A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant

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In feeding, aphids inject saliva into plant tissues, gaining access to phloem sap and eliciting (and sometimes overcoming) plant responses. We are examining the involvement, in this aphid–plant interaction, of individual aphid proteins and enzymes, as identified in a salivary gland cDNA library. Here, we focus on a salivary protein we have arbitrarily designated Protein C002. We have shown, by using RNAI-based transcript knockdown, that this protein is important in the survival of the pea aphid (*Acyrthosiphon pisum*) on fava bean, a host plant. Here, we further characterize the protein, its transcript, and its gene, and we study the feeding process of knockdown aphids. The encoded protein fails to match any protein outside of the family Aphididae. By using *in situ* hybridization and immunohistochemistry, the transcript and the protein were localized to a subset of secretory cells in principal salivary glands. Protein C002, whose sequence contains an N-terminal secretion signal, is injected into the host plant during aphid feeding. By using the electrical penetration graph method on c002-knockdown aphids, we find that the knockdown affects several aspects of foraging and feeding, with the result that the c002-knockdown aphids spend very little time in contact with phloem sap in sieve elements. Thus, we infer that Protein C002 is crucial in the feeding of the pea aphid on fava bean.


The authors declare no conflict of interest.

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protein and on this basis tentatively classifies the Protein C002 program of Rost content interrupted by loops or turns. For instance, the PROF programs for prediction of secondary structure predict a high helix glycosylation sites (www.cbs.dtu.dk/services/NetNGlyc/). Promature protein is 21.8 kDa. There are no potential O-protein (www.cbs.dtu.dk/services/SignalP/), with cleavage predicted by SignalP to be a signal peptide for an extracellular contains 219 aa residues. The N-terminal sequence of the protein predicted encoded amino acid sequence. The predicted protein cleavage site as predicted by SignalP.

Assembly of the c002 assembled from a single gene (locus) encoding Protein C002 (Fig. 2). In Southern analysis, we probe revealed a single band of 1,100 bases (Fig. 2). Right). Northern blot for the c002 transcript (Fig. 2). Left) Northern blot for the c002 transcript. (Right) Southern blot of pea aphid genome DNA digested by: XbaI (first lane), Ncol (second lane), EcoRV (third lane), and EcorI (fourth lane).

Fig. 1. Nucleotide sequence of transcript c002 and the inferred amino acid sequence of Protein C002. The nucleotide sequence is a unigene sequence assembled from c002 ESTs from NCBI. It agrees with the sequence obtained from the c002 gene (see Fig. S1). The arrow indicates the signal peptide cleavage site as predicted by SignalP.

studies we conclude that Protein C002, a secreted component of salivary glands, is essential for feeding on fava bean by the pea aphid.

Results

Sequences of Transcript c002 and Protein C002. In Fig. 1 we present the unigene nucleotide sequence of transcript c002 and its predicted encoded amino acid sequence. The predicted protein contains 219 aa residues. The N-terminal sequence of the protein is predicted by SignalP to be a signal peptide for an extracellular protein (www.cbs.dtu.dk/services/SignalP/), with cleavage predicted between residues 23 and 24. The mass of the predicted mature protein is 21.8 kDa. There are no potential O-glycosylation sites (www.cbs.dtu.dk/services/NetOGlyc/) or N-glycosylation sites (www.cbs.dtu.dk/services/NetNGlyc/). Programs for prediction of secondary structure predict a high helix content interrupted by loops or turns. For instance, the PROF program of Rost et al. (9) predicts 62% helix in the mature protein and on this basis tentatively classifies the Protein C002 as “all-α.”

c002 is an abundant EST in our salivary gland cDNA library. In ~4500 ESTs from the library, there are 17 occurrences of the c002 EST, a 14-fold higher frequency than among head and salivary glands, is essential for feeding on fava bean by the pea aphid.

Immunohistochemical localization of Protein C002, using rabbit polyclonal antibodies raised against the recombinant protein, revealed staining in a subset of several cells in the principal salivary gland (Fig. 3C).

