Characterization of two adenine nucleotide translocase paralogues in the stink bug, Plautia stali

Ryohei Sugahara, Masaomi Minaba, Akiya Jouraku, Toyomi Kotaki, Takenori Yamamoto, Yasuo Shinohara, Hideto Miyoshi and Takahiro Shiotsuki

Institute of Agrobiological Sciences, NARO, Tsukuba, Ibaraki 305-8634, Japan
Institute for Genome Research, University of Tokushima, Tokushima, Japan
Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

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Adenine nucleotide translocase (ANT) is a nuclear-coded mitochondrial protein that exchanges ATP for ADP across the mitochondrial inner membrane. Most organisms possess several ANT paralogues, and functional differences among these paralogues remain largely unknown. In the present study, we identified ANT paralogue genes in hemipteran species: the stink bug, bean bug, pea aphid, and Japanese mealybug. The ANT paralogues of the stink bug, Plautia stali, are encoded by two genes, PsANTI1 and PsANTI2. PsANTI1 was constantly expressed at all developmental stages and in all tissues analyzed. In contrast, the expression levels of PsANTI2 were undetectable in first instar nymphs and adult antennae. Gene silencing of each paralogue in P. stali revealed that PsANTI1 plays an important role in homeostasis, whereas the depletion of PsANTI2 failed to result in lethality. Thus, we concluded that PsANTI1 is a good target gene for developing novel pesticides. © Pesticide Science Society of Japan

Keywords: adenine nucleotide translocase, Hemiptera, stink bug, gene expression, RNAi.

Electronic supplementary materials: The online version of this article contains supplementary material (Supplemental Table S1), which is available at http://www.jstage.jst.go.jp/browse/jpestics/.

Introduction

Insecticide resistance is a significant and worldwide agricultural threat and is mainly caused by the continuous application of pesticides with the same target molecule. Accordingly, there have been strong demands to develop new pesticide compounds with different modes of action. We recently established a screening method to discover substances that inhibit the function of the insect adenine nucleotide translocase (ANT), a protein that is evolutionarily conserved in vertebrate species and also known as the ADP/ATP carrier (AAC) in yeasts. In this screening program, Saccharomyces cerevisiae lacking its AAC genes was complemented by the expression of insect or vertebrate ANT genes, and the inhibitory activities of substances for each ANT were easily monitored by investigating the survival of the transformants. Accordingly, this system enables us to explore a compound that is harmful to some insect species but not to vertebrates or other insects. The details of its design and procedure will be published soon. The application of this screening method is expected to be an efficient tool in the development of novel insecticides.

ANT is a membrane protein that catalyzes the exchange of ADP and ATP across the inner mitochondrial membrane and appears to play an essential role in cell energy metabolism. Therefore, ANT is an attractive candidate as a target gene in the development of new pesticide compounds. However, most insects harbor several ANT paralogues, and the functional differences among these paralogues remain largely obscure.

We previously reported that the silkworm, Bombyx mori, possesses two ANT paralogues, BmANT insect 1 and 2 (BmANTI1 and BmANTI2); the former appears to play a role in homeostasis, while the latter is specifically expressed in the testis. Paralogues evolutionarily similar to these two genes have been detected in the lepidopteran species, Plutella xylostella, Adoxophyes honmai, and Danaus plexippus. The red flour beetle, Tribolium castaneum, appears to possess the homeostatic ANT paralogue because its knockdown has been shown to significantly increase the risk of larval lethality. In the fruit fly Drosophila melanogaster, the knockdown or overexpression of DmANT1 led to developmental lethality, suggesting that this paralogue is the homeostatic ANT paralogue. On the other hand, the testis-specific ANT paralogue has only been detected in mammals, reptiles, and flies. This paralogue appears to be necessary for the continuous supply of large amounts of ATP to meiotic cells. In the desert locust, Schistocerca gregaria, the expression patterns of
two ANT paralogues were analyzed, with neither being identified as a testis-specific gene. The detailed roles of hemipteran ANT paralogues remain unknown.

Since hemipteran species are significant pests for fruits and crops, the aim of the present study was to determine whether hemipteran ANT has potential as a target gene for pesticides. We cloned ANT-encoding genes for the stink bug, *Plautia stali*, the bean bug, *Riptortus pedestris*, the pea aphid, *Acyrthosiphon pisum*, and the Japanese mealybug, *Planococcus kraunhiae*. We identified two ANT genes, *PsANT1* and *PsANT2* in *P. stali*. In order to characterize the *PsANT* paralogues, the expression profiles of the genes were compared in the whole body at different developmental stages and in different tissues of the adult. *PsANTI2* or *PsANT1* was also silenced by RNAi at the third nymphal instar in order to elucidate the biological functions of the paralogues.

