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Update section

Short communication

The resistance of cowpea seeds to bruchid beetles is not related to levels of cysteine proteinase inhibitors

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Abstract

A cDNA encoding a cysteine proteinase inhibitor was isolated from a cDNA library prepared from developing seeds of an insect-resistant line of cowpea. The sequence of the encoded protein was homologous with those of other plant cysteine endoproteinase inhibitors, and with Type 2 cystatins from animals. Southern blot analyses indicated that small gene families were present in both resistant and susceptible lines of cowpea, while northern blot analyses showed similar levels of expression. It is concluded that the levels of expression of the inhibitor do not account for the differences in insect resistance of the two lines.

Cowpea (*Vigna unguiculata*) is a major crop in many tropical countries, but much of the production may be lost due to post-harvest damage by bruchid beetles such as the cowpea weevil (*Callosobruchus maculatus*). In order to reduce such damage genetic sources of resistance were identified by screening a cowpea germplasm collection [1] and have been incorporated into breeding programmes. Although the resistance in one of these lines (TVu 2027) was initially thought to be related to high levels of trypsin inhibitors [2], Xavier-Filho *et al.* [3] have since shown no correlation between the levels of these or other proteinase inhibitors and resistance or susceptibility to *Callosobruchus*. In addition it has been shown that *C. maculatus* utilizes aspartic and cysteine proteinases for digestion rather than serine proteases such as trypsin [4, 5], in common with

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Database under the accession number Z21954, cowpea cysteine protease inhibitor cDNA.

other Coleoptera [6, 7]. This has stimulated interest in inhibitors of cysteine proteinases, both in relation to natural defence mechanisms and as potential sources for engineering resistance. Fernandes *et al.* [8] have reported that inhibitors of cysteine proteinases are expressed in developing seeds of a susceptible line of cowpea, although at levels too low to have an impact on the digestive enzymes of insect pests. We describe here the molecular cloning of one such inhibitor from developing seeds of a cowpea line resistant to *C. maculatus* (IT81D 1045), and compare its levels of expression and genomic organization in this line and a susceptible cultivar (Pitiuba).

A cDNA library was constructed using poly(A)$^+$ RNA isolated from IT81D 1045 seeds at 16 days after pollination. The cDNAs were directionally cloned into λgt11 vector digested with *Sfi* I and *Not* I (Promega). About 4×10^6 pfu were obtained with inserts ranging from 500 to 1000 bp. About 8×10^4 plaques were screened for cDNAs related to the cowpea cysteine proteinase inhibitor by probing with a specific antiserum utilizing the Stratagene *pico* Blue immunoscreening kit. Three positive clones were identified, one of which contained an insert of about 520 bp following polymerase chain reaction (PCR) amplification with λgt11 sequencing primers. The insert hybridized on Southern blotting with a cDNA encoding the rice cysteine proteinase inhibitor oryzacystatin [9]. The *Eco* RI site was lost during the cloning procedure, therefore the cloned cDNA was amplified by PCR and subcloned into M13 vectors [10] for sequencing. Four independent M13 clones were sequenced and all consisted of the same 496 bp insert which included a short poly(A) tail and an open reading frame encoding a protein of 97 residues with a calculated mass of 10750 Da. The sequence of this protein showed strong homology with the sequences of other plant cysteine proteinase inhibitors (Fig. 1). In particular, all except the *Wisteria* protein contain a conserved region of five residues (QVV[SAG]G) thought to be important for binding to cysteine proteases (underlined in Fig. 1A). Among the full length plant cystatin sequences, the highest similarity is with the maize cystatin with 60% identical residues and 85% similarity

