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Comparative Biochemistry and Physiology, Part C 140 (2005) 227–235

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Effectiveness of recombinant soybean cysteine proteinase inhibitors against selected crop pests

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Received 25 June 2004; received in revised form 8 February 2005; accepted 10 February 2005

Abstract

Three recombinant soybean cysteine proteinase inhibitors (rSCPIs), L1, R1 and N2, were assessed for their potential to inhibit the growth and development of three major agricultural crop pests known to utilize digestive cysteine proteinases: Western corn rootworm (*Diabrotica virgifera virgifera,* WCR), Colorado potato beetle (*Leptinotarsa decemlineata,* CPB) and cowpea weevil (*Callosobruchus maculatus,* CW). In vitro experiments showed that cysteine proteinase activities in the crude gut extracts of the WCR, CPB, and CW were inhibited to various degrees by the three rSCPIs. Of the three rSCPIs tested, N2 was most effective in inhibiting the crude gut extract of WCR, CPB, and CW (50% inhibition at 5×10^{-8}, 5×10^{-8}, and 3×10^{-7} M, respectively). The L1 was the least potent of the three CPIs tested, with 50% inhibition at 5×10^{-6} M of the crude gut extracts of WCR. Results of in vivo experiments conducted to assess the effect of the three rSCPIs on the vital growth parameters of WCR, CPB and CW were consistent with results of the in vitro experiments.

Keywords: Colorado potato beetle; Cowpea weevil; Cysteine proteinase inhibitors; Western corn rootworm; Insect digestive enzymes; Insecticides; Plant resistance to insects; Soybean protease inhibitors; Beetle larvae

1. Introduction

Beetle larvae like those of the Western corn rootworm on maize, the Colorado potato beetle on potatoes, and the cowpea weevil or cowpea beetle on cowpeas pose major problems for agriculture and food industries. Among the corn rootworm species, the Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte) poses the greatest threat as a pest because of its higher reproductive capacity and greater mobility (Piedrahita et al., 1985; Coats et al., 1986; Riedell et al., 1991; Godfrey et al., 1993; Davis, 1994). Some 80% of the root damage is caused by third instar (last instar) larval feeding. Corn plants with heavily damaged root systems exhibit moisture stress (Riedell, 1990), an imbalance in nitrogen uptake and nutrient acquisition (Kahler et al., 1985), and plant stalk lodging. In parts of Africa, where cowpeas [*Vigna unguiculata* (L.) Walp.] are a major source of human dietary protein, the cowpea weevil (CW) [*Callosobruchus maculatus* (F.)] causes serious losses to stored cowpea grains (Jackai and Singh, 1983). Cowpea infestation begins in the field, where CW eggs are laid on the pods. After the crop is harvested and put into storage, the CW populations expand rapidly. CW larval feeding within the cowpea seeds during this time causes substantial damage (Van Alebeek, 1996). Often, on-farm storage for 6 months is accompanied by about 30% loss in weight, and up to

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1532-0456/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cca.2005.02.007

70% of the seeds are infested and unfit for human consumption (Gatehouse et al., 1989). The Colorado potato beetle (CPB) [*Leptinotarsa decemlineata* (Say)] is the most troublesome pest of potato (*Solanum turberosum* L.) in the northeastern U.S. It has developed resistance to all currently registered synthetic insecticides (Forgash, 1981). The lack of new insecticides, along with environmental pollution associated with attempts to control the CPB, WCR, and CW, has stimulated renewed interest in their non-insecticidal control (Ng and Lashomb, 1983; Lashomb, 1994).

Failure of traditional insect control methods to reduce crop losses has stimulated research to develop alternative means to manage crop pests. Better understanding of insect digestive enzymes may prove to be a key in making crops insect resistant. Insects show a high diversity in digestive proteases (Wolfson and Murdock, 1990). The potential exists for naturally occurring cysteine proteinase inhibitors to be introduced by genetic engineering to create crop plants that are resistant to infestation by these pests. Therefore, enhancing plant resistance to insects using cysteine proteinase inhibitor genes from donor plant species may be an alternative to using conventional pesticides.