Detection of Protein C002 in Plants Fed upon by Aphids. Five hundred aphids were placed on fresh fava bean plants for 24 h and then removed. Leaf tissue was then extracted and Western blot analysis was performed using polyclonal anti-C002 rabbit antibodies. As shown in Fig. 4 (lane 3), a band was detected in plants fed upon by aphids that matches the position of recombinant Protein C002 (Fig. 4, lane 1). On the other hand, a Protein C002 band was not detected in plants that had not been exposed to the Baylor College of Medicine Human Genome Sequencing Center (www.hgsc.bcm.tmc.edu/projects/aphid/) indicates a single gene, containing a single intron [see supporting information (SI) Fig. S1].

Localization of Transcript c002 and Protein C002 Within Salivary Glands. Most of the volume of aphid salivary glands is comprised of large secretory cells, 4 in each accessory gland and 21 in each lobe of the bilobed principal gland (11, 12). In situ hybridization with digoxygenin-labeled c002 DNA revealed the presence of transcript c002 only in the principal salivary glands and in only a few of the secretory cells in each lobe (Fig. 3 A and B). The unstained (differential interference contrast) image shows the overall morphology expected of an accessory salivary gland and its associated (bilobed) principal salivary gland (11, 12). (These authors’ diagrams of the histology of aphid salivary glands can be seen online, on pages 51 and 53 in ref. 10). Propidium iodide staining revealed the large nuclei expected within the secretory cells, and the c002 transcript was largely confined to a handful of such cells in each lobe of the principal gland.

In situ hybridization and immunohistochemical localization of Protein C002 in dissected pea aphid salivary glands. (A) Differential interference contrast image. PG, principal gland; AG, accessory gland. (B) Differential interference contrast image overlaid with in situ hybridization for transcript c002. Green is the positive signal for antisense c002; nuclei are red. (C) Immunohistochemistry with anti-Protein C002 staining. Nuclei are red; green is the positive signal for anti-C002 antibody. (Scale bar: 50 μm.) Negative controls (sense probe for in situ hybridization and preimmune serum for the immunohistochemistry) did not show any positive staining.
aphids (lane 2). These results indicate that Protein C002 is transferred from aphid to plant during feeding. Protein C002 was also detected in extracts from pea aphid heads and salivary glands (lanes 4 and 5, respectively). No bands were detected in any of these samples in a Western blot with secondary antibody only (result not shown).

A band of about 75 kDa detected in the plant extracts, both before and after aphid feeding (Fig. 4, lanes 2 and 3), was also detected in plant extracts when a preimmune serum was used (result not shown). Thus, it appears that the rabbit used for raising antibodies against Protein C002 had previously developed antibodies against a plant protein of 75 kDa and that these antibodies were not fully removed in passing the anti-C002 antiserum over a column of immobilized Protein C002 for antibody purification.

**Effects of c002 Transcript Knockdown on Feeding Behavior.** EPG provides a powerful method to study probing behavior by an aphid during foraging and feeding (3, 13, 14). Three main phases during styllet penetration have been defined from EPG studies: the pathway phase, the xylem phase, and the phloem (or sieve element) phase (3, 14), and details within each of these phases can be discerned (3, 13–15).

We have used EPG to assess the effect in the foraging and feeding behavior of pea aphids in which transcript c002 has been knocked down (8). Representative EPG traces are shown in Fig. 5. These (and EPGs of nine other insects in treatment and control groups) revealed dramatic differences in feeding (or attempted feeding) by the two groups. The EPGs as shown in the figure (and their continuations for 8 h) differ in several ways, most strikingly in that the c002-knockdown insect never entered a phloem (or sieve element) phase. In fact, of the 10 knockdown aphids, only one exhibited a sieve element phase (and then only once and for just 30 min). The control insect, however (Fig. 5), not only entered the sieve element phase, but remained in that phase for 5 of the 8 h of observation (data not shown in the figure, where we include only the first 3 h of the trace). There are other, more subtle differences between the treatment and the control insect, for instance, a lower rate of occurrence of cell puncture signals during the probing phase.

