Molecular cloning of [Thr⁴, His⁷]-corazonin (Apime-corazonin) and its distribution in the central nervous system of the honey bee *Apis mellifera* (Hymenoptera: Apidae)

Ladislav ROLLER,^{1,2} Seiji TANAKA,³ Kiyoshi KIMURA,⁴ Honoo SATAKE⁵ and Yoshiaki TANAKA^{1,*}

¹ Developmental Biology Department, National Institute of Agrobiological Science (NIAS); Tsukuba 305–8634, Japan

² Institute of Zoology, Slovak Academy of Sciences; Dúbravská 9, 84506 Bratislava, Slovakia

³ Division of Insect and Animal Sciences, NIAS at Ohwashi, Independent Administrative Institution; Tsukuba 305–8634, Japan

⁴ Department of Animal Breeding and Reproduction, National Institute of Livestock and Grassland Science; Tsukuba 305–0901, Japan

⁵ Suntory Institute for Bioorganic Research; Osaka 618–8503, Japan

(Received 3 December 2005; Accepted 28 February 2006)

Abstract

Corazonin (Crz) is a neuropeptide with a conserved structure and distribution pattern in the central nervous system (CNS) of many insects. We cloned the Crz gene from brains of the honey bee workers (*Apis mellifera* L., Apime). The ApimeCrz cDNA comprises 502 base pairs and encodes a new form of corazonin displaying Thr⁴ substitution of [His⁷]-corazonin. In workers of *A. mellifera*, corazonin immunoreactivity was found in a group of four to five lateral neurosecretory cells (LNSC) in each hemisphere of the protocerebrum and in lateral paired neurons segmentally distributed in the ventral ganglia. However, the ApimeCrz mRNA was transcribed only in the LNSC. The Crz LNSC projected into the ipsilateral *corpus cardiacum*. The pattern of distribution of corazonin immunoreactive material in the CNS suggests that Apime-corazonin plays roles as a neurohormone as well as a neurotransmitter or neuromodulator. The effects of this neuropeptide on the induction of dark color in albino nymphs of *Locusta migratoria* and spinning rate in spinning larvae of *Bombyx mori* were assayed. The results indicate that Apime-corazonin shows biological activities comparable to those for [Arg⁷]- and [His⁷]-corazonins.

Key words: Corazonin; *Apis mellifera*; neuropeptides; immunohistochemistry; *in situ* hybridization

INTRODUCTION

Corazonin (Crz) is a neuropeptide originally isolated from the cockroach *Periplaneta americana* (Veenstra, 1989). Two forms of the peptide have been found in the central nervous system (CNS) of insects. The corazonin amino acid sequence in cockroaches, a cricket and two species of moths is the same (Veenstra, 1991, 1994; Hua et al., 2000; Hansen et al., 2001) and named [Arg⁷]-corazonin. The other form with a substitution at position 7, [His⁷]-corazonin, has been isolated from three species of locusts and a stick insect (Veenstra, 1991; Predel et al., 1999; Tawfik et al., 1999). Both forms of corazonin induce dark coloration in the

epidermis of an albino mutant of *Locusta migratoria* (Tawfik et al., 1999; Hua et al., 2000). There is direct, immunohistochemical or the albino locust bioassay based evidence that corazonin-like substance occurs in the CNS of 18 insect orders (for review see Roller et al., 2003; Tanaka, 2005). Recently, corazonin has been detected in the pericardial organ or sinus gland of crustaceans (Li et al., 2003; Fu et al., 2005). These data indicate that corazonin is widespread in the Arthropoda.

The Crz gene has been characterized only in a few insects including the fruitfly *Drosophila melanogaster* (Veenstra, 1994 and Gene ID 41742 an automated genome annotation), other three *Drosophila* species (Choi et al., 2005) and two

* To whom correspondence should be addressed at: E-mail: yoshiaki@affrc.go.jp

The nucleotide sequence reported in this paper has been submitted to the GenBank™ with accession number NM001012963.

DOI: 10.1303/aez.2006.331

moths *Galleria mellonella* (Hansen et al., 2001) and *Bombyx mori* (GenBank accession no. AB106876). However, these Crz cDNAs all encode only [Arg⁷]-corazonin.

