Characterization and Localization of the Vacuolar-Type ATPase in the Midgut Cells of Silkworm (*Bombyx mori*)

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Z. Naturforsch. **64c**, 899–905 (2009); received May 20/June 20, 2009

The vacuolar ATPase (V-ATPase) is a multifunctional enzyme that consists of several subunits. Subunit B is a part of the catalytic domain of the enzyme. The result of the RT-PCR suggested that the V-ATPase B subunit is a ubiquitous gene. 24 h after the larvae were infected with the *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV), the expression level of the V-ATPase B subunit in the midgut of the resistant strain NB was about 3 times higher than in the susceptible strain 306, and then the expression level of the V-ATPase B subunit decreased rapidly to a very low level. This indicated that the virus may cause a lot of changes of physiological conditions in the midgut. Localization of the V-ATPase B subunit was attempted in midgut cells of *Bombyx mori* by immunohistochemistry. The immunohistochemical localization with the antibody against the B subunit revealed a positive staining in goblet cell apical membranes of *Bombyx mori* midgut cells as well as in the midgut of *Manduca sexta*. This sequence has been registered in GenBank under the accession number EU727173.

Key words: Bombyx mori, Midgut, V-ATPase B Subunit

Introduction

The vacuolar-type H^+-ATP synthetase (V-ATPase) is found ubiquitously in endomembranes of eukaryotic cells. Furthermore, it is also found in the plasma membranes of a variety of eukaryotic cells (Wieczorek *et al*., 1999). V-ATPase is a multisubunit enzyme that consists of two complexes, a peripheral V_1 complex and an integral V_0 complex. The V_1 complex is responsible for hydrolysis of ATP, and the V_0 complex is responsible for translocation of protons across the endomembrane or plasma membranes (Stevens and Forgac, 1997; Forgac, 2000; Nishi and Forgac, 2002). Studies on the midgut of the tobacco hornworm *Manduca sexta* have provided the basis for our understanding of the role of V-ATPases in plasma membrane energized by the proton-motive force (Wieczorek, 1992). In this system, V-ATPase plays a vital role in the generation of a transmembrane voltage and in energizing secondary active transport processes such as $K^+/2H^+$ antiport and amino acid/K^+ symport across the apical plasma membrane (Lepier *et al*., 1994; Azuma *et al*., 1995).

In the study of mosquito (*Aedes aegypti*), the pH value of the midgut was 8 to 11 (Zhuang *et al*., 1999); it can exceed 12 in the midgut of caterpillars (Dow, 1984), and the alkalization mechanism may be mediated by an H^+ V-ATPase. In the model lepidoptera insect *Manduca sexta*, the V-ATPase generates a transmembrane voltage which drives an electrophoretic exchange (antiport) of luminal H^+ for cellular K^+, for the alkalinity of the lumen and acidity of the cell (Azuma *et al*., 1995; Wieczorek *et al*., 1999). The high pH value of the lumen is very important; many proteins in the midgut are alkaline proteins, so the alkaline environment could make these proteins exertive functions. The pH value of the midgut also plays a very important role in the resistance against baculoviruses (Keating *et al*., 1989, 1990; Neuvonen *et al*., 1990). Intracellular V-ATPases are also important in the entry of many viruses and toxins, including influenza virus and anthrax toxin (Nishi and Forgac, 2002; Abrami *et al*., 2004).

0939–5075/2009/1100–0899 $ 06.00

Material and Methods

Insects

The silkworm *Bombyx mori* was inbred in our laboratory. The highly susceptible silkworm strain 306 (LD_{50}, $4.8 \cdot 10^5$ ODV/ml) and the resistant silkworm strain NB (LD_{50}, $>4.8 \cdot 10^8$ ODV/ml) were used for this study. All larvae were reared with fresh mulberry leaves at (25 ± 2) °C under a 12 h light/12 h dark photoperiod.