**Materials and Methods**

1. **Insects**

A colony of *P. stali* was established from adult individuals collected in Ibaraki, Japan, in 2001 and maintained in the National Institute of Agrobiological Sciences (NIAS), Tsukuba. The details of colony maintenance and the rearing method for *P. stali* have been described previously.11) *R. pedestris* was collected in a soybean field in Tsukuba, Japan, in 2012. *A. pisum* and *P. kraunhiae* were supplied by the National Institute of Advanced Industrial Science and Technology (AIST)12) and the Fukuoka Agriculture and Forestry Research Center,13) respectively.

2. **Identification and cloning of ANT genes**

Total RNAs were extracted using ISOGEN (Nippon Gene) and 2. Identification and cloning of ANT genes Total RNAs were extracted using ISOGEN (Nippon Gene) and the SV Total RNA Isolation System (Promega) from the whole bodies of *P. stali*, *R. pedestris*, *A. pisum*, and *P. kraunhiae*. Each RNA was reverse transcribed into cDNA using Superscript III and oligo(dT) primers (Invitrogen). ANT gene open reading frames (ORFs) were identified in the NCBI database and the sequencing data from our RNA-seq analysis in the present study. The full-length ORFs of *RpANTI1*, *RpANTI2*, and *PkANTI3* were determined by the 5′ and 3′ rapid amplification of cDNA ends (RACE) using a GeneRacer Kit (Invitrogen). The 5′ ends of the other ANT genes were subcloned and sequenced in order to confirm whether they were correctly predicted. The full-length ORFs of ANT genes were amplified using the primer pairs listed in Supplemental Table S1 and inserted into the pENTR11, pCR2.1 (Invitrogen), or T-vector, following which their nucleotide sequences were determined.

3. **RNA-seq analysis**

The total RNAs of *P. stali*, *R. pedestris*, and *P. kraunhiae* were extracted using ISOGEN from the whole bodies of nymphs and purified using the SV Total RNA Isolation System. The preparation of cDNA libraries from total RNAs and sequencing by the Illumina HiSeq 2000 sequencer were performed by Hokkaido System Science Co., Ltd. for *P. stali* and *R. pedestris*, and Macrogen Japan Corp. for *P. kraunhiae*. The RNA-seq reads of *P. stali*, *R. pedestris*, and *P. kraunhiae* were de novo assembled by Trinity, and 38790, 59306, and 50672 contigs were generated, respectively. The RNA-seq data obtained for *P. stali*, *R. pedestris*, and *P. kraunhiae* have been deposited in the DDBJ (the DNA databank of Japan) sequence read archive, DRA, under accession number DRA004114.

4. **Nucleotide sequence submission**

The nucleotide and amino acid sequences identified in the present study have been submitted to the DDBJ. The accession numbers are shown in Table 1.

**Table 1.** A list of hemipteran ANTs examined

<table>
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<th>Order</th>
<th>Family</th>
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5. Phylogenetic analysis
The amino acid sequences of hemipteran and human ANT genes were aligned using the CLUSTAL-W program, and the phylogenetic tree was constructed with GENETYX software version 11.0 (Genetyx) using the neighbor-joining method (bootstrap trials, 1000 times; TOSSGAPS, on).

6. cDNA preparation for the analysis of gene expression profiles of PsANTs
A series of P. stali RNAs were extracted from the whole bodies of first, second, third, fourth (penultimate), and fifth (last) instar nymphs and of adults. The numbers of individuals used were 11, 10, 10, 5, and 4, respectively, for each stage. Regarding tissue samples, adults on day 4 were dissected, and the antennae, central nervous system, testes, ovaries, integument, fat body, muscle, gut, and Malpighian tubules were separated in saline solution. The central nervous system in this experiment was composed of the brain, subesophageal ganglion, corpora cardiaca, corpora allata, thoracic ganglia, and abdominal ganglia. The numbers of individuals used for tissue samples were at least four for each. Regarding the daily mRNA expression profiles of PsANTs at the third instar, three insects were collected for each sample. Total RNA was extracted using ISOGEN and purified using the SV Total RNA Isolation System. RNA concentrations were estimated by a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), and the firststrand of cDNAs were subsequently synthesized from 0.5 µg of isolated total RNA using SuperScript III reverse transcriptase (Invitrogen, Life Technologies) and a mix of oligo(dT) primers and random hexamers.