A search in the *A. mellifera* genome database allowed us to suggest the presence of a third corazonin form in this insect (Tanaka, 2005). In the present work we cloned the cDNA encoding a new form of corazonin in *A. mellifera* (Apime-corazonin). We also localized the neurons that expressed ApimeCrz gene. To examine its relation to the known corazonin forms, Apime-corazonin was tested for the ability to reduce spinning rate in the silkworm as well as for the dark color-inducing activity in the albino strain of *L. migratoria*.

MATERIALS AND METHODS

Insects. The honey bees of a commercial strain were obtained from a rearing stock at National Institute of Livestock and Grassland Science in Tsukuba, Japan. Albino locusts and the larvae of silkworm, *B. mori* were reared at NIAS under conditions as described previously (Tanaka et al., 2002).

Cloning of Apime-corazonin cDNA. Using the amino acid sequence of [Arg⁷]-corazonin (QT-FQYSRGWTNGKR) as a query, BLAST analysis (TBLASTN) was performed against *A. mellifera* genomic database (The TIGR Gene Indices, <http://tigrblast.tigr.org/tgi/>). Based on the putative coding sequence of the obtained genome contig (ref|NW_048393.1|AmeUn_WGA708_1 currently placed in ref|NW_627027.1|AmeLGUn_WGA505_2), primers were designed as follows: (BeeCRZ1: 5'-CGTTTACGTACAGTCACGGTTG-GACA-3' and BeeCRZ2: 5'-CGTTGGAGGAAC-TTGCGAATAGGAA-3') and 5'RACE (BeeCRZ3: 5'-TTGGCAAAAACATTGTCCGATTGAA-3' and BeeCRZ4: 5'-GCATTCCTATTCGCAAGT-TCCTCCA-3'). RACE was performed using the above primers and SMART RACE cDNA Amplification Kit (BD Biosciences, CA, USA) according to the manufacturer's instructions. The first-strand cDNA prepared from adult brain total RNA was used as a template. PCR products were gel-filtrated with a spin column of Sephacryl S-300 HR (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and cloned into pGEM-T vector (Promega, WI, USA). Insert sequences were determined by

BigDye Terminator cycle sequencing method with a 3700 DNA analyzer (PE Applied Biosystems, CA, USA).

Nucleotide and protein sequences were analyzed by free bioinformatics tools at JustBio web site (<http://www.justbio.com/>, Clustal W 1.8 and Translator), by BLAST at NCBI web site and by SignalP 3.0 (Dyrlov et al., 2004).

Immunohistochemistry. Whole-mount immunofluorescent immunohistochemistry was performed, as described by Roller et al. (2003). A rabbit polyclonal antibody against [His⁷]-corazonin developed by Wako Co. (Nagano, Japan) and characterized by Roller et al. (2003) was employed at dilution 1:4,000. For visualization of bound antigen-primary antibody complex a goat anti-rabbit IgG labelled with Alexa Fluor 488 (Molecular ProbesTM, CA, USA) or a donkey anti-rabbit Cy3 labelled IgG (Jackson ImmunoResearch, PA, USA) was used at dilution 1:1,000. Stained tissues were washed sequentially with PBS, 50%, 70% and 90% glycerol in PBS and mounted in glycerol.

Whole-mount *in situ* hybridization. Tissues were dissected out from bees in 4% paraformaldehyde (PA), fixed in PA at 4°C overnight and subjected to *in situ* hybridization procedure as described by Tautz and Pfeifle (1989). Several modifications of the procedure were adopted. We used hybridization buffer and custom-designed probes provided with HybriProbe TriSeq Kit (Biognostik, Göttingen, Germany). A mixture of three fluorescein labelled DNA oligonucleotides (30 nucleotides each) designed from the Apime-preprocrazonin cDNA was hybridized with target mRNA in the bee CNS at 32°C overnight. A mixture of three sense probes supplied in the HybriProbe TriSeq Kit was used for a negative control experiment. Hybridized probes were detected using a rabbit anti-fluorescein alkaline phosphatase labelled antibody (Sigma, MO, USA) at dilution 1:40 and visualized using the NBT/BCIP color substrates (Roche Diagnostics, Penzberg, Germany). Stained tissues were mounted or subjected to the immunohistochemical procedure as described above.

