. Identification of C1A cysteine peptidases and trypsins in selected pathogen fungi and bacteria

Bioinformatics searches were done to find putative C1A cysteine proteases and S1 trypsins in the selected microorganisms. The genomic sequences of the fungi *M. grisea* and several *Fusarium* species as well as that of the bacteria *D. dadantii* and different *P. syringae* patovars are available in the web [37–40]. The necrotrophic fungus *P. cucumerina* has not been still sequenced but several gene sequences are available in the databanks. From these searches, we found that there are not C1A protein sequences in the selected fungi, which is consistently with extensive searches in databanks in which we only found C1A sequences in the fungi *Podospora anserina* and *Chaelomium globosum*. Trypsins were also absent in the genome of *M. grisea* whereas *Fusarium* species have one trypsin gene. In contrast, we found both, one C1A protein and three trypsin genes in both *D. dadantii* and *P. syringae* bacteria.

2.2. Inhibitory in vitro activity of barley cysteine and serine protease inhibitors on phytopathogenic microorganisms' growth

We have previously reported the toxic effects of seven barley cystatins (HvCPI-1 to HvCPI-7) exerted on the fungal growth [17]. To complete this study, we analyzed the antifungal properties of the barley cystatins HvCPI-8 to HvCPI-13 and the serine protease inhibitor BTI-CMe against *F. oxysporum*. Besides, we tested the growth inhibition exerted by the entire barley cystatin family and the BTI-CMe protein on the phytopathogenic fungi *P. cucumerina* and *M. grisea*. The antifungal dose of each protein was quantified by adding increasing amounts of each inhibitor to the fungal culture medium. The effective concentration for 50% growth inhibition (EC_{50}) was calculated for each case (Table 1). Most of the barley cystatins and the trypsin inhibitor BTI-CMe were able to inhibit the spore germination and the mycelial development of the three fungal species in a similar manner (Fig. 1). Then, there were no morphological differences in the microscopical images obtained from cystatin or BTI-CMe fungal inhibition. However, a varied inhibitory level was observed (Table 1). The in vitro growth of *M. grisea* was strongly inhibited for most cystatins at low concentration values ($EC_{50} < 1.5$ μM). The strongest inhibitory effects on *F. oxysporum* mycelium growth were produced by HvCPI-2, -3 and -6 proteins

Table 1
Inhibition of the fungal growth of phytopathogenic fungi by barley cysteine and serine protease inhibitors.

Inhibitor	EC_{50} (μM)[a]		
	M. grisea	*P. cucumerina*	*F. oxysporum*
HvCPI-1	4.51	5.97	2.14
HvCPI-2	5.08	n.i.	1.02
HvCPI-3	0.89	5.25	0.99
HvCPI-4	1.10	1.88	2.59
HvCPI-5	2.75	5.7	4.15
HvCPI-6	0.18	1.17	1.09
HvCPI-7	0.76	4.9	n.i.
HvCPI-8	0.85	3.55	5.33
HvCPI-9	0.41	5.69	5.92
HvCPI-10	1.25	2.25	n.i.
HvCPI-11	0.41	0.83	6.0
HvCPI-12	0.77	2.82	5.46
HvCPI-13	1.34	4.8	1.56
BTI-CMe	1.23	2.5	1.52

n.i. = no inhibitory activity detected at concentrations ≤6 μM.
[a] Effective μM concentration for 50% inhibition (EC_{50}) was calculated with three replicates of each experiment. Standard errors were lower than 10%.