Soldier caste-specific gene expression in the mandibular glands of *Hodotermopsis japonica* (Isoptera: Termopsidae)

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Edited by Bert Hölldobler, University of Wurzburg, Wurzburg, Germany, and approved September 14, 1999 (received for review July 7, 1999)

Although ''polymorphic castes'' in social insects are well known as one of the most important phenomena of polyphenism, few studies of caste-specific gene expressions have been performed in social insects. To identify genes specifically expressed in the soldier caste of the Japanese damp-wood termite *Hodotermopsis japonica*, we employed the differential-display method using oligo(dT) and arbitrary primers, compared mRNA from the heads of mature soldiers and pseudergates (worker caste), and identified a clone (PCR product) 329 bp in length termed *SOL1*. Northern blot analysis showed that the *SOL1* mRNA is about 1.0 kb in length and is expressed specifically in mature soldiers, but not in pseudergates, even in the presoldier induction by juvenile hormone analogue, suggesting that the product is specific for terminally differentiated soldiers. By using the method of 5′- and 3′-rapid amplification of cDNA ends, we isolated the full length of *SOL1* cDNA, which contained an ORF with a putative signal peptide at the N terminus. The sequence showed no significant homology with any other known protein sequences. *In situ* hybridization analysis showed that *SOL1* is expressed specifically in the mandibular glands. These results strongly suggest that the *SOL1* gene encodes a secretory protein specifically synthesized in the mandibular glands of the soldiers. Histological observations revealed that the gland actually develops during the differentiation into the soldier caste.

In social insects such as bees, wasps, ants, and termites, there are variations of individuals in terms of their behavior and/or morphology. These different types of individuals in a colony are called "castes" (1). In these social insects, reproductive divisions of labor occur, in which some individuals become sterile. All of the castes, including reproductives and neuters, have the genomic potential to become any caste, namely they have the genetic information for all caste types present in the genome. In the course of the individual ontogeny, the caste of an individual is determined in a process called "caste differentiation." It is thought that some genes should be expressed in a caste-specific manner. The phenomenon of the occurrence of several distinct phenotypes in a given species, each of which develops facultatively in response to some cue from the internal or external environment, is known as polyphenism (2, 3). However, few genes expressed in a caste-specific manner have been reported, although some researchers have started to identify such genes (e.g., see refs. 4 and 5 for studies in *Apis mellifera*).

Termites are one of the major groups of eusocial insects and have a well-organized social system. So far, several reports have suggested that some genes are expressed specifically in a certain caste in several species of termites (6). For example, development of eyes and wings arises when workers start reproduction by themselves in *Nasutitermes takasagoensis* (7). Furthermore, some differences in juvenile hormone titer and protein composition in the hemolymph have been reported in some termite species (e.g., ref. 8). However, no such genes have been identified in termites.

In the study described here, genes expressed specifically in soldiers of the Japanese damp-wood termite *Hodotermopsis japonica* Holmgren were identified by means of the differential-display method (9). We chose soldiers because soldier castes are seen in all of the termite species and have distinctive phenotypes. We used total RNA from the heads of mature soldiers and pseudergates (worker caste), as the head of the soldier is the most distinctive in various morphological characters when compared with pseudergates. In addition, expression of the genes was analyzed by Northern blot and *in situ* hybridization analyses. Histological analysis of the organ where the specific gene is expressed was also performed. This termite species is thought to be a relatively primitive termite and is distributed in natural evergreen forests of the Nansei Archipelago in Japan (10–13). It has a worker caste that is totipotent to differentiate to all castes and is called a "pseudergate" (defined in ref. 14). Through two exuviations, pseudergates differentiate into presoldiers, then into soldiers (15).

Materials and Methods

RNA Extraction from Insects. Colonies of *H. japonica* were sampled from Yaku-shima Island in Kagoshima Prefecture, Japan. Heads of the soldiers and pseudergates were dissected, frozen in liquid nitrogen, and preserved at −80°C for RNA extraction. Total cellular RNA was extracted by the guanidine isothiocyanate/phenol/chloroform method (16).