Date extraction of cDNA sequence of the B. mori V-ATPase B subunit gene

The NCBI (http://www.ncbi.nlm.nih.gov/) EST database and silkworm cDNA database BGI (http://silkworm.genomics.org.cn/) were used in this study. The V-ATPase B subunit mRNA sequence (GenBank no. X64354) of *Manduca sexta* was used to search for the cDNA sequence of the *B. mori* V-ATPase B subunit gene. We used the ExPASy translate tool (http://au.expasy.org/tools/dna.html) to deduce the cDNA's amino acid sequence. Using BLAST search for similarity in GenBank, several V-ATPase B subunit sequences from different insects were identified and aligned using the ClustalW method. In order to establish the genomic organization, the cDNA of the V-ATPase B subunit was searched to the *B. mori* genome in BGI (http://silkworm.genomics.org.cn/), and the SilkDB (http://silkworm.genomics.org.cn/silksoft/silkmap.html) was used to predict the chromosomal localization of the V-ATPase B subunit.

Protein expression, purification, and polyclonal antiserum production

One encoding fragment (1503 bp) of the V-ATPase B subunit was amplified using the polymerase chain reaction (PCR). A sense primer, 5'-ggatatcaaggtgatctctcacgcc-3' (containing the *Eco*RV site), and an antisense primer, 5'-ggtcgaccacgagcactccaaggtt-3' (containing the *Sal*I site), were used for the PCR. The amplified products were cloned to the pMD18-T vector, then verified by digesting with *Eco*RV and *Sal*I restriction enzymes.

Recombinant protein was used as antigen to generate antibodies against the V-ATPase B subunit. A DNA fragment encoding the full-length V-ATPase B subunit protein was cloned into the pET30a (Novagen, Darmstadt, Germany) vector. The 6× His-tagged V-ATPase B subunit fusion protein was then expressed in *E. coli* strain BL21 (DE3) and purified with Ni–NTA Agarose (Qiagen, Duesseldorf, Germany). Antibodies against the V-ATPase B subunit fusion protein were raised in New Zealand white rabbits. Specific antibodies were affinity-purified with Antibody Purification Kit and Spin Columns with ROSEP®-A Media (Millipore, Billerica, USA) from the obtained antisera and stored in 0.1% BSA containing 0.05% NaN_3 at 4 °C.

Cell extracts, electrophoresis, and Western blot analysis

E. coli cell extracts were prepared by sonicating in lysis buffer [0.1 M Tris-HCl, pH 6.8, 2.5% sodium dodecyl sulfate (SDS), and 10 mM EDTA], heating in a boiling water bath for 5 min, centrifuging at 12,000 rpm for 5 min at 4 °C, and collecting supernatants. After 2× SDS-PAGE sample buffer (200 mM Tris-HCl, pH 8.3, 4% SDS, 400 mM DTT, 20% glycerol, 2 mM EDTA, 0.05% bromophenol blue) was added, each protein was resolved on 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane (Millipore). The membranes were briefly washed with PBST (phosphate-buffered saline containing 0.05% Tween 20) and incubated at 25 °C for 3 h in PBST containing 5% fat-free dry milk solids. Then they were incubated at room temperature for 1.5 h with affinity-purified anti-V-ATPase B subunit antibody and anti-6× His antibody, washed twice in PBST for 15 min each, washed five times in PBST for 5 min each, and incubated at room temperature for 1.5 h with secondary antibody (1:500 horseradish peroxidase-conjugated goat anti-rabbit IgG). After washing by following the same steps as for the primary antibody, the signals were detected by incubation with DAB solution (0.6 mg/ml diaminobenzidine and 0.01% H_2O_2 in PBS).

Quantitative real-time PCR and data analysis

Larvae of the strains 306 and NB were raised to the fifth instar. The larvae of newly metamorphosed 5th instar were fed with mulberry leaves treated with *Bm*NPV ($2 \cdot 10^8$ ODV/ml). The midgut was taken from silkworm larvae 24 h, 48 h and 72 h post *Bm*NPV infection (p.i.); then total RNA was extracted from the midgut with Rneasy Mini Kit (Qiagen). We used 2 μg total RNA as a