To observe labelled preparations, we used a microscope, Nikon Eclipse 600 with a Nikon Coolpix 990 digital camera (Nikon, Tokyo, Japan) and Leica MZ16 and Leica DMLB microscopes with Leica DC300 photographic equipment (Leica Microsystems, Wetzlar, Germany). Images were

processed using Adobe Photoshop 7.0 software (Adobe, CA, USA).

Synthetic peptides. Synthetic corazonins were all custom-synthesized at Suntory Institute for Bioorganic Research and further purified by reverse phase high-performance liquid chromatography (HPLC) before the experiment. These peptides were dissolved in rapeseed oil (Hayashi Chemicals, Tokyo, Japan) before being used for bioassays.

Bioassays. Various amounts of the synthesized Apime-corazonin were tested for the dark-color inducing activity in albino nymphs of *L. migratoria* according to the method described previously (Tanaka et al., 2003). In brief, newly ecdysed 4th stadium nymphs were injected with the peptide mixed with rapeseed oil (2 μ l) and photographed 4 d later still in the same stadium by a scanner (ES 2000, Epson, Tokyo, Japan) connected to a personal computer (Mate MA10T, NEC, Tokyo, Japan), and the mean luminance of the lower part of the pronotum was measured with computer software (Adobe Photoshop 7.0) for each individual (Tanaka, 2004). For comparison, [Arg⁷]- and [His⁷]-corazonins were also tested in the same way.

Bombyx spinning rate bioassay was performed as previously described by Tanaka et al. (2003). The peptide was injected into fifth stadium larvae between 22:00–23:00 on day 7 of the fifth stadium, and then the cocoon layers were weighed between 12:00–13:00 on day 8 as a significant reduction in cocoon weight was observed at 12:00 on day 8 (Tanaka et al., 2002).

RESULTS

Structure of the Apime-corazonin cDNA and protein

The cloned Apime-corazonin cDNA (Apime-Crz) contains 502 base pairs (bp) including an open reading frame (ORF) beginning with a translation start codon ATG at bp 71 and ending with a stop codon TAA at bp 392 (Fig. 1). The presence of three purines upstream from the ATG fulfils criteria for a translation start site (Kozak, 1991). The 3' untranslated region (3'UTR) contains an AATAAA motif that represent putative polyadenylation signal (Proudfoot and Brownlee, 1976). The predicted ApimeCrz precursor is a polypeptide of 107 amino acids with a deduced molecular mass of 12,305 Da. Its first 21 amino acids compose a signal peptide with cleavage site VMC-QT as was predicted by computer analysis. The signal peptide is immediately followed by the sequence Gln-Thr-Phe-Thr-Tyr-Ser-His-Gly-Trp-Thr-Asn-Gly, which is identical to [His⁷]-corazonin except for Thr at 4th residue. It represents a new form named Apime-corazonin. Corazonins are known to be N-terminally blocked and C-terminally amidated. Gln at N-terminus and Gly followed by cleavage site (two dibasic residues Lys-Arg) at C-terminus facilitate the expected modifications. The last part of the precursor is a 72 residue C-terminal peptide, the corazonin precursor related peptide (CPRP, *sensu* Veenstra, 1994). BLAST analysis of available databases did not find significant hits to Apime-CPRP sequence.

Fig. 1. cDNA and deduced amino acid sequence of *A. mellifera* preprocorazonin. The putative translation start and stop codons are bold typed and polyadenylation signal is underlined in the nucleotide sequence. Signal peptide is typed in italics and corazonin sequence is grey highlighted. The amidation site and adjacent cleavage site are underlined.

Fig. 2. Preprocorazonin structure and corazonin gene organization in *A. mellifera*. Domains of Crz gene transcript are boxed and an open reading frame is shaded in grey. Amino acid lengths of the preprocorazonin domains are indicated. A signal peptide (SP) is followed by corazonin (Crz), posttranslational processing site (GKR) and corazonin precursor related peptide (CPRP). The Crz genomic sequence (vertical line) contains two introns and their positions and sizes are shown. Corresponding sequences of the final transcript are indicated for exons.

Organization of the Apime-corazonin gene

A search for genomic sequences corresponding to ApimeCrz cDNA revealed that ApimeCrz gene is split into three exons localized in unplaced genomic contig NW_627027. The 5' untranslated region (5'UTR) of Crz mRNA (bp 1 to 70) corresponds to bp 6,039–6,108 of the contig, main part of the ORF (bp 71–289) corresponds to bp 7,711–7,931 and the rest ORF with 3'UTR (bp 290–476) are coded at bp 8,020–8,206 (Fig. 2). Both introns contain canonical splicing acceptor (GT) and donor (AG) sites. These results are supported by automated genome annotation of the Crz gene (ID 503862) reported in the genome database (NCBI web site).