Differential Display. Differential display was performed essentially as described previously (9, 17–19). Briefly, 2-μg samples of RNA from soldiers and pseudergates were treated with 0.5 unit of RNase-free DNase I in 20 mM Tris·HCl (pH 8.4) containing 50 mM KCl, 0.5 mM dNTPs, 10 mM DTT, and 2.5 mM $MgCl_2$ in a total volume of 40 μl, and then reverse transcribed with or without 200 units of Super Script II (GIBCO/BRL) with 40 pmol of an anchored oligo(dT) primer with a *Bam*HI site (*Bam*-TG primer, 5′-CCC GGA TCC T_{15} G-3′). The resulting samples were amplified by the PCR with 20 combinations of arbitrary 10-mers with a *Hin*dIII site (*Hin*d-1 to -20 primers, 5′-CGG GAA GCT TN_{10}-3′, where N is any base) and *Bam*-TG primer. The reaction mixtures (10 μl) contained 1/10th of the volume of the reverse transcription products, 0.2 μl of 10× PCR buffer, 0.5 μl of [α-^{32}P]dCTP (>37 GBq/μl), 0.05 μl of *Taq* Gold polymerase (5 units/μl; Perkin–Elmer). The PCR conditions were: (95°C for 10 min + 37°C for 5 min + 72°C for 5 min) for 1 cycle + (95°C for 15 s + 55°C for 1 min + 72°C for 1 min) for 25 cycles + 72°C for 5 min. The PCR products were separated on a

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: JH, juvenile hormone; JHA, JH analogue; RACE, rapid amplification of cDNA ends; DIG, digoxigenin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB031871).

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denaturing 6% polyacrylamide gel and exposed to Kodak x-ray film. Reactions without reverse transcriptase were used as control experiments to detect bands amplified from a genomic DNA contaminating the RNA preparations. To ensure that the electrophoresis profile was reproducible, duplicate reverse transcription PCR reactions were performed.

Subcloning and Sequencing. Bands of interest were excised, the gel was boiled in TE buffer [10 mM Tris·HCl (pH 7.4)/1 mM EDTA], and the DNA was reamplified by PCR with the primer combination used in the differential-display method under the same PCR conditions, except that the concentration of the dNTPs was 200 μM, the first cycle was omitted, and no radioisotope was used. The reamplified DNA was subcloned into a pGEM-3Zf(+) vector at the *Sma*I site and transfected into *Escherichia coli* JM109. The nucleotide sequence of each strand was determined by the DNA-sequencing reaction with dideoxynucleotide cycle-sequencing procedure with a Dye-Terminator cycle-sequencing kit (Perkin–Elmer). Electrophoresis was performed on 6% polyacrylamide gels (Super Reading DNA Sequence Solution; Toyobo, Osaka), and the data were obtained with an automatic DNA sequencer (Perkin–Elmer, model 373S).

Northern Hybridization. Total RNA was extracted from the heads of termites by the guanidine isothiocyanate/phenol/chloroform method, as described above. To examine the gene expressions in the course of the soldier differentiation, RNA was also extracted from pseudergates exposed to juvenile hormone (JH) analogue (JHA; pyriproxyfen) for 1 or 2 wk. JHA treatment to induce presoldier differentiation was performed as described in ref. 20. Extracted total RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane (GeneScreen*Plus*, DuPont). The 264-bp fragment of the *SOL1* cDNA was used as a probe. The probe was labeled with ^{32}P by random priming, using Random Primer DNA Labeling Kit Version 2 (Takara Shuzo, Kyoto). Prehybridization and hybridization were carried out according to standard methods at 42°C in 50% formamide, 5× standard saline phosphate/EDTA [SSPE; 1 × 0.18 M NaCl/10 mM phosphate (pH 7.4)/1 mM EDTA], 5× Denhardt's solution (1 × 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% BSA), 1% SDS, and 200 μg/ml of single-stranded salmon sperm DNA. After hybridization, membranes were washed in decreasing concentration of SSC [0.15 M sodium chloride/0.015 M sodium citrate (pH 7)] and exposed to Kodak x-ray film.

