Peptides 30 (2009) 825–831

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Protective effects of a cysteine proteinase propeptide expressed in transgenic soybean roots

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ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 25 January 2009
Accepted 26 January 2009
Available online 6 February 2009

Keywords:
Nematode
Cysteine proteinase
Propeptide
Transgenic plants
Heterodera glycines

ABSTRACT

Sedentary endoparasitic nematodes cause extensive damage to a large number of ornamental plants and food crops, with estimated economical losses over 100 billion US$ worldwide. Various efforts have put forth in order to minimize nematode damage, which typically involve the use of nematicides that have high cost and enhanced toxicity to humans and the environment. Additionally, different strategies have been applied in order to develop genetically modified plants with improved nematode resistance. Among the strategies are anti-invasion and migration, feeding-cell attenuation, and anti-nematode feeding. In the present study, we focus on anti-nematode feeding, which involves the evaluation and potential use of the cysteine proteinase (CPs) propeptide as a control alternative. The cysteine proteinase prodomain, isolated from *Heterodera glycines* (HGCP prodomain), is a natural inhibitory peptide used to transform soybean cotyledons using *Agrobacterium rhizogenes.* Genetically modified soybean roots expressing the propeptide were detected by Western blot and expression levels were measured by ELISA (around 0.3%). The transgenic roots expressing the propeptide were inoculated with a thousand *H. glycines* at the second juvenile stage, and a remarkable reduction in the number of females and eggs was observed. A reduction of female length and diameter was also observed after 35 days post-inoculation. Furthermore, the *H. glycines* mature protein was detected in females fed on soybean transformed root expressing or not expressing the propeptide. The data presented here indicate that the HGCP propeptide can reduce soybean cyst nematode infection and this strategy could be applied in the near future to generate resistant crop cultivars.

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1. Introduction

Proteinase inhibitors (PIs) are small proteins commonly found in a variety of organisms [28]. In plants, PIs are constitutively produced and/or synthesized in response to wounding, abiotic stresses, and attack by insect pests and pathogens [11,49,50]. PIs are able to inhibit proteinases synthesized in abundance by insect and pathogens, reducing the availability of free essential amino acids, which are extremely necessary for their growth and development [16]. Several reports have described cysteine-, serine-, aspartyl- and metallo-proteinase inhibitors in plants [7,26,43,49]. Most PIs interact with their target proteinases in a canonical fashion, resulting in the formation of a stable and inactive proteinase inhibitor complex [1,40]. A similar mechanism of action also occurs for the propeptides present in precursors of cysteine proteinases, which are active as molecule inhibitors with the ability to inhibit proteolytic enzymes [24,67]. Different reports indicate that CPs propeptides are potent inhibitors of their cognate enzymes [6,22,34,46,53–55].

On one hand, plants synthesize several protective compounds, but, on the other, phytonematodes exhibit a remarkable mechanism of parasitism and host feeding [15,23], causing enormous worldwide agricultural losses, particularly in soybean production. The economical losses caused by plant parasitic nematodes in

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0196-9781/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2009.01.022