Localization of corazonin immunoreactive cells

We employed a polyclonal antibody against [His⁷]-corazonin to detect corazonin immunoreactive cells in the CNS of *A. mellifera* workers. In the lateral protocerebrum a group of four to five cells was stained (Fig. 3). Somata of these neurons were about 20 μ m in diameter. The cells displayed two branches of immunoreactive fibers, the very fine ipsilateral projections into the retrocerebral neurohemal organs and their collaterals extending into the ventromedial protocerebrum (Fig. 3A). Corazonin immunoreactive fibers were found to terminate with numerous varicosities in the *corpora cardiaca* (Fig. 3B). No other immunoreactive cells were detected in the brain. In the ventral ganglia, segmentally distributed bilateral somata were immunoreactive (data are not shown, see below).

Expression of the ApimeCrz gene

Patterns of localization for the ApimeCrz mRNA in the bee CNS were observed using whole-mount *in situ* hybridization. In the worker brain, a group of lateral cells was labelled in each

hemisphere of the protocerebrum (Fig. 3C). These cells were identical to those showing immunoreactivity for [His⁷]-corazonin (Fig. 3D). The same situation was found in the brain of the queen (data not shown). The ApimeCrz mRNA signal was not detected in the ventral ganglia of the worker and queen.

Albino locust and silkworm spinning rate bioassays

To determine if Apime-corazonin shows the same effects as [Arg⁷]- and [His⁷]-corazonins, we tested synthesized peptides with the albino locust and silkworm spinning rate bioassays. Apime-corazonin induced dark color in albino locusts (Fig. 4). Its dark-color inducing activity was as strong as that for the other corazonins (ANOVA, $p > 0.05$ at each dosage; Fig. 5). Apime-corazonin at 1 pmol was significantly effective (the Student's *t*-test) in reducing the spinning rate in spinning larvae of *B. mori*. No significant difference was observed in the effect among the three tested forms (ANOVA, $p > 0.05$ at each dosage; Fig. 6). These data suggest that Apime-corazonin is a bioactive form of corazonin.

DISCUSSION

A cDNA encoding Apime-corazonin preprohormone was cloned using data of the *A. mellifera* genome assembly. The ApimeCrz gene includes two introns and seems to be present in the genome in a single copy. Crz gene containing two introns has also been found in *Drosophila virilis* (Choi et al., 2005). The position of the first intron within the 5'UTR of mRNA is similar between *A. mellifera* and *D. virilis*. The second intron is located within the ORF coding CPRP and it seems to be a well conserved feature among insects. ApimeCrz tran-

Fig. 3. Neurons producing corazonin in an *A. mellifera* adult brain (whole-mounts; A, B, D: composite images). A: Anterior view of the lateral protocerebrum showing corazonin immunoreactive cells (asterisk) and their axonal patterns with processes projecting to the medial protocerebrum (arrow) and ipsilateral retrocerebral neurohemal organ (arrowhead, Alexa Fluor 488). The medial line of the brain is indicated by a vertical bar. B: Immunoreactive fibers with terminal branches in *corpora cardiaca* (CC) which are attached to the foregut (squares). No staining was detected in *corpora allata* (CA, Cy3). C: Anterior view of the brain showing neurons (asterisk) containing corazonin mRNA in the region of lateral neurosecretory cells (NBT/BCIP). D: Localization of the ApimeCrz mRNA (arrow, dark dots, NBT/BCIP) and corazonin peptide (fluorescent yellow, Cy3) in the identical protocerebral cells (asterisk). Scale bars, 50 μ m.

Fig. 4. Photographs showing albino nymphs of *L. migratoria* injected with oil or different doses of Apime-corazonin 4 d earlier.

script is 478 bp long (without poly-A tail) and does not show any significant sequence similarity with known Crz mRNAs. The Crz transcript base pair lengths vary from about 500 in *A. mellifera* to about 950 in *G. mellonella* (Hansen et al.,

Fig. 5. Average luminance values for the pronotum of 4th stadium albino nymphs of *L. migratoria* after injection of various doses of corazonins and oil. Injections were made at the beginning of the 4th nymphal stadium and the nymphs were photographed 4 d later. $N=5-7$ each. Vertical lines indicate SD.