5′- and 3′-Rapid Amplification of cDNA Ends (RACE). The sequence of the total length of the *SOL1* mRNA was determined by 5′- and 3′-RACE (21). Samples of 2 μg of RNA from soldier heads were treated with 1 unit of RNase-free DNase I (GIBCO; amplification grade) in 20 mM Tris·HCl (pH 8.4) containing 50 mM KCl, 0.5 mM dNTPs, 10 mM DTT, and 2.5 mM $MgCl_2$ in a total volume of 10 μl. First- and second-strand synthesis was performed with the Marathon-Ready cDNA Kit (CLONTECH), then Marathon cDNA Adaptor was ligated. PCR was done with Klen *Taq* polymerase (CLONTECH). Primers used in the PCR were adaptor primer 1 included in the CLONTECH kit (5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′) and specific primers for the *SOL1* gene (S13c-r2: 5′-CCG ACC TTC CAC CGT TTG CAT CC-3′ for 5′-RACE, and S13c-f2: 5′-GCC TGA AGG GCT GGG ACA AAC CAG-3′ for 3′-RACE). The amplified DNA fragments were subcloned into pGEM-T vectors (Promega) and transfected into *E. coli* JM109. The nucleotide sequence of each strand was determined by DNA sequencing as described above. Hydrophilicity/hydrophobicity of the amino acid sequence encoded by the *SOL1* gene was examined by Genetics Mac Version 9.0 Software, based on the Kyte and Doolittle method (22). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB031871.

***In Situ* Hybridization.** *In situ* hybridization was carried out as described previously (23) with minor modifications. Frozen sections (10 μm) of the soldier head were fixed with 4% paraformaldehyde in PBS and then hybridized with digoxigenin (DIG)-11-UTP-labeled single-strand sense or antisense RNA probes. The DIG-labeled RNA probes were prepared with the DIG RNA Labeling Kit (Roche Molecular Biochemicals). A 373-bp fragment of clone *SOL1* was amplified by PCR and subcloned into a pGEM-T vector and was used as a template to generate RNA probes. This plasmid was linearized with *Nco*I and *Sal*I and transcribed with SP6 RNA polymerase and T7 RNA polymerase to generate 386- and 400-nt antisense and sense probes, respectively.

Histological Observations. To observe the mandibular glands histologically, paraffin sections were made and stained with hematoxylin and eosin. The whole head or dissected gland of an individual termite was dehydrated in increasing concentrations of EtOH, transferred to xylene, then embedded in paraffin. Successive sections were made 5 μm thick, then put on a glass slide coated with egg-white/glycerol. Staining was done after removing paraffin by xylene, and slides were transferred into the decreasing concentrations of EtOH, then into hematoxylin solution [0.1% hematoxylin/0.02% $NaIO_3$/5% $AlK(SO_4)_2$/0.1% citric acid/5% trichloroacetaldehyde] and destained in running water, and then transferred to 0.5% eosin solution and destained

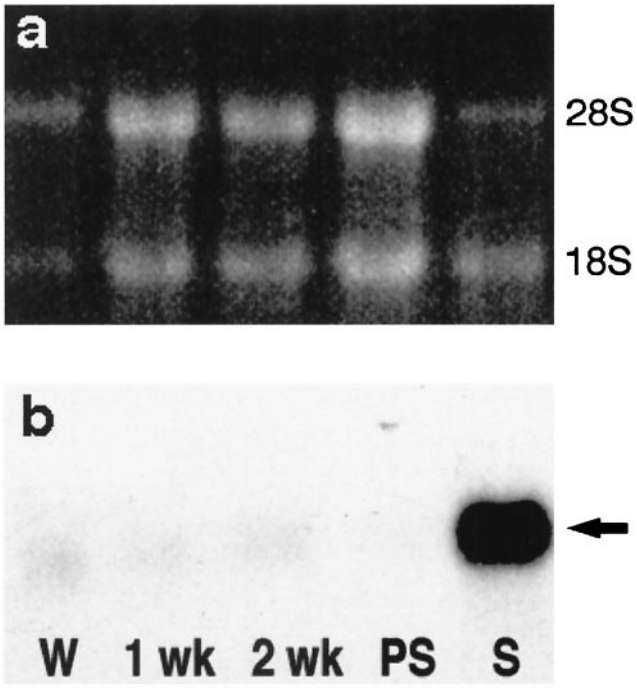

Fig. 1. Northern blot analysis of *SOL1* expression in termite heads. Pseudergates (W) were exposed to JHA for 1 or 2 wk to induce differentiation into the soldier caste (PS, JHA-induced presoldier; S, soldier). Total RNAs from heads were resolved on denaturing agarose gels, blotted, and probed. (*a*) 18S and 28S rRNA bands stained with ethidium bromide indicate that approximately equal amounts of RNA were loaded in each blot. (*b*) Hybridization with the labeled *SOL1* cDNA fragment. An arrow indicates the *SOL1* transcript (≈1 kb), which was strongly expressed only in the soldier caste. Little or no expression was found in other castes, even during the course of differentiation.