7. Double-stranded RNAs
Two dsRNAs targeted to different regions in PsANTI1 and PsANTI2 were generated using pLit plasmids by in vitro transcription using the T7 RiboMAX large-scale RNA production kit (Promega) according to the manufacturer's protocol. Venus is a variant of the green fluorescent protein, and dsVENUS was used as a negative control.

8. Semi-quantitative reverse-transcription PCR
The mRNA levels of genes were evaluated with semi-quantitative reverse-transcription PCR (semi-qRT-PCR) using a KOD-FX DNA polymerase (TOYOBO Co.). RT-PCR primer pairs for PsANTI1 and PsANTI2 are listed in Supplemental Table S1. The PCR cycle numbers were optimized to avoid saturation of the reaction product.

9. RNAi experiment in P. stali
In order to reduce the mRNA levels of PsANTI1 and PsANTI2 for P. stali, newly ec lysed third instar nymphs (within 24 hr of emergence) were anesthetized with CO2 gas and placed on ice. The immobilized nymphs were aligned on double-sided adhesive tape and injected with approximately 0.3 µL of dsRNA solution (2.5 µg/µL) into the ventral part of the second abdominal segment using a capillary tube pulled by a Narishige needle puller. Bugs that died within four days post-injection were eliminated from the analysis. Total RNA was extracted from the whole bodies of dsRNA-injected nymphs four days later. Three bugs were sacrificed for each treatment. cDNAs were synthesized using a ReverTra Ace reverse transcriptase (TOYOBO Co.) and oligo(dT) primers.

Results and Discussion
1. Isolation of some hemipteran ANT genes and analysis of amino acid sequences
In order to obtain transcriptome data for P. stali, R. pedestris, and P. kraunhiae, an RNA-seq analysis was conducted on total RNA extracted from these species (see "Materials and Methods"). We then carried out the de novo assembly of reads and searched data for the ANT-coding genes of these insects. The sequencing data of A. pisum were open to the public, and BLAST searches were also performed against these data. The full-length ORF clones of ANT genes for P. stali, R. pedestris, A. pisum, and P. kraunhiae were amplified and inserted into cloning vectors. The nucleotide sequences of ANT were determined by sequencing these plasmids (Table 1), and their amino acid sequences were predicted.

The phylogeny of the ANT paralogues was investigated by constructing a phylogenetic tree of full-length amino acid sequences for some of the hemipteran species. Most hemipteran ANTI2 and ANTI3 formed a branch with human ANTs, a group that is distinct from hemipteran ANTI1 (Fig. 1). These results implied that the hemipteran ANTs examined evolved into at least two different lineages.

Fig. 1. Phylogenetic tree of ANT proteins.
A phylogenetic analysis of hemipteran ANT proteins. The neighbor-joining tree was generated by Genetyx software with multiple sequence alignment. The reliability of the tree was evaluated by bootstrapping 1000 replicates. The numbers at the nodes denote bootstrap values (%), and values <50% are not indicated. The sequences of human ANT genes, HsANTI1 (NP_001142), HsANTI2 (NP_001143), HsANTI3 (NP_001627), and HsANTI4 (NP_112581) were retrieved from the NCBI/GenBank databases. Abbreviations: Ap, Acrystosiphon pisum; Nl, Nlapparvata lugens; Pk, Planococcus kraunhiae; Ps, Plautoia stali; Rp, Riptortus pedestris; Sr, Stenotus rubrivittatus; Hs, Homo sapiens; Bt, Bemisia tabaci.
2. PsANT1 is constantly expressed during the course of development in P. stali

In order to analyze the gene functions of hemipteran ANT paralogues, gene expression profiles were made for PsANT1 and PsANT2 during the development of P. stali. The mRNA levels of each gene were measured for the whole body using semi-quantitative PCR (Fig. 2A). The amplification of P. stali elongation factor 1-alpha (PsEF1α) cDNA was used as an internal control. The expression levels of PsANT1 and PsANT2 were similar between females and males at the last nymphal instar and adult stages. PsANT1 was constantly expressed at all developmental stages. In contrast, the mRNA level of PsANT2 was extremely low or undetectable in first nymphal instars. These results implied that PsANT1, but not PsANT2, was required for developmental homeostasis.

In mammals, the gene expression of ANT paralogues is known to be strictly controlled in a tissue-dependent manner.8,17,18 We subsequently compared the distribution patterns of PsANT1 and PsANT2 for various tissues, namely, the antennae, central nervous system, testes, ovaries, integument, fat body, muscles, gut, and Malpighian tubules on day 4 of P. stali adults. A semi-quantitative PCR analysis revealed that PsANT1 mRNA was present in all tissues tested, whereas the expression of PsANT2 was almost absent from the antennae (Fig. 2B). Thus, PsANT paralogues were expressed differently in the tissues of P. stali tested in the present study.