world agriculture represent over 100 billion US$ annually [14]. The root-knot nematode *Meloidogyne* spp. and the cyst nematodes *Heterodera* spp. and *Globodera* spp. are the most harmful [2]. The control method is mainly based on resistant cultivars, crop rotation, and nematicides [14,17]; the latter are often highly expensive and toxic, with environmental risk association [41]. Genes encoding anti-nematode bioactive proteins and peptides have been transferred to host plants using transgenic methodologies. This recent and attractive strategy has been frequently researched due to its efficiency, as well as low cost and reduced environmental risk [15]. Different reports show the efficiency of anti-feedant genes, such as those coding for proteinase inhibitors (oryzacystatin, sporamine) that confer resistance in *Arabidopsis* to *Meloidogyne incognita*, *Globodera* spp., and *Heterodera schachtii* [10,35,36,61]. Several other isolated genes clearly show potential application for nematode control, including the glutamic acid decarboxylase (*GAD*), encoding a biosynthetic enzyme for GABA (γ-aminobutyric acid) synthesis (an inhibitory neurotransmitter at neuromuscular junctions [38]), lectins [45], and the Nem-R genes Gro1-4 [42], *Mi* [52], Hs1pro-1, and Hsa-1(Og) [37,57]. Bt toxins, toxic peptides, plant secondary metabolites, antibodies, and RNA interference have also been appraised for application in phytonematode control [13,18,20,21,48,51,66]. Proteinases normally have been related to different steps of parasite–host interactions including invasion, survival, and nutrition [59]. Among these proteolytic enzymes, several studies have demonstrated the importance of cysteine proteinases for the vital processes of phytonematodes [35,61]. Despite the large number of proteinase inhibitors now available, a novel strategy has been used based on an inhibition mechanism that occurs naturally in cells. In this mechanism, proteinases are synthesized as inactive pro-enzymes or zymogen molecules, in which the inappropriate proteolytic activity is blocked by a short peptide named propeptide. When the proteinase is directed to the correct cell compartment, the propeptide is cleaved and the enzyme becomes active toward the substrates. The effect of the proteinase propeptides in protein folding and inhibition have been reported in several works [3,12,56]. Recently, the use of cysteine proteinase (CPs) propeptides was explored as an anti-nematode effector, in which the product of the prodomain of *Heterodera glycines* CPs exhibited high *in vitro* inhibitory activity toward its cognate enzyme and to other related nematode proteinases, but does not inhibit cysteine proteinases of insect–pest species [53]. Nevertheless, this strategy was also successfully explored for use in the control of insect pests. Silva et al. [53], using a recombinant propeptide (PCPAo-proregion from *Acanthoscelides obtectus* proteinase), have demonstrated an efficient inhibition of cysteine proteinases from the bean weevil, *A. obtectus*, as well as the digestive CP of other related bean bruchids. All of these data suggest the prodomain of proteinases as a powerful tool for nematode cysteine proteinase inhibition, and allow for the design of new strategies to be used in soybean cyst nematode control. In this context, this report shows the expression of a HGCP prodomain in soybean roots, and evaluates its effectiveness to confer resistance against the plant–parasitic nematode *H. glycines*.

2. Materials and methods

2.1. Expression of HGCP-Iv and HGCP proregion domain in Escherichia coli

Genes encoding HGCP-Iv (*H. glycines* cysteine proteinase promature protein) and PROHGCP (*H. glycines* cysteine proteinase proregion) from *H. glycines* were cloned in pET 102-D TOPO vector (Invitrogen®) and the proteins were expressed in an *E. coli* (Strain BL21 DE3) system according to [53]. The expression of recombinant proteins was induced with 0.5 mM isopropyl-1-thiol-β-D-galactopyranoside (IPTG) for 3 h at 37 °C with continuous shaking (200 rpm). Recombinant proteins were purified using an affinity Ni-NTA (Qiagen®) column and used to produce antiserum in mice (BALB/c) according to [25].