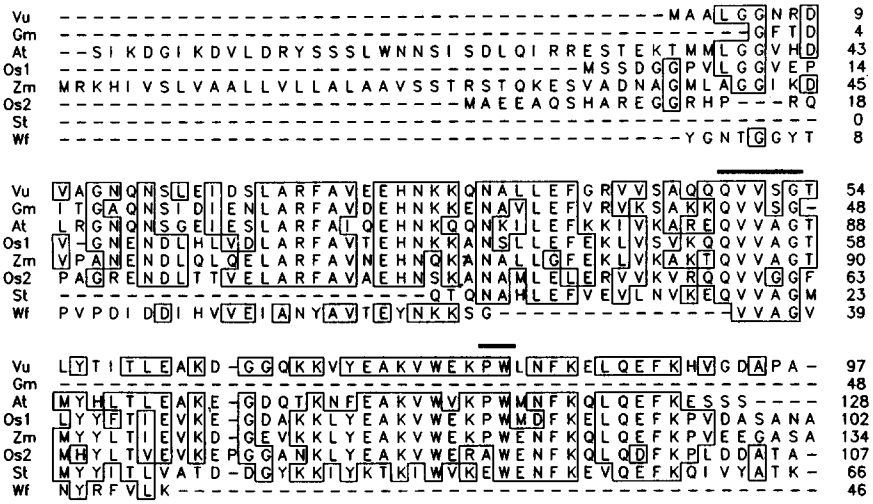

Fig. 1. Alignment of the cowpea cysteine protease inhibitor sequence (Vu) with those of other inhibitors of cysteine proteinase from different plant sources: soybean (Gm) [18]; *Arabidopsis* (At; EMBL accession number Z17618); rice oryzacystatin 1 (Os1) [9]; maize (Zm; EMBL accession number D10622); rice oryzacystatin 2 (Os2) [19]; potato (St) [20]; and *Wisteria* (Wf) [21]. The soybean, *Arabidopsis*, potato, and *Wisteria* sequences are incomplete at the N-terminus. The alignment was made using the programme 'PILEUP', from the University of Wisconsin Genetic Computer Group [11]. Identical residues are boxed. The overlined pentapeptide sequence QVVSG is characteristic of inhibitors of cysteine proteinases, and the dipeptide sequence PW of the Type 2 cystatins [see 12].

(calculated with the 'GAP' program [10]). Comparisons with other cysteine protease inhibitors from animals show similarities to the Type 1 cystatins (stefins) in the absence of disulphide bonds, but a stronger sequence similarity to Type 2 cystatins, including the motif PW at positions 79–80 [see 12]. The cowpea cystatin lacks a signal sequence, in common with the oryzacystatins but in contrast to the maize inhibitor. The *Wisteria*, soybean, potato and *Arabidopsis* sequences in Fig. 1 are incomplete and the presence or absence of signal peptides has not been established.

Northern blots of total RNA fractions from developing seeds of the resistant cowpea line IT81D 1045 and a susceptible cultivar (Pitiuba) showed similar patterns and levels of expression (Fig. 2). In both cases expression was highest at 8–10 days after pollination and then declined, although transcripts were still present at 18 days. Southern blot analysis of genomic DNA (Fig. 3) showed single hybridizing fragments of about 1.4 kb in both lines when digested with *Hin*d III, but differences when *Eco* RI was used. A single hybridizing fragment of about 8·1 kb was observed in Pitiuba, but several more weakly hybridizing fragments (1·8, 2·1 and 2·7 kb) in IT81D 1045. Comparisons with copy number reconstructions indicated 4–5 gene copies per haploid genome in both lines. Several more weakly hybridizing bands were observed in all tracks when the filters were washed at lower stringency or exposed for a longer period, indicating the presence of other less closely related genes in both lines.