3. PsANT1 is required for larval development in P. stali

In order to determine whether PsANT1 and PsANT2 are indispensable for larval development, we performed a knockdown analysis of these genes for P. stali nymphs and their effects on nymphal lethality were evaluated. Two dsRNAs were generated

Fig. 2. Comparison of expression profiles of PsANT1 and PsANT2 genes.

The temporal and tissue-specific expression patterns of PsANT genes. (A) PsANT1 and PsANT2 mRNA levels were measured in the whole body during the course of development. Total RNAs were extracted from first instar nymphs to adults, and subjected to a semi-quantitative reverse transcription (semi-qRT)-PCR analysis. The bugs were divided into females and males on the last nymphal instar and adults when collected. The amplification of elongation factor 1-alpha (EF1α) cDNA was used as an internal control. (B) The mRNA levels of PsANT1 and PsANT2 were measured in various tissues. The antennae (AN), central nervous system (CNS), testes (TE), ovaries (OV), integument (IN), fat body (FB), muscle (MS), gut (GU), and Malpighian tubules (MT) were retrieved from adults on day 4, and their total RNAs were subjected to a semi-qRT-PCR analysis.

Fig. 3. Effects of gene silencing of PsANT1 and PsANT2 on insect survival.

(A) Lines on a schematic diagram of PsANT1 and PsANT2 show the relative positions of dsRNA-targeted regions. The lengths of dsRNAs are shown in parentheses. (B) The indicated dsRNAs were injected into newly ecdysed third instar nymphs. Four days after the dsRNA injection, three were sampled for each treatment and the whole bodies were subjected to a semi-qRT-PCR analysis. A transcript for P. stali EF1α served as an internal control. (C) Photographs show two typical carcasses that were treated with PsANT1 dsRNAs. White and black arrowheads indicate the new and old cuticle, respectively. (D) The mRNA levels of PsANT1 and PsANT2 were examined on each day (D0–4) of the third instar and during ecdysis. Day 4 of the third nymphal instar was a developmental stage just before ecdysis. Whole bodies were used in the experiment.
against parts of the untranslated region (UTR) and ORF of each PsANT (Fig. 3A) and subsequently injected into third instar nymphs. VENUS dsRNA was concurrently injected into similarly prepared nymphs as a negative control. A semi-qRT-PCR analysis indicated that all dsRNAs efficiently knocked down their target genes (Fig. 3B).

We then examined the effects of the dsPsANT injection on nymphal development in P. stali. Third instar nymphs were injected with dsPsANT or dsVENUS, and their risks of nymphal lethality were recorded until adult eclosion. Importantly, most dsPsANT1-a- or dsPsANT1-b-injected individuals died during ecdysis to the last instar and adult stages, whereas this phenomenon was not observed with injections of dsPsANT2-a, dsPsANT2-b, or dsVENUS. For the carcasses caused by the treatment with dsPsANT1, the newly formed cuticle had started appearing at the ventral thoracic midline (Fig. 3C, upper photograph), or the old cuticle had separated from the new cuticle at the head capsule but still covered the legs and abdomen (Fig. 3C, lower photograph). Injections of dsRNAs for PsANT11 accordingly caused markedly lower eclosion rates than those caused by PsANT2 or dsVENUS injections in P. stali (Table 2). Knockdown of the PsANT12 expression did not result in increased lethality, suggesting that this paralogue is dispensable for nymphal development. However, there remains a possibility that the efficiencies of PsANT12-silencing were not sufficient to display the phenotype.

The failure to molt induced by dsPsANT11-injections indicates that PsANT11 specifically plays an important role in ecdysis. In order to examine this possibility, PsANT mRNA expression levels were assessed in third instar nymphs from day 0 (within 24 hr of ecdysis) to the next ecdysis. A semi-qRT-PCR analysis showed that PsANT11 and PsANT12 were constantly expressed during the third instar (Fig. 3D). These results suggest that PsANT11 is required for a continuous supply of ATP to bugs during nymphal development rather than a temporal supply. A chronic shortage of ATP caused by the silencing of PsANT11 may have severe effects on ecdysis, because molting may be accompanied by the consumption of a large amount of ATP.

Taken together, these results suggest that PsANT11 is the homeostatic ANT paralogue for P. stali. Hence, we concluded that PsANT11 has potential as an appropriate candidate gene for a new target for pesticides against this species. The application of the yeast screening system with homeostatic ANT paralogues may be an efficient tool in the development of new insecticides to control hemipteran pest insects.

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