2.2. Expression of PROHGCP in soybean roots via Agrobacterium rhizogenes

The CP proregion from *H. glycines* (PROHGCP) was cloned in pGPTV-Kan vector [4] under control of the cauliflower mosaic virus (CaMV) 35S gene promoter generating a new vector designated as p100HG (Fig. 1). This vector was introduced in *A. rhizogenes* by electroporation according to [30]. The soybean genotypes (Br16, Doko, Conquista, and Mandarin) and the *A. rhizogenes* strains (2659, LBA 9401, and 8196) were evaluated in relation to root induction and transformation levels. The soybean genotypes Mandarin and Br16 (susceptible to *H. glycines*) and the *A. rhizogenes* 2659 strain were chosen and subsequently used in the soybean root transformation with the p100HG vector. Soybean seeds were treated with 70% ethanol for 1 min following 1% sodium hypochloride for 20 min. Seeds were washed with sterile milliQ H_2O and cultivated in Petri dishes containing 50 ml (MS) medium [1,39] with 3.0% sucrose (pH 5.7) in dark chamber growth. After 4 days, Petri dishes were submitted to a photoperiod of 16 h at 26 °C and cold light, until reaching the vegetative stage V1 according to [19]. The seedling cotyledon abaxial faces were excised, scratched out, and rapidly immersed in Luria-Bertani medium containing *A. rhizogenes*. After this procedure, the treated cotyledons were transferred to Petri dishes containing a humid filter paper with the abaxial face returned upward. This system was submitted to photoperiod of 16 h at 26 °C and cold light over 3 days. Later on, the cotyledons were put in MXB medium containing MS salts, B5 vitamins, 3% sucrose, agar (2 g l^{-1}), and claforan (500 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) antibiotics. After 15–25 days, adventitious roots appeared, showing a hair-full root symptom. When the primordial root reached 5–7 cm, it was excised and transferred to Petri dishes containing MXB medium and antibiotics. Liquid MXB medium (5 ml) with a lower concentration of claforan (150 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) was added to the Petri dishes at 15-day intervals.

2.3. Detection and analysis of transformed soybean roots expressing PROHGCP

Roots (0.5 g) were homogenized in 50 mM Tris–HCl buffer pH 6.8 containing 1% β-mercaptoethanol and 0.2% polyvinylpyrrolidone (PVP) in order to obtain the protein crude extracts. The homogenate was centrifuged at $9000 \times g$ for 20 min at room temperature. The pellet was removed and supernatant (crude extract) kept at −20 °C for further analyses. Protein concentration was determined using the method described by Bradford with minor modifications [5]. A sample containing 30 μg was analyzed by SDS-PAGE 12% [31]. An electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membrane was performed according to [58]. Western blot analysis was carried out as previously described by [8] by using antiserum raised against PROHGCP diluted at 1:500. In this assay, purified recombinant protein PROHGCP was used as a positive control and crude extract from *E. coli* (strain BL21 DE3) as a negative control for expression analyses in roots. The PROHGCP expression level was determined by ELISA technique according to [29]. In this assay, the propeptide was used as a positive control, and protein crude extract of transformed roots without the vector was used as a negative control. The crude extract obtained from transformed roots (20 ng) was analyzed in triplicate using antiserum raised against PROHGCP.

2.4. Nematodes extraction

Living *H. glycines* females were extracted manually (using forceps and a blade) from soybean plants (Mandarin genotype) maintained in a greenhouse. Pre-selected females containing eggs were disrupted and immediately was removed in a Baermann funnel. After 3–5 days, the *H. glycines* second-stage juveniles (J2) were obtained and quantified using a Neubauer chamber. After this procedure, J2 were disinfested with mercury chloride (0.01%) and streptomycin (0.02%) according to [33].

2.5. Proteolytic activity and proteinase inhibitory assays

The reaction was performed in 100 μl including 2.5 mM DTT, 5% DMSO, and 0.1 M Na_2HPO_4 buffer, with 2.0 mM EDTA, pH 6.5. The refolded protein solution (HGCP-Iv) (10 ng per assay) was pre-warmed at 37 °C for 1 h prior to adding 10 μM substrate. Incubation was continued for an additional 15 min and fluorescence of the released amido-methylcoumarin was measured in a DyNA Quant 500 fluorescence reader (Pharmacia-Biotech), with 360 nm excitation and 460 nm emission. The reaction was stopped after 15 min with 95% ethanol. In the inhibitory assay, nematode extract was pre-incubated with the recombinant propeptide (80 ng) for 15 min at 37 °C. Remaining activity was determined using the same fluorogenic substrate. A similar procedure was utilized for the inhibitory assay with synthetic inhibitor E-64 [Trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane]. Antiserum was used as a control to abolish any inhibitory activity. Each sample was assayed in triplicate.