In Table 1, we present a summary of the EPGs recorded from 10 c002-knockdown and 10 control-injected insects (a total of 80 h of EPGs from each group). Several foraging or feeding parameters can be seen to be affected in a statistically significant way by the knockdown. First, an aphid’s ability to identify a suitable location for initiating probing is significantly reduced because it takes c002-knockdown aphids >6 times longer than the control insects to identify such a site and begin probing ($P = 0.0073$; Table 1). Second, once probing is initiated, the c002-knockdown aphids probe individual epidermal and mesophyll cells at only half the rate of control aphids ($P = 0.031$). Third and most strikingly, c002-knockdown aphids are far less likely to initiate sieve element phase feeding ($P < 0.0001$) than the control insects. The control aphids fed 52 times longer than the c002-knockdown aphids ($P < 0.0001$), which could be due either to an inability of the knockdown insects to identify and remain in a sieve element once a sieve element is probed or to simply not coming into contact with a sieve element. Finally, the c002-knockdown aphids spend 4-fold more time not engaged in probing behavior than do control insects ($P = 0.0001$).

**Discussion**

Here and in and a previous article (8), we have identified a protein, Protein C002, that appears to play an essential role (or roles) in the foraging and feeding of the pea aphid on fava beans, a typical host plant for this aphid species.

Protein C002 can, on the basis of our results, be considered a specialized, salivary gland protein, which does not exclude the possibility that it is synthesized in other organs in small amounts. Indeed, we have a preliminary indication that transcript c002 occurs in gut, but at 100-fold lower amounts than in salivary gland (10). The organ distribution of transcript c002 (and Protein C002) is one of the many aspects of this transcript and protein that will be under continued investigation in our laboratories.

Both Protein C002 and its transcript occur in the principal salivary glands in the pea aphid but apparently in only some of those cells (~5 of the 21 cells within each lobe). This restriction of expression of the c002 gene to a subset of secretory cells is intriguing. In unpublished work, we have found that the enzyme laccase is also restricted in its distribution in the principal salivary gland, but to a different subset of secretory cells than is Protein C002 (Q. D. Liang and G.R.R., unpublished observations). Thus, it appears that individual secretory cells in the principal salivary gland have different “assignments”; that is, each secretory cell will produce a particular set of proteins (many of which will be enzymes), and thus different subsets of the secretory cells could in principle produce salivas of different compositions.

Our EPG studies on c002-knockdown insects (and control insects) demonstrated striking effects of the knockdown. Our earlier work (8) demonstrated premature deaths of the knockdown insects feeding on fava bean leaves. The EPG results...
reported here offer a good deal of insight into that observation. It is not much of an oversimplification to state that the knockdown aphids do not feed. They attempt to feed, but are, for all intents and purposes, unsuccessful in doing so. Thus, of 10 knockdown insects studied by EPG, only one showed a sieve element phase, and that was quite short (only 30 min). The knockdown aphids exhibit a probing phase, a typical behavior as aphids search for a sieve element for feeding (3, 13–15), but they do either not find sieve elements, do not penetrate them, or, if they do, do not maintain their penetration. In other words, the c002-knockdown insects essentially do not ingest phloem sap. This lack of feeding is presumably responsible for the premature death that we observed in these knockdown insects (8).