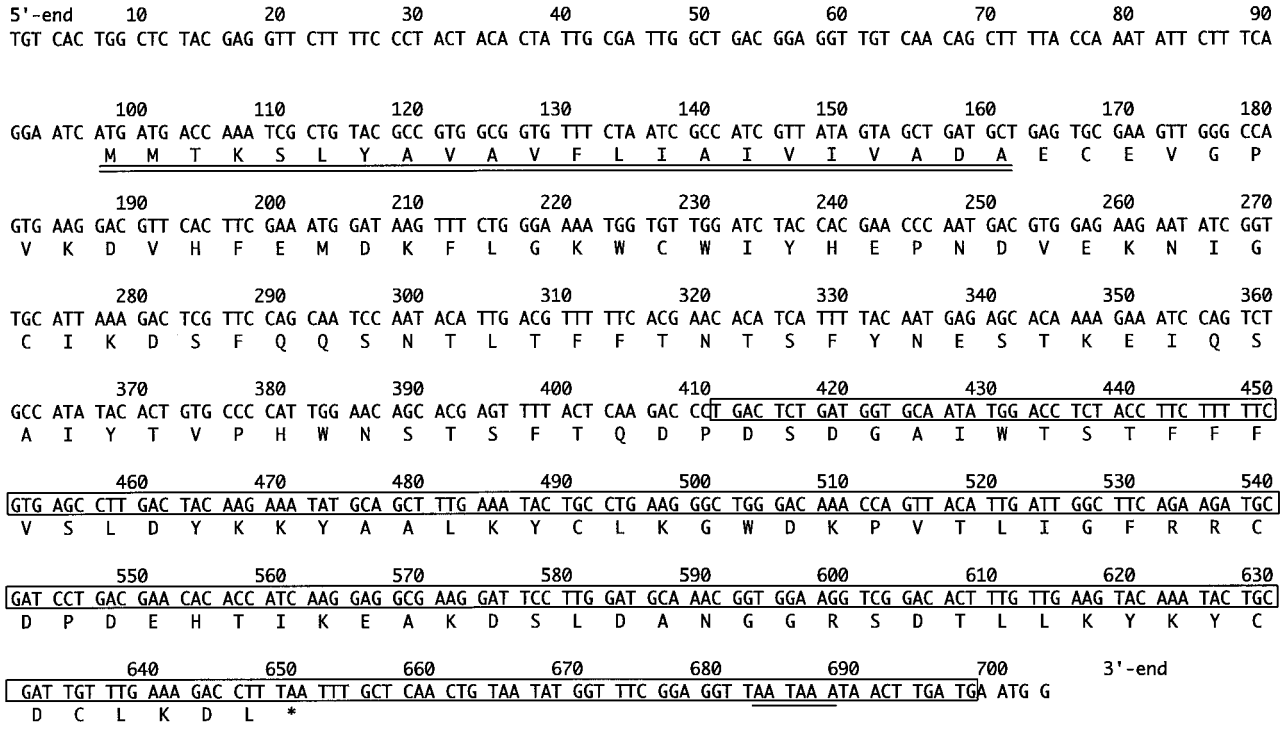

Fig. 2. cDNA and putative amino acid sequences for *SOL1*. The 703-bp cDNA, obtained by 5′- and 3′-RACE, contains a putative ORF that encodes a polypeptide of 184 amino acid residues. An asterisk indicates the termination codon. Boxed sequence indicates the DNA fragment found by differential display. The putative signal peptide at the N terminus is double underlined. A potential polyadenylation signal at the 3′ end of the sequence is underlined. The *SOL1* sequence will appear under the DDBJ/EMBL/GenBank accession number AB031871.

as well. After staining, slides were dehydrated and enclosed with Canada balsam under cover glasses.