2.6. Nematode assay

Transformed soybean roots were cultivated in Petri dishes containing MXB medium and infected with one thousand J2, distributed randomly. The infected roots were maintained in Petri dishes with the same medium at 26 °C in the dark. In each plate, 5 ml of this medium were added for root nutrition in 100-day intervals. Transformed roots without the vector were used as a positive control. The number of females per plate, number of eggs per female, and female length (μm) and width (μm) were evaluated after 25 days of inoculation.

2.7. Detection of HGCP protein in female crude extract of H. glycines

Crude extract of the feeding females was obtained as performed for transgenic roots. Aliquots of this crude extract were subjected to SDS-PAGE 12.5% separating polyacrylamide gel under reducing conditions [31]. A nitrocellulose membrane was used to transfer proteins by a semidry procedure, using a Pharmacia-LKB Multiphor II transfer system for a period of 45 min. *H. glycines* cysteine proteinase was detected using antiserum against mature protein (HGCP-Iv) A goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Bio-Rad) was used as a secondary antibody and the color developed using an HRP color development reagent (Bio-Rad).

Table 1
Frequency induction of transformed soybean root genotypes BR 16 and Mandarin via *A. rhizogenes* strain 2659 in the presence and absence of p100HG vector.

A. rhizogenes	Strain 2659		Strain 2659+ p100HG	
Soybean genotype	BR 16	Mandarin	BR 16	Mandarin
Cotyledons infected/genotype	120[a]	120[a]	120[a]	120[a]
Cotyledons infected/strain	240[a]	240[a]	240[a]	240[a]
Induced roots after infection	84[a]	70[a]	25[b]	16[b]
Efficiency in transformation (%)	70[a]	59[a]	24[b]	13[b]

Values marked by same letter do not show statistical differences when evaluated by Tukey test at the 5% significance level.

3. Results and discussion

3.1. Soybean root transformation

Approximately 10 days after cotyledons infection with *A. rhizogenes*, calluses of globular tissues were observed, and with 20-day development, roots were excised and a root system established in Petri dishes containing MXB medium. The root growth rate was between 0.1 and 0.3 cm per day and the majority demonstrated plagiotropic growth. Transgenic lines did not show reliable phenotypic differences in comparison to non-transgenic lines. The Agrobacterium strain 2659 carrying the vector p100HG showed efficiency of transformation varying from 22% to 34% in the infected cotyledons for all soybean genotypes analyzed (Table 1). Moreover, no difference in callus formation, root origin, and time of root growth were observed between the wild type strain and the strain containing the vector.

The soybeans Br16, Doko, Conquista, and Mandarin and the *A. rhizogenes* strains 2659, LBA 9401, and 8196 were also evaluated in relation to root induction and transformation levels, and BR 16 and Mandarin plus *A. rhizogenes* 2659 strains exhibited the most efficient combination. *Glycine max* genotype selection used in this work was facilitated by the previous studies of *A. rhizogenes* susceptibility [44]. Data reported here corroborate the results described by [9], which showed that *A. rhizogenes* cucumopine family (strain 2659) is more efficient than manopine (strain LBA9401) and agropine (strain 8186) families. Moreover, in contrast to different plants belonging to the Solanaceae family, the *G. max* transformed roots did not exhibit spontaneous regeneration in the absence of hormone. Other plant species, including leguminous, have demonstrated regeneration with no hormones from transformed roots produced by *A. rhizogenes* infection, such as *G. canescens* and *G. argyrea* [44].