2001) and even sizes of 5'UTR, coding region and 3'UTR display interspecific differences. The Crz transcripts of *A. mellifera* and *Drosophila* species contain canonical polyadenylation signal

AATAAA. Different putative polyadenylation signal ATTAATAA could be found in Crz mRNA of the moths *B. mori* and *G. mellonella* in two copies.

The corazonin precursor of *A. mellifera* displays structural similarity to the other known Crz precursors (Fig. 7). A putative signal peptide (SP) is immediately followed by corazonin itself and a corazonin precursor related peptide (CPRP). A similar structure occurs in precursors of the adipokinetic/red pigment concentrating hormones family (AKH/RPCH; Veenstra, 1994). Only a few identities could be found among aligned SPs and CPRPs, and the CPRP appears to be the most variable and rapidly evolving domain in the Crz preprohormone (Choi et al., 2005). However, this alignment seems to correlate with phylogenetic relatedness of the analysed insects as indicated by the comparatively high sequence similarity between *G. mellonella*

and *B. mori* (Fig. 7).

The mature corazonin peptide represents the most conserved part of the precursor (Fig. 7). But in the case of *A. mellifera* two substitutions of amino acid residues were found. His residue at the 7th position occurs in several orthopteroid insects (Veenstra, 1991; Predel et al., 1999; Tawfik et al., 1999) and it has not yet been detected in any representative of the Holometabola. A substitution at the 4th position, Thr residue, was found in corazonin for the first time. Apime-corazonin represents a new form of this peptide presumably produced by *A. mellifera*. Recently, the structure of Apime-corazonin was confirmed by tandem mass spectrometry (MS) analysis (Verleyen et al., 2006).

To confirm the presence of corazonin and the ApimeCrz gene transcript in the CNS of *A. mellifera*, we performed whole-mount immunohistochemistry and *in situ* hybridization experiments. A group of lateral neurosecretory cells (LNSC) in the protocerebrum and lateral paired neurons segmentally distributed in the ventral ganglia were found to contain Crz immunoreactive material. A similar staining pattern has been found in representatives of 7 insect orders (e.g. Veenstra and Davis, 1993; Cantera et al., 1994; Roller et al., 2003). In *A. mellifera*, the immunoreactive cells are very similar in number and distribution to those in a primitive hymenopteran *Arge nigrinodosa* (Roller et al., 2003). We showed that the ApimeCrz transcript is present in the LNSC and the mature corazonin is delivered to the possible releasing site in the ipsilateral *corpus cardiacum*. The Crz immunoreactive cells in

Fig. 6. Effects of graded doses of corazonins on the spinning rate in *Bombyx mori*. ■: Oil (control), ●: Am-corazonin; ◇: [His⁷]-corazonin, △: [Arg⁷]-corazonin. *N*=6–12 each. Vertical lines indicate SD. Asterisks indicate significant differences from the value of control (the Student's *t*-test, **p*<0.05, ***p*<0.01).

Fig. 7. Alignment of corazonin preprohormone amino acid sequences using Clustal W (1.8). Four species are compared: *Bombyx mori* (Bm, protein ID BAC66443.1), *Galleria mellonella* (Gm, protein ID AAF87082.1), *Apis mellifera* (Am) and *Drosophila melanogaster* (Dm, protein ID NP_524350.1). Black represents sequence identity, yellow sequence similarity and grey additional sequence identity between moths. Thr substitution in the Apime-corazonin is red highlighted.

the *pars lateralis* of the protocerebrum have also been found to contain Crz mRNA in *G. mellonella* and four *Drosophila* species (Hansen et al., 2001; Choi et al., 2005). These data support previous suggestions on conserved expression of corazonin in the ipsilateral neurosecretory cells of the protocerebrum in insects (Roller et al., 2003).

