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

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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.

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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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 × *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.