3.2. Detection and analysis of soybean roots transformed expressing the PROHGCP

Soybean root (genotype BR 16) wild type (Fig. 2A) and that further transformed via *A. rhizogenes* strain 2659 (Fig. 2B) were established in Petri dishes with selective MXB medium, showing clear morphological differences. Immunoblotting analysis showed that a single band near 30 kDa was detected in *E. coli* crude extracts. A slower band was detected in soybean roots expressing PROHGCP

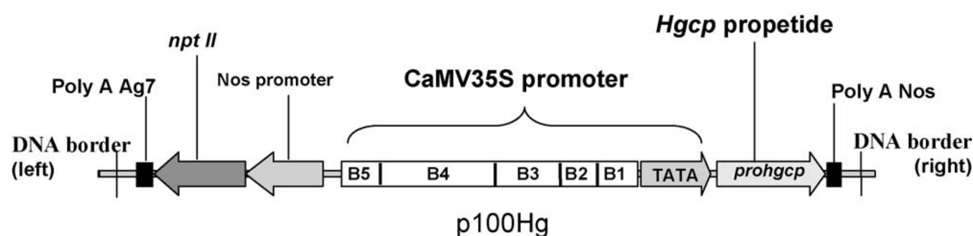

Fig. 1. Cysteine proteinase proregion from *H. glycines* (PROHGCP) in the pGPTV-Kan vector carrying the CaMV35S promoter control utilized here.

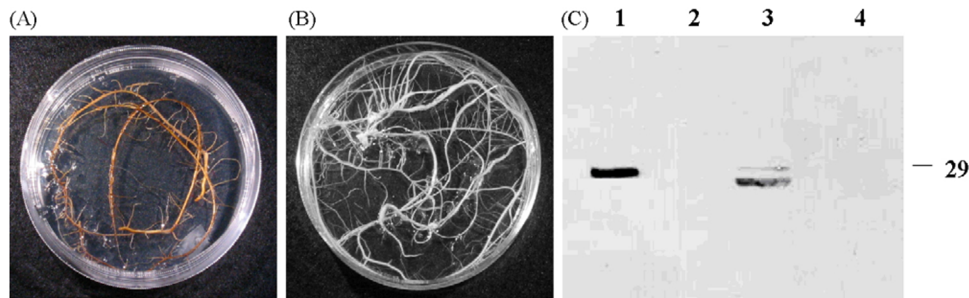

Fig. 2. Soybean root (genotype BR 16) wild type (A) and further transformed via *A. rhizogenes* (strain 2659), established in Petri dishes with selective MXB medium (B). Western-blot analysis (C) of transformed soybean roots using PROHGCP anti-serum: (1) *E. coli* crude extract containing propeptide, (2) crude extract from *E. coli* without vector, (3 and 4) crude extract from soybean roots transformed with p100HG vector expressing or non-expressing PROHGCP.

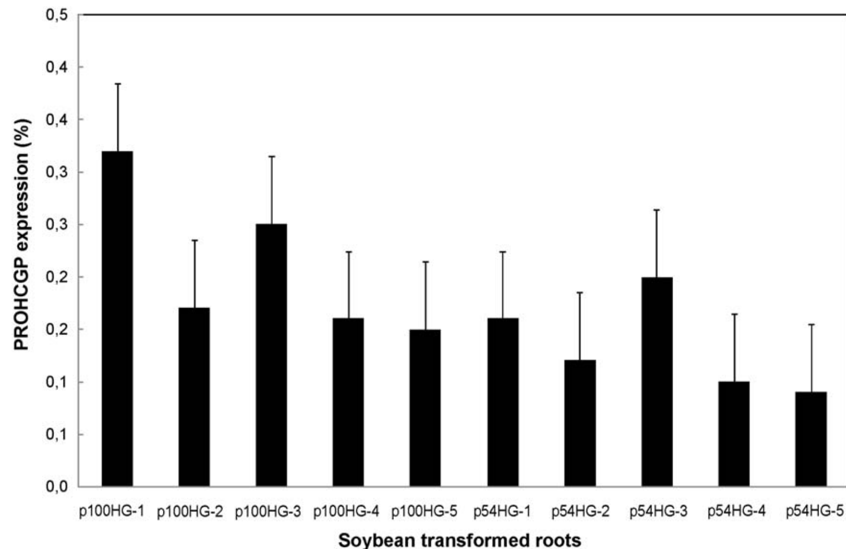

Fig. 3. Percentage of PROHGCP on total protein extracts from soybean transformed roots performed by ELISA. p100HG1-5, roots transformed with p100HG; p54HG1-5, roots transformed with p54HG.