Limited success with traditional plant breeding methods to create host plant resistance to insects (or nematodes) has made it imperative to use molecular genetic approaches to impact needed traits. Development of efficient crop transformation systems has made it feasible to design and conduct experiments transferring genes whose products are insecticidal. Protease inhibitors that target specific insect proteinases are candidates for use in this application. One such vulnerable protease system is the proteases in the digestive tract of the crop pests described above. Many insects and pests of crop plants utilize cysteine proteinases to digest dietary protein. The growth and development of these pests are seriously affected by the inhibition of cysteine proteinases (Murdock et al., 1987; Hines et al., 1991; Orr et al., 1994; Urwin et al., 1995). Reduced proteolysis within the gut of the insects, caused by inhibition of cysteine proteinases, results in a lack of free amino acids, leading to detrimental effects on insect growth and development (Urwin et al., 1995; Michaud et al., 1996).

Accordingly, work in our interdisciplinary group has explored the possibility of vectoring genes encoding CPIs into crop plants for the purposes of imparting protection against insects. A cysteine proteinase inhibitor isolated in our laboratory (Hines et al., 1991) has shown promise in inhibiting the gut proteolytic activity of numerous insects known to utilize digestive cysteine proteinases. Initial studies on soybean in our laboratory were followed by isolation of a 12 kDa soybean CPI (SCPI), shown to be a member of the cystatin superfamily of CPIs based on immuno-crossreactivity data and N-terminal sequence analysis (Hines et al., 1991). This native SCPI was heat labile, inhibited papain and ficin, but did not inhibit the CP bromelain, the serine proteinases bovine trypsin or bovine α-chymotrypsin, or porcine pepsin. Because SCPI did not inhibit mammalian digestive enzymes, it became more interesting as a candidate for use in transforming plants to confer insect resistance (Hines et al., 1991). The soybean CPI isolated by Hines et al. (1991) strongly inhibited the crude gut extracts of certain insect larvae that are dependent on CPs for digestion: cowpea weevil, red flour beetle (*Tribolium castaneum*), Mexican bean weevil (*Epilachna varivestis*), and common bean weevil (*Acanthoscelides obtectus* Say).

Three unique cDNA clones (pL1, pR1 and pN2) that encode the SCPI were isolated from an immature embryo ZAPII library by polymerase chain reaction (PCR) of poly (A)+RNA from soybean (*Glycine max* L. Merr.) embryos, indicating the presence of CPI isoforms in this genotype (Zhao et al., 1996). From the amino acid sequence analyses, it was deduced that L1, R1 and N2 not only shared 60–70% sequence homology with one another but showed overlapping conserved amino acid sequences with oryzacystatin and chicken egg white cystatin. All the soybean CPIs have essential residues conserved in the first and second hairpin loops, but at the N-terminus, N2 is one residue longer than oryzacystatin and R1 and L1 are 7 and 9 residues shorter, respectively. Recombinant N2 and R1 inhibit papain activity to a degree comparable to other plant CPIs, but L1 to a much lower degree (Zhao et al., 1996). Zhao et al. (1996) showed with in vitro assays that recombinant N2 and R1 have greater inhibitory activity than L1 against gut proteinases of WCR and CPB larvae, and that compared to the commercial CPI, E-64 [epoxide trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane], N2 and R1 were more effective inhibitors against WCR proteinases, but less effective inhibitors against CPB.

The objective of this study was to use in vivo studies to assess the potential of the three recombinant CPIs to inhibit the growth and development of WCR, CPB, and CW, three major agricultural crop pests known to utilize cysteine proteinases.

2. Materials and methods

2.1. Preparation of crude gut extracts of WCR, CPB and CW

Digestive tracts from larvae (third instar of WCR (*D. virgifera virgifera* LeConte); third and fourth instar of CPB (*L. decemlineata* (Say)); third instar of CW (*C. maculatus* (F.)) were dissected into 100 μL of 0.2 M sodium acetate (pH 5.5) to prepare crude gut extracts (Murdock et al., 1988; Zhao et al., 1996). The supernatant was diluted appropriately to give a 3-fold difference in counts between the blanks and the controls (i.e., normalized to show a 3-fold spread) with 0.2 M sodium acetate buffer (pH 5.5) for measurement of cysteine