template in the first-strand cDNA synthesis. PCR was performed on the resulting cDNAs using the following primers for the V-ATPase B subunit: V-ATPase-B-F (5'-tcacccatcccattccc-3') and V-ATPase-B-R (5'-ccttgccgatagcgtagc-3') (GenBase, Shanghai). The constantly expressed gene, actin A3, was used as the internal control. Two primers, AF (reverse: 5'-ggatgtccacgtcgcacttca-3') and AR (forward: 5'-gcgcggctactcgttcactacc-3'), were designed on the basis of the sequence of the *B. mori* actin gene (accession no. BMU49854). Quantitative real-time PCR (QRT-PCR) information was obtained using Mx 3000P (Stratagene, La Jolla, USA) for thermal cycling, real-time fluorescence detection and subsequent analysis. The two-step amplification protocol was a follows: 2 min at 94 °C, followed by target amplification via 40 cycles at 94 °C for 15 s, at 58 °C for 20 s and at 72 °C for 20 s. After PCR, the absence of unwanted byproducts was confirmed by automated melting curve analysis and agarose gel electrophoresis of the products. The transcript levels of the target fragment were normalized with actin A3 transcript levels in the same samples. The comparative *C*t method is also known as the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$ (Livak and Schmittgen, 2001). Here, ΔCt_{sample} is the *C*t value for any sample normalized to β-actin and $\Delta Ct_{reference}$ is the *C*t value for the calibrator also normalized to β-actin. The data was analyzed by SPSS 13.0.

RT-PCR

We investigated the tissue distribution of the V-ATPase B subunit in strain NB. Total RNA was isolated from the midgut, fat bodies, silk gland, testis, ovary, malpighian tube, egg and hemocyte of 5th instar larvae using Rneasy Mini Kit (Qiagen) according to the user manual. 2 μg total RNA was used as a template in the first-strand cDNA synthesis. PCR was performed on the resulting cDNAs using two pairs of primer in the QRT-PCR. The PCR was carried out with Taq polymerase for 35 amplification cycles (94 °C/45 s, 58 °C/45 s, and 72 °C/30 s). The PCR product was examined by electrophoresis in 1% agarose gel with ethidium bromide staining.

Immunocytochemistry

Histological tissues used in this study were identical to those described previously (Zhuang *et al.*, 1999). The 4th and 5th instar larvae were fixed 4 h on ice in fixation solution (PBS containing 4% paraformaldehyde) and then cryoprotected in 30% sucrose. They were then embedded in OCT compound (Tissue-Tek) and stored at –70 °C until they solidified. Frozen sections (10–14 μm) were cut. The slices were subsequently incubated at 37 °C for 30 min in 3% H_2O_2 solution to block endogenous peroxidase, and incubated at 37 °C for 40 min with normal goat serum diluted 10 times in PBS containing 0.5 mg/ml BSA. The slices were further incubated with primary antibody, *i.e.*, the anti-V-ATPase B subunit rabbit antibody (diluted 1000 times in PBS), for 3 h at 37 °C and then washed five times in PBS. Fixed and mounted slices of larvae were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG for 1.5 h at 37 °C, washed five times in PBS, and developed for 5 min at room temperature using a substrate solution (0.005% H_2O_2, 0.02% diaminobenzidine in PBS), and then counter-stained in Mayer's hematoxylin. Meanwhile, the negative control was prepared using PBS to replace primary antibody. Images were photographed.

Results

Cloning of the V-ATPase B subunit from B. mori

A 1939-bp cDNA was identified by data-mining techniques which included an 1485-bp open reading frame, coding for the subunit B of V-ATPase from *B. mori*; this sequence has been submitted to GenBank (accession number EU727173). This sequence was different from the previously sub-

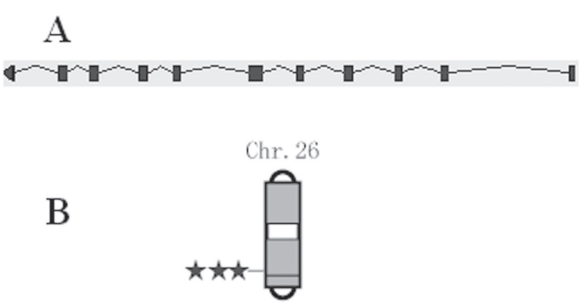

Fig. 1. Genomic organization and chromosomal localization of the V-ATPase B subunit. (A) Genomic organization of V-ATPase B subunit extrons are black framed and introns are the line. (B) Chromosomal localization of the V-ATPase B subunit; three shading stars mean best hit.