Results

To identify genes expressed specifically in the soldier caste of the termite, the differential-display method was employed using total RNA extracted from soldiers and pseudergates of the Japanese damp-wood termite. RNA from pseudergates was used as a control RNA to detect genes expressed ubiquitously in individual termites. By screening approximately 1,000 bands on sequence gels with 20 combinations of arbitrary primers (*Hin*d-1 to -20 primers) and *Bam*-TG primers, 11 candidate bands were identified reproducibly. Among them, a band termed *SOL1* was detected almost specifically in the soldier heads by a combination of the *Hin*d-12 primer (5′-CGG GAA GCT TAC TCC ATG ACT C-3′) and the *Bam*-TG primer. The band was excised from the gel, and the DNA was reamplified by PCR with the same primers and subcloned into a pGEM vector. The nucleotide sequences of two independent clones were determined and found to be identical. The isolated clone was 329 bp long, and the sequence showed no homology to any other known DNA sequences.

To examine the expressions of the candidate genes, Northern blot analysis was performed using RNA extracted from the termite heads. Only one candidate (*SOL1*) gave a discrete signal in the analysis, showing that this gene is strongly expressed in the soldier heads. Expressions of the other candidates were not detected in this analysis. From the results, the size of the *SOL1* mRNA was shown to be about 1 kb. In some termites, including the focal species, JH and/or its analogue (JHA) can induce soldier differentiation; therefore, we extracted RNA from induced termites after exposure to the JHA pyriproxyfen for examination of the expression of *SOL1* mRNA. The result was that no bands were detected in any other stages during the presoldier induction by JHA, indicating that the *SOL1* gene is specifically expressed in the mature soldiers' heads (Fig. 1).

Next, to isolate the full-length *SOL1* cDNA, 5′- and 3′-RACE was performed. As a result, 703 bp of the cDNA sequence, which contained an ORF encoding 184 amino acid residues, was obtained (Fig. 2). The signal of polyadenylation (AATAAA) was found near the 3′ end of the cDNA sequence. None of the nucleotide and amino acid sequences were found to be homologous to any other known protein sequences. However, the N terminus end of the SOL1 protein sequence encodes a putative signal peptide composed of hydrophobic amino acid residues, suggesting that the SOL1 protein is a secretory protein. The presumed cleavage site is between the 22nd (Ala) and 23nd (Glu) amino acids (Fig. 3).

To examine the localization of the expression of the *SOL1* gene, *in situ* hybridization analysis was performed using frozen

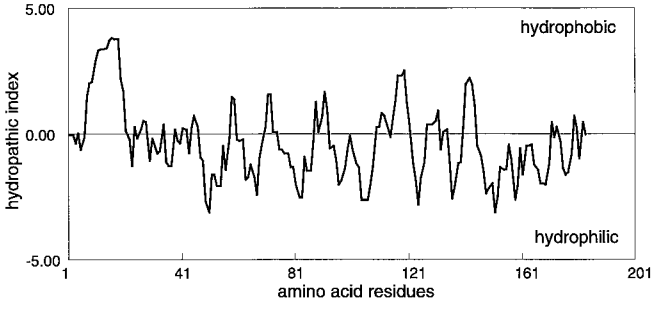

Fig. 3. Hydrophilicity/hydrophobicity plot of the putative amino acid sequence of SOL1. The profile was generated by the method of Kyte and Doolittle (22). Tracings above the center line indicate hydrophobic regions, and below the line indicate hydrophilic regions.