and may be due to a post-translational modification (Fig. 2C). The frequency of transformed roots expressing the propeptide was around 24% using the strain soybean 2659 carrying the vector p100HG. Furthermore, propeptide expression levels were estimated by ELISA at up to 0.308% of the total protein root crude extract (Fig. 3). The vector p54HG (which is the same p100HG vector without the B5 domain) was also evaluated in order to compare expression efficiency. No significant difference was observed using the Tukey test (5% significance level) for the propeptide expressed in the roots, leading to the choice of p100HG vector.

3.3. Effects of the propeptide on H. glycines

In vitro inhibitory fluorimetric assays showed that nematode crude extract was completely inhibited by propeptide expressed in soy roots, as well as by the CP-specific synthetic inhibitor E-64 (Table 2).

H. glycines from the J2 stage were inoculated in transformed roots 5 days after inoculation. Mature females were detected in the roots approximately 3–4 weeks post-inoculation. The female developmental rate was around 43% in the control and 30% for the p100HG vector treatment. Marked differences were observed in the number of females and number of eggs per female according to the Tukey test (5% significance level) (Table 3). In summary, the propeptide expression reported here caused a remarkable reduction in egg number (58%) and also a decrease of females by 31% in relation to the control. The level of propeptide expressed in the roots was statistically correlated to the number of females and eggs per female. On the other hand, the negative correlation coefficient ($R = -0.480$) suggests that if the propeptide expression levels were higher, the number of females and eggs per female would most likely be lower. Despite these differences, the mature proteinase (HGCP-Iv) was found in females feeding on transgenic and non-transgenic soybean roots (Fig. 4). These results indicate that propeptide inhibits the proteolytic activity, however it does not promote the proteinase digestion in *H. glycines* feeding females. Additionally, reduction of nematode reproductive success caused by PROHGCP can be a good strategy for nematode control in soybean cultivars. Similar results can be observed in nematode feeding of transgenic plants that express proteinase inhibitors. As an example, potato plants expressing oryzocystatin, a natural cysteine proteinase inhibitor, reduced the number of

Table 2
Inhibitory activity of PROHGCP toward *H. glycine* proteolytic extract.

Treatments	Proteolytic activity (relative units/ng protein)
HgCE	440[a]
HgCE + E64	47[b]
HgCE + propeptide	38[b]

Fluorimetric assays were performed in a DyNA Quant 500 fluorescence reader (Pharmacia-Biotech), using Z-Phe-Arg-AMC as substrate (10 μM). Inhibitors were pre-incubated with the enzymes before measuring proteolytic activity. Assays were performed in triplicate.

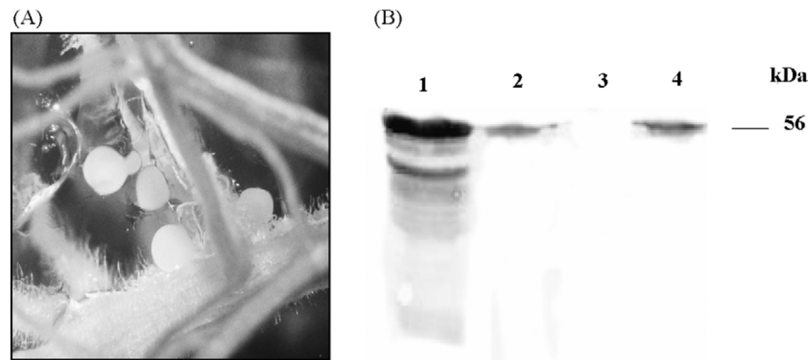

Fig. 4. (A) *H. glycines* females feeding in soybean transformed root expressing PROHGCP, (B) Western blot analysis of the female crude extracts using the mature protein HGCP-Iv antiserum: (1) *E. coli* crude extract containing the mature protein expressed, (2) female crude extract that fed transformed roots without propeptide PROHGCP, (3) *E. coli* crude extract not containing the mature protein expressed, (4) female crude extract that fed transformed roots expressing PROHGCP.