The lateral paired neurons in the ventral ganglia were not detected by the hybridization probe, suggesting that the used antibody cross-reacts with a corazonin-related substance. Corazonin expression in the ventral ganglia is variable among insects and it may be stage specific. Eight pairs of the lateral neurons in the ventral ganglia produce Crz mRNA in larvae but not in adults of *Drosophila* (Choi et al., 2005). On the other hand, no cells express Crz gene in the ventral ganglia of larvae, pupae and adults in *G. mellonella* (Hansen et al., 2001). An expression of ApimeCrz in the ventral ganglia of juvenile *A. mellifera* may be interesting to test in the future.

Corazonin exhibits multiple effects in different species of insects and crustaceans. It is known to affect phase-related characters including body color, morphometric ratios and abundance of antennal sensilla in locusts (Tanaka, 2005), to reduce spinning rate in the spinning larvae of *B. mori* (Tanaka et al., 2002), to induce release of pre-ecdysis and ecdysis triggering hormones from the Inka cells in the tobacco hornworm, *Manduca sexta* (Kim et al., 2004; Žitňan and Adams, 2005) and to promote tegumentary pigment migration in the crayfish, *Procambarus clarki* (Porrás et al., 2003). However, similar structure-activity relationships were exhibited in *L. migratoria* and *B. mori* despite the different physiological effects (Tanaka et al., 2003). The new form shows basically the same levels of activity as found with [Arg⁷]- and [His⁷]-corazonins in the two bioassay systems, suggesting that Apime-corazonin is a biologically active form. In Apime-corazonin Gln at the 4th residue is substituted with Thr, but the biological activity was not reduced. This supports our previous result that the residue at position 4 is not so important as compared with the residues at positions 1, 3 and 5 (Tanaka et al., 2003).

The presence of a dark-color inducing factor in *A. mellifera* has been indicated by implantation of a brain and *corpora cardiaca* taken from adult bees into albino nymphs of *L. migratoria* (Tanaka,

1993). The present results demonstrating the localization of corazonin in the brain of adult bees provide strong evidence that the factor suggested by Tanaka (1993) is identical to Apime-corazonin.

Recent progress of the genome research in several insect species allowed us to search for the presence of neuropeptide homologs easily. Corazonin appears to be widespread among insects except for the Coleoptera (Tanaka, 2000, 2005; Roller et al., 2003). BLAST analysis suggested that only [Arg⁷]-corazonin is encoded in *D. melanogaster*, *B. mori*, and the mosquito, *Anopheles gambiae*, and [Thr⁴, His⁷]-corazonin seems to be the only form encoded in *A. mellifera*. The absence of [Arg⁷]- and [His⁷]-corazonin in this species is also suggested by MALDI-TOF-MS analysis (Verleyen et al., 2006). Interestingly, [Thr⁴, His⁷]-corazonin does not seem to be a sole form of corazonin in Hymenoptera because only [His⁷]-corazonin was detected in the wasp *Vespula saxonica* (Verleyen et al., 2006). However, BLAST analysis against *Tribolium castaneum* genomic sequence data (www.bioinformatics.ksu.edu/BeetleBase) did not reveal significant hits for corazonin in the beetle genome, which may support our previous findings (Tanaka, 2000, 2005; Roller et al., 2003).

The physiological function of Apime-corazonin in the honey bee remains to be elucidated, although its distribution in the retrocerebral neuroendocrine system indicates a possible neurohormonal role. The dense branching patterns of the Crz immunoreactive fibers within the brain suggest that Apime-corazonin may act also as a neurotransmitter or neuromodulator. The Crz producing LNSC coexpress the circadian protein *Per* in *M. sexta* (Wise et al., 2002) and *Drosophila* (Choi et al., 2005), which indicates that they appear to be involved in the regulation of circadian rhythms. Furthermore, these cells may have a function related to photoperiodic time measurement, since their surgical ablation suppressed diapause in photoperiodically diapause-destinated pupae of *M. sexta* (Shiga et al., 2003). A putative function for Apime-corazonin may be deduced from future studies on the function of corazonin expressing neurosecretory cells.

ACKNOWLEDGEMENTS

The authors thank Dr. Dušan Žitňan (Institute of Zoology,

Bratislava) for providing an excellent whole-mount *in situ* hybridization protocol. Thanks are also due to Dr. H. Noda and his team (NIAS) for assistance in DNA sequencing and to Ms. N. Kemmochi, Ms. S. Ogawa and Mr. K. Maeno (NIAS) for rearing locusts. The grass used for rearing locusts was raised by Field Management Station of NIAS at Ohwashi, Tsukuba.

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