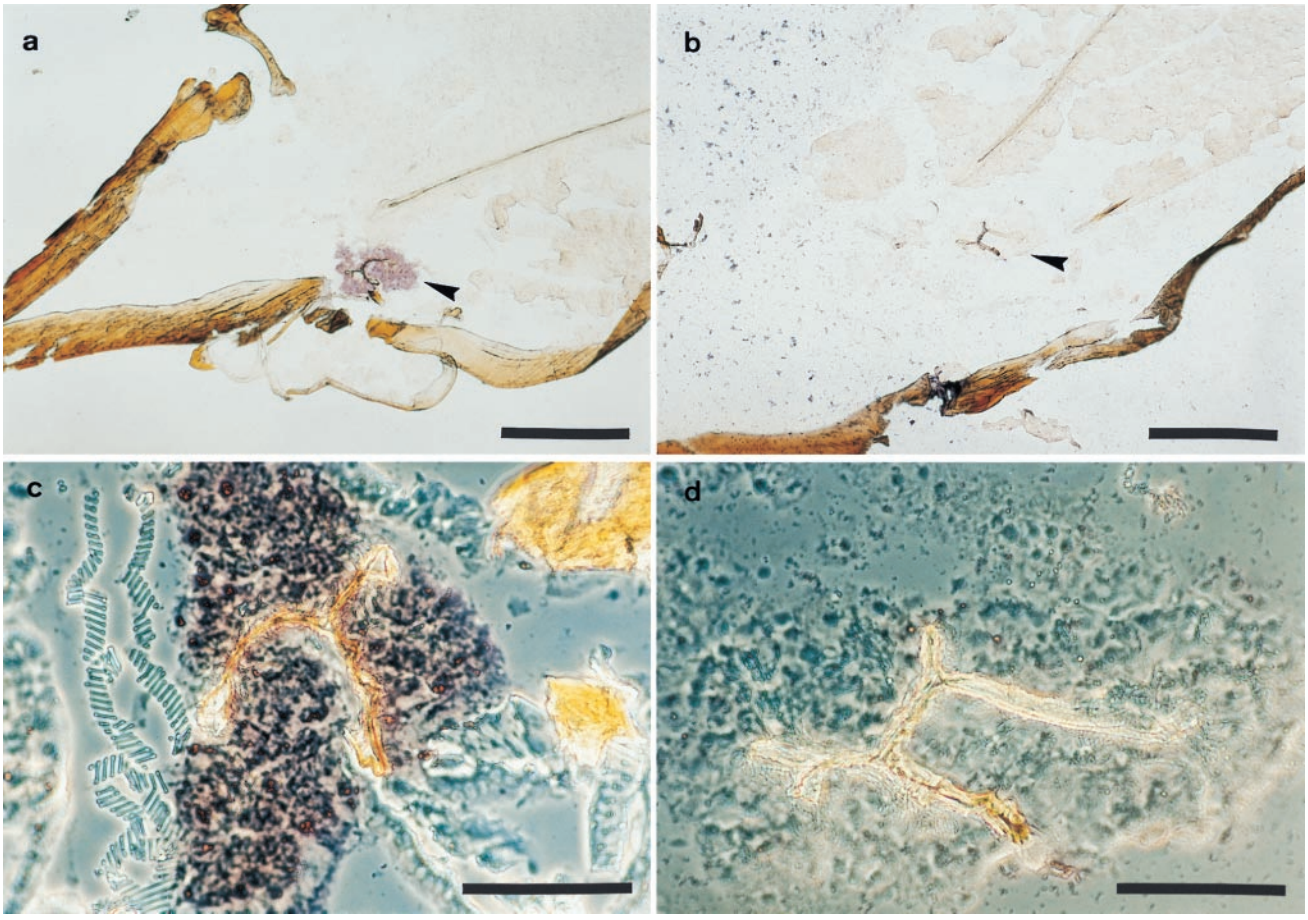

Fig. 4. *In situ* hybridization of the *SOL1* mRNA in the mandibular glands at the bases of the mandibles in soldiers. Frozen sections (10 μm) of the head of the soldier termite were subjected to *in situ* hybridization with antisense (*a* and *c*) and sense (*b* and *d*) DIG-labeled RNA probes. The probes were then detected with peroxidase-conjugated anti-DIG Ab. The mandibular glands, which are stained in purple, are indicated by an arrowhead (*a* and *b*). (*c* and *d*) Magnified photos of the mandibular glands shown in *a* and *b*, respectively. The yellow ducts of the mandibular glands were seen in the center of the glands. [Bars indicate 0.5 (*a* and *b*) and 0.1 mm (*c* and *d*).]

sections of soldier heads with a DIG-labeled antisense RNA probe. The expression of the *SOL1* gene was clearly detected in an organ located at the base of the mandibles but not in any other part of the head (Fig. 4). Many references describe that this organ is a mandibular gland (24–28). The pair of mandibular glands are characteristically well developed and therefore easily found in the heads of soldiers (Fig. 5), but are not well developed in the heads of pseudergates. According to histological observations, the cells of these glands became larger as differentiation into soldiers occurred. Secreted substances were observed in the duct (reservoir) of the glands in soldiers (Fig. 6).