Table 3
Effects of propeptide PROHGCP expressed in soybean roots on *Heterodera glycines* development.

	Female	Eggs/female	% PROHGCP	Female length (μm)	Female width (μm)
Control	43.7a[a]	99.2a	0.0a	282.4a	184.4a
p100HG	30.1b	41.6b	0.3b	246.8b	157.6b
Reduction	31.0%	58.0%	–	12.6%	14.5%

[a]Values marked by same letters (a and b) do not show statistical difference when evaluated by Tukey Test at the 5% significance level.

eggs and the female size of the phytoparasite *Globodera pallida* [61] (Table 3).

Nematode control with PIs expressed in transgenic tomato [60], *Arabidopsis thaliana* [63], and rice [64] has been very well demonstrated [28]. Cysteine proteinase inhibitors affect both the growth rate and the development of several nematodes, such as *pallida* [60], *H. glycines* [62], *H. schachtti*, and *M. incognita* [61,64], suggesting the importance of these inhibitors in the parasite digestion process. Among the different classes of proteinase inhibitors, cysteine proteinases prodomains have been described as potent inhibitors with the ability to control nematodes [53,54]. Recombinant expressed papaya proteinase IV propeptide (a plant enzyme) inhibits Colorado potato beetle cysteine peptidases [65], and synthetic propeptides inhibit *Manduca sexta* midgut trypsin [56]. Inhibition of *Fasciola hepatica* cysteine proteinase by its propeptide was also demonstrated [27]. Several studies exploring the relationship between proteinases and propeptides have possibly developed a new generation of specific peptide inhibitors, which can be used strategically in plant sedentary nematodes control.

Therefore, the prodomain peptide of cysteine proteinase from *H. glycines* can inhibit recombinant cognate enzyme as well as crude soluble extract of *H. glycines*. Additionally, our data are also in agreement with the report described by [53], which demonstrated, by *in vitro* experiments, that propeptide inhibits proteinases of *H. glycines* juveniles. Furthermore, not only nematodes have been controlled by prodomain inhibitor peptides. As an example, proregion-derived peptide acts as a weak and reversible inhibitor of congopain, the major cysteine proteinase of *Trypanosoma congolense* and inhibits the native cruzipain (cysteine proteinase of *T. cruzi*) [32]. Moreover, previous work [65] also demonstrated the inhibition of cysteine proteinase Colorado potato beetle by papaya proteinase IV propeptide as well as papain. The cysteine proteinase of *F. hepatica* (animal parasite) also was inhibited by its propeptide [27,47]. Recently, it was demonstrated that the CP propeptide of *A. obtectus* inhibits midgut crude soluble extract of several bruchids, such as *A. obtectus*, *Zabrotes subfasciatus*, and *Callosobruchus maculatus* [54], showing a broad spectrum of inhibition of the propeptide against pests of similar species.

An understanding of the interaction mechanisms involving propeptides and digestive proteinases can favor the development of complementary inhibitors that can achieve broad-spectrum inhibition of nematode proteinase systems and thus minimize the occurrence of compensatory or degradation processes in target pests. Our results also point out the possible usefulness of plant propeptides as a regulator of cysteine proteinases in biotechnological systems, and further show the ability of proteinase inhibitors to preserve the integrity of 'companion' defense-related proteins from the action of insensitive proteinases in target pests. However, propeptide stability in plants should be studied, as well as the increase of propeptide expression rates by using more efficient promoters. Therefore, the aim of this study was to demonstrate the effectiveness of a cysteine proteinase propeptide (PROHGCP) as an anti-feedant factor for *H. glycines* control in soybean roots. Novel experiments have been done in order to obtain plants with enhanced resistance showing higher PROHGCP expression levels.

Acknowledgments

This report was supported by grants from the Brazilian government, Embrapa, UCB, FAP-DF, CAPES, and CNPq.

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