Although we have shown that a novel cysteine

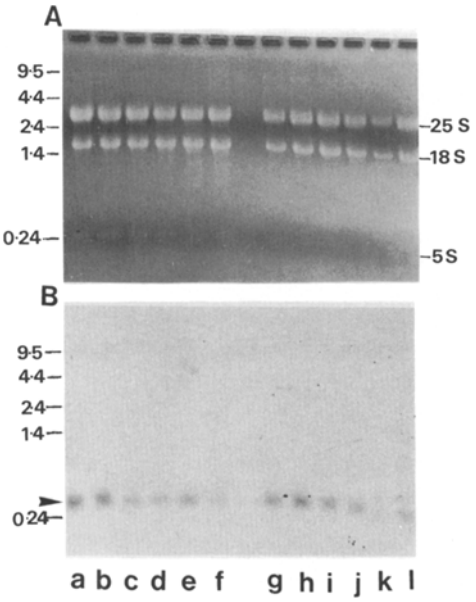

Fig. 2. Northern blot analysis of total RNA fractions from the susceptible cultivar Pitiuba (tracks a–f) and the resistant line IT81D 1045 (tracks g–l) using the cowpea cysteine proteinase inhibitor cDNA. Fractions are from 8 (tracks a, g); 10 (tracks b, h), 12 (tracks c, i), 14 (tracks d, j), 16 (tracks e, k) and 18 (tracks f, l) days after pollination. The top part shows the stained gel before transfer and the bottom part an autoradiograph of the filter. The final wash was carried out in 0.5 × SSC, 0.1% SDS at 65 °C. The positions of size markers are indicated to the left of the figure and the major ribosomal RNA bands to the right of part A.

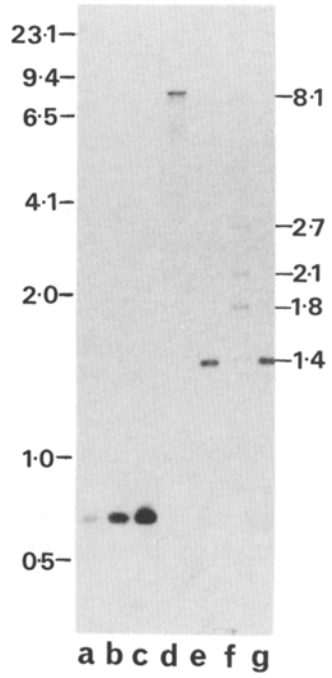

Fig. 3. Southern blot analysis of genomic DNA from cotyledons of the susceptible cultivar Pitiuba (tracks d, e) and the resistant line IT81D 1045 (tracks f, g), digested with *Eco* RI (tracks d, f) or *Hin*d III (tracks e, g) and probed with the cowpea cysteine proteinase inhibitor cDNA. Tracks a–c are reconstructions representing 1, 5 and 10 copies per haploid genome respectively. Conditions used for hybridizations and washing were as in Fig. 2. The positions of size markers are shown to the left of the figure, and the calculated sizes of the hybridizing bands to the right.

protease inhibitor is synthesized in developing seeds of cowpea, two lines of evidence indicate that this does not contribute to natural resistance to bruchid beetles. Firstly, the levels of cysteine proteinase inhibitors present in cowpea seeds and in other plant tissues are very low compared with the levels of serine proteinase inhibitors [8]. It has therefore been suggested that they play a role in metabolic regulation rather than in defence [8]. Secondly, the levels of cysteine proteinase inhibitors [3, 13] and the populations of corresponding mRNAs (this study) are similar in seeds of susceptible and resistant lines of cowpea. This indicates that cysteine proteinase inhibitors do not contribute to resistance to *Callosobruchus* and other bruchid beetles. However, the fact that these pests do utilize cysteine proteinases [4–7] which are inhibited by plant proteinase inhibitors of the cystatin type [7] indicates a potential for conferring resistance in transgenic plants. The low levels of the cowpea cysteine proteinase inhibitor present in seeds means that it has not been possible to purify sufficient protein to determine its inhibitory properties in detail. It is therefore not possible to predict the levels which would be required to inhibit insect feeding *in vivo*.

What then is the mechanism of resistance of cowpea to *Callosobruchus* and other bruchid beetles? Early work suggested that differences in the levels of cowpea trypsin inhibitor (CPTI) were responsible [2], and this protein was indeed shown to confer resistance to the lepidopteran pest tobacco budworm (*Heliothis virescens*) in transgenic tobacco plants [14]. However, *H. virescens* is a lepidopteran not a colepteran, and a related species (*H. zea*) has been shown to use serine proteinases for digestion [15]. In addition the soybean trypsin inhibitor (related to CPTI) inhibits these enzymes *in vitro* and the growth of larvae when incorporated in artificial diets [15]. This is not the case with coleopterans such as *Callosobruchus*. In fact, more recent studies indicate that the resistance of IT81D 1045 is due not to the presence of specific inhibitors but to a variant form of vicilin storage protein which is resistant to digestion by midgut proteinases [16, 17], thereby limiting the food supply to the larva.

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