Discussion

This study identifies a gene that is expressed in a caste-specific manner in termites. The morphology and function of the soldier caste of termites is very distinctive in comparison with other castes, such as reproductives or workers (29). Therefore, the gene-expression pattern has long been supposed to differ in mature soldiers to some extent. As an initial step in analyzing the molecular mechanisms of caste differentiation, identification of genes expressed specifically in the mature soldiers was examined by using the differential-display method.

As differential display is a technique that detects differences in gene expression between tissues based on PCR amplification (9), it sometimes yields not only the gene-specific product, but also a number of "false" or nonspecific products (30). The *SOL1* mRNA identified in this study was detected in the analysis reproducibly. Furthermore, Northern blot analysis showed that this mRNA was strongly expressed specifically in soldiers (Fig. 1). Although expression of the other candidate genes identified in the differential display were not confirmed by Northern blot analysis, there may be some genes expressed at a low level whose products play important roles in soldiers.

The *SOL1* gene obtained here is expressed only in the soldier caste, but not in any other stages of the differentiation into presoldiers (Fig. 1), indicating that the expression begins after the completion of the differentiation into the terminal functional soldier caste. Although there should be a number of genes expressed during the course of the soldier differentiation, the *SOL1* gene is probably one of the most downstream genes in the gene-expression cascade. The localization of the expression of *SOL1* in heads of soldiers was shown to be restricted in mandibular glands by *in situ* hybridization analysis (Fig. 4), suggesting that the SOL1 product plays a specific role in the gland. The *SOL1* cDNA of 703 bp contains a putative ORF that has a signal peptide at the N terminus end (Figs. 2 and 3), suggesting that the translation of the gene occurs at the surface of the endoplasmic reticulum, and then the products are secreted into the extracellular region (cf. ref. 31). With these assumptions, the SOL1 protein is presumably secreted into the duct of the mandibular glands; however, direct evidence from immunohistochemical techniques are required to confirm this.

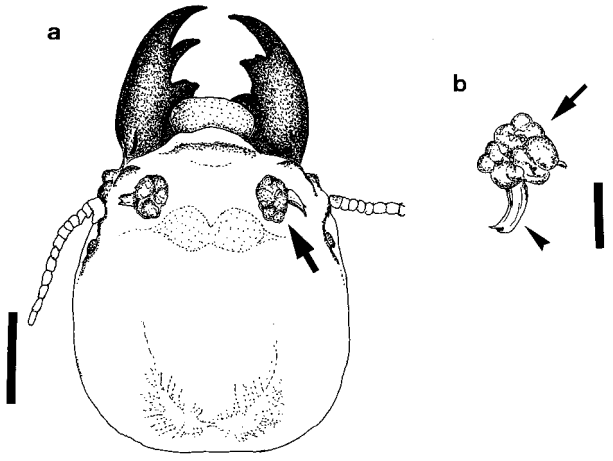

Fig. 5. Schematic representation of the position and structure of the mandibular glands that are located at the bases of each mandible. (*a*) The mandibular glands were a pair of tubular organs with many acini and are easily observed when a soldier head is dissected anatomically. (*b*) Illustration of mandibular gland (arrow) and accessory mandibular gland (arrowhead), which form the mandibular gland complex.

Judging from the specificity of expression in the soldier caste, there are two possibilities for the function of the SOL1 protein: (*i*) a defense substance against predators, or (*ii*) a substance affecting interactions among individuals (i.e., pheromone-like substance). If the SOL1 protein plays the role of a pheromone, it may be an alarm pheromone or a pheromone that regulates caste differentiation, for example, to inhibit the soldier differentiation of other colony members. In other social insects, such as ants and honeybees, the mandibular gland is known to be a pheromone gland, which secretes alarm pheromones in ant workers (32, 33) or queen substances in honeybees. Queen substances are known to be involved in the attraction of worker bees to the queen bee, and they suppress the development of ovaries in workers, inhibit the construction of queen cells, and affect foraging behavior (34, 35). Pheromones secreted from mandibular glands are mixtures of volatile organic compounds (35–37). In termites, however, there are few reports on the function of the mandibular gland secretions, and only histological studies have been published (24, 26, 27). The accessory mandibular gland and its hydrocarbon secretions have been described in the related genus *Zootermopsis*, and these substances were suggested to work as recognition pheromones (28). Thus, no proteins have been reported as mandibular gland secretions so far. Pheromone substances are generally volatile; therefore, if SOL1 functions in the communication among individuals, it is probably transferred by means of direct contacts among individuals, such as stomodeal and proctodeal trophallaxis or grooming.