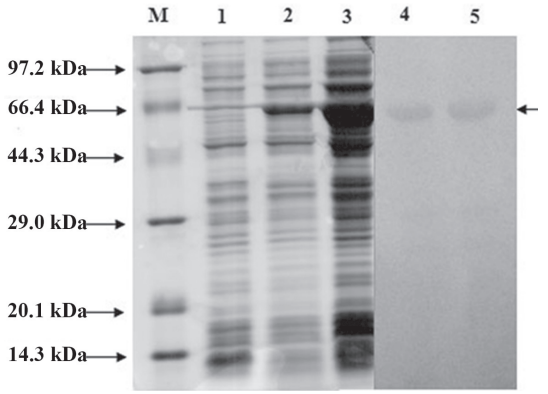

Fig. 2. SDS-PAGE of fusion protein and Western blot analysis of *E. coli*-expressed protein using the rabbit antiserum. M, protein marker; lane 1, total cellular extracts from *E. coli* BL21 not transformed with plasmid after IPTG-induction; lane 2, total cellular extracts from *E. coli* BL21 transformed with pET30a plasmid after IPTG-induction; lane 3, total cellular extracts from *E. coli* BL21 transformed with the V-ATPase B subunit/pET30a plasmid after IPTG-induction; lane 4, Western blot of *E. coli*-expressed V-ATPase B subunit fusion protein reacted with anti-anti-6× His sera; lane 5, Western blot of *E. coli*-expressed V-ATPase B subunit fusion protein reacted with anti-V-ATPase B subunit sera.

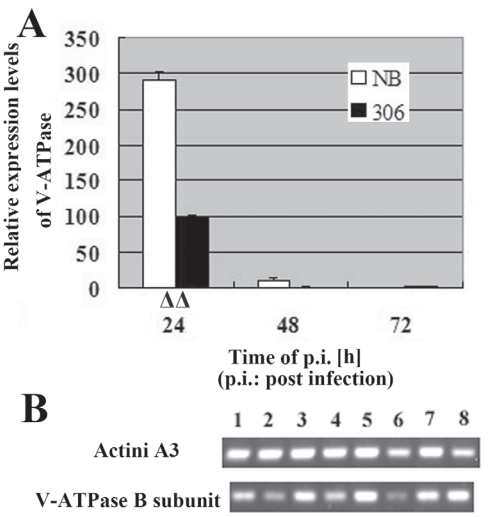

Fig. 3. V-ATPase B subunit mRNA levels in the midgut of the resistant strain NB and the susceptible strain 306 at different times after infection with *Bm*NPV and mRNA expression of the *B. mori* V-ATPase B subunit gene in different tissues. (A) Comparative copies of the V-ATPase B subunit in the resistant strain NB and the susceptible strain 306 24 h, 48 h and 72 h p.i. Quantitative real-time PCR data were evaluated using the $2^{-\Delta\Delta Ct}$ method to calculate the comparative copies. ΔΔ denotes that the relative expression of NB is markedly higher than that of 306 ($P < 0.05$). The data was analyzed by SPSS 13.0. (B) Agarose gel electrophoresis of the RT-PCR products in different tissues. Lane 1, midgut; lane 2, hemocyte; lane 3, fat bodies; lane 4, silk gland; lane 5, ovary; lane 6, testis; lane 7, egg; lane 8, malpighian tube.

mitted V-ATPase B subunit sequence of *B. mori* (accession number EF107513); our sequence has 12 bp more in the encoding region (data not shown). Searching for the deduced amino acid sequence in the NCBI database, two conserved motifs SAAGLPHN and ILTD, were obtained (data not shown). From the BGI database we found that eleven extrons form the ORF (Fig. 1A) and the V-ATPase subunit B is localized on the