Although salivary secretions have long been recognized as vitally important in the interaction of aphids and plants (3, 7), this work describes a body of evidence that has been marshaled to demonstrate the essentiality of an individual, unambiguously identified salivary protein or enzyme and to provide direct evidence for that essentiality and for its role in feeding. The molecular mechanism by which Protein C002 acts in aphid feeding is not clear from the current results. Because Protein C002 matches no annotated protein in sequence, it is a total unknown, functionally, at the molecular level. Thus, we can exclude little at this stage. For instance, we cannot exclude that it might correspond to an enzyme activity detected by others in diluted saliva but that the enzyme occurs in the pea aphid in an unrecognizable form (nonmatching amino acid sequence); we cannot exclude that it could be a calcium-binding protein. Indeed, we cannot entirely exclude the possibility that it is a structural component of the stylect sheath, although this possibility seems unlikely given the rather normal probing of epidermal and mesophyll cells exhibited by the knockdown aphids. Finally, we certainly cannot exclude a role for Protein C002 produced in another organ, especially gut, but this role would have to be in addition to that of the salivary protein, which contacts plant tissue. One possibility that intrigues us is that Protein C002 aids in identifying or maintaining contact with sieve elements and that its depletion causes a defect analogous to the inability of Macrosiphum euphorbiae to establish sieve element phases in transgenic potato plants having half the normal sieve element content (16). This and other possible mechanisms will be the subject of ongoing investigations in our laboratories.

**Materials and Methods**

**Aphids, Plants, Standard Procedures, and cDNA Library Construction.** The aphid colony and growth of soybean are as described (8, 10), as are the standard methods used in this present work. The PCR-based cDNA library was made following instructions with the SMART cDNA library construction kit (Clontech), starting with RNA isolated from 250 dissected salivary glands. ESTs are posted at NCBI, accession numbers DV747494–DV752010.

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**Table 1. Analysis of the effects of c002-knockdown on foraging and feeding behavior in the pea aphid**

<table>
<thead>
<tr>
<th>Behavior examined</th>
<th>Control group mean (SD)</th>
<th>Treatment group mean (SD)</th>
<th>P, Ho: 1 = 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to first pathway phase, min</td>
<td>7 (13)</td>
<td>45 (45)</td>
<td>0.0073*</td>
</tr>
<tr>
<td>Rate of PD during initial cell-probing phase, no. per min</td>
<td>0.50 (0.23)</td>
<td>0.28 (0.27)</td>
<td>0.031*</td>
</tr>
<tr>
<td>Total duration of pathway phase, min</td>
<td>260 (75)</td>
<td>260 (96)</td>
<td>0.52</td>
</tr>
<tr>
<td>Total time mouth parts not touching the plant, min</td>
<td>56 (40)</td>
<td>210 (110)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Time to first SEP feeding, min</td>
<td>130 (87)</td>
<td>430 (140)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Total duration of SEP feeding in 8 h, min</td>
<td>156 (90)</td>
<td>3 (8.4)</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

EPGs were recorded for 8 h in insect in each of two groups of 10: the knockdown insects, injected with siC002-RNA; and control insects injected with siGFP-RNA (see Materials and Methods). The statistical analysis is based on a randomization test with 10,000 iterations. PD, potential drop, caused by styllets puncturing cells; SEP, sieve element phase, observed during feeding in a sieve element.

*Significant at α = 0.05.
antisense probes were synthesized by asymmetric PCR. Dissected salivary glands were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. After washing three times with PBS containing 0.2% Triton X-100 (PBST), the glands were treated with protease K (10 μg/ml) and fixed again with 4% paraformaldehyde for 1 h at room temperature. Prehybridization was performed, without probe, in hybridization solution (50% formamide, 5× SSC, 50 μg/ml heparin, 0.1% Tween 20, and 100 μg/ml salmon sperm DNA) at 48°C for 30 min. The fixed glands were exposed to DIG-labeled single-strand sense or antisense DNA probes (200 ng) in hybridization solution at 48°C for 20–30 h and washed successively in hybridization solution and in PBST. The glands were then blocked with 1% BSA by incubating at room temperature for 30 min, washed, and incubated with anti-digoxigenin horseradish peroxidase overnight at 4°C. After another wash in PBST, the samples were exposed to the Tyramide signal amplification kit (Molecular Probes).

Nuclei were counterstained with 1.5 μM propidium iodide (Molecular Probes) for 30 min at room temperature. Finally, the glands were mounted in glycerol and examined under a fluorescence microscope (Nikon Eclipse E800) with a triple bandpass filter for FITC (green color) and Cy3 (red color). Photographs were taken with a digital camera attached to the compound microscope, and pictures were edited with Adobe Photoshop 7.0.

Immunohistochemistry. Pea aphid salivary glands were dissected in PBS, washed three times in PBST, and fixed in Bouin’s fixative (71% saturated picric acid, 24% formaldehyde, 5% glacial acetic acid) for 10 min at room temperature in a humidified chamber. The glands were washed extensively with PBST and incubated with primary antibody (raised in rabbit against recombinant C002) at 1:100 dilution overnight at 4°C. The glands were then washed three times at 15-min intervals with PBST and blocked with 5% normal goat serum in PBST for 1 h, washed three times at 15-min intervals with PBST, and incubated with secondary antibody Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories) at 1:500 dilution overnight at 4°C. The glands were then washed extensively with PBST at 15-min intervals. Nuclei were stained by using TO-PRO-3 (Molecular Probes, Invitrogen) at 5 μM for 30 min in dark at room temperature. Glands were washed extensively with PBST and mounted on mounting media (Gel/Mount; Biomeda Corporation) on a glass slide. Photographs were taken with a Nikon Zeiss LSM 5 Pascal (laser scanning confocal microscope).

Analysis of Aphid Feeding Behavior. EPG measurements were carried out in an electrically grounded Faraday cage to shield the setup from external electrical noise. All experiments were carried out at room temperature (22°C–24°C). A gold wire, 2 cm long and 10 μm in diameter, was glued to the dorsum of the aphid by using conductive silver paint (Colloidal Silver; Ted Pella, Inc.). The wire was connected to the EPG monitor, and current flows through the insect and into the monitor, thus generating an interpretable signal. The feeding behavior of individual aphids on fava bean plants was monitored for 8 h with the help of a four-channel amplifier (two channels for siGFP-RNA-injected aphids and two for siC002-RNA-injected aphids). Ten replications were completed for each of two groups. The target group was C002-knockdown insects, injected as described in ref. 8 and maintained for 3 days on an artificial diet. These conditions produce ~60% knockdown of transcript C002 (figure 3 in ref. 8). The control group consisted of insects injected with siGFP-RNA (8) and otherwise treated the same as the target group. Waveform recordings were analyzed with the EPG analysis software PROBE 3.0 installed on a PC.

The parameters measured in this work included: (i) time to the first pathway phase (a measure of how quickly an aphid can identify a suitable location for probing individual cells); (ii) the rate of occurrence of potential drops during the initial pathway phase (a measure of how quickly an aphid probes individual plant cells with its stylet); (iii) the total duration of the pathway phase (a measure of time spent searching for a nutrient-rich sieve element); (iv) time to the first sieve element phase feeding (a measure of how quickly an aphid can find a nutrient-rich cell for feeding); and (v) total duration of sieve element phase feeding (a measure of time spent feeding on a nutrient-rich source). If the sieve element phase is not reached during the entire experiment time, the time to first sieve element phase feeding was considered to equal the total observation time (15), i.e., 8 h for this work. We tested for significant differences between our two treatments by using a randomization approach with 10,000 iterations. Specifically, we tested the null hypothesis that HO(mopteran) = H1(mopteran) by randomizing the data and determining the probability of getting the observed H1(mopteran). We used the SAS platform (18) and α = 0.05.

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Fig. S1. The nucleotide sequence of co02 cDNA was used to BLAST the *Acyrthosiphon pisum* genomic reads at the Baylor College of Medicine Genome Sequencing Center (www.hgsc.bcm.tmc.edu/projects/aphid). The 20 matching sequences were identified and assembled into two nonoverlapping contigs with the CAP3 Sequence Assembly Program [Huang X, Madan A (1999) CAP3: A DNA sequence assembly program. *Genome Res* 9:868 – 877]. The C002 coding region is divided into two exons with an intervening intron of 1.7 kb. The dotted line in the intron denotes the gap between the two contigs. Putative TATAA boxes are boxed in gray, and polyadenylation signal sequences (AATAAA) are underlined. The proximal poly(A) signal is likely to be the functional signal because this would give a transcript of the observed size.