As shown in Fig. 6, mandibular glands already exist in the preceding castes and instars, such as pseudergates and presoldiers; however, *SOL1* mRNA is expressed only in mature soldier caste, indicating that the gene is not the target gene of JH. As there is congruence between the timing of the development of the gland and that of the *SOL1* expression, this gene product is presumed to be a protein that is secreted only in soldiers. SOL1 does not have any homology of sequence and structure with milk protein of honeybee and salivary protein of *Drosophila*, thus we cannot speculate on the origin and phylogeny of the gene. Further investigations on the effects of the SOL1 protein are needed to reach conclusions about its functions. Studies with recombinant protein will be useful for analyzing the effects on individual termites.

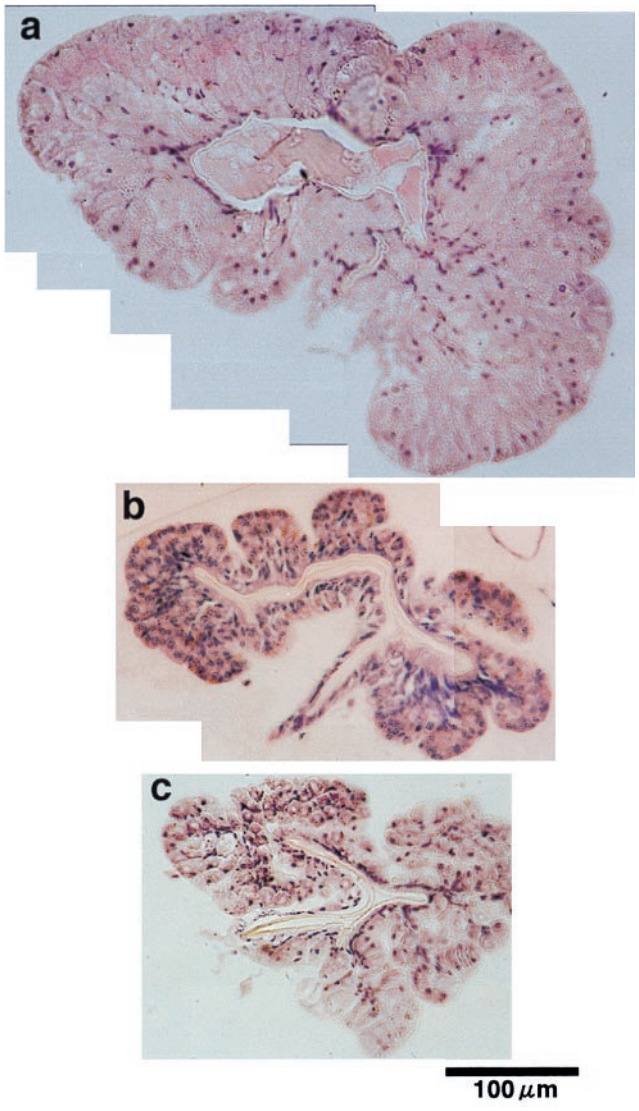

Fig. 6. Histlogical observations of mandibular glands. Paraffin sections of the mandibular gland were stained with hematoxylin and eosin. Cells of the mandibular glands become larger during differentiation into soldiers. (*a*) Soldier; (*b*) presoldier, and (*c*) worker (pseudergate). Note that secretory substances are seen in the duct of the glands of soldiers.

The most serious problem of the identification of novel genes in termites is the difficulty in the studies on the function of the genes, because the generation time of termite individuals and colonies is too long to study genetics and to make transgenic organisms. To avoid these problems, several methods of investigation may be viable, for example, studies on the homologous genes in *Drosophila*, or using experimental systems developed in related insects such as cockroaches. In the case of the *SOL1* gene product, however, the function of the protein could be studied directly, by the examination of the effects on the individuals, if the protein is found to be one of the secretions from the mandibular glands.

We thank K. Maekawa, H. Katoh, M. Machida, and O. Kitade for assistance in the field sampling. We also thank N. Lo who kindly corrected the manuscript. This study was supported by Grant-in-Aid Scientific Research Program (nos. 10440231 and 11142204) from the Ministry of Education, Science, and Culture of Japan. T. Miura, A.K., and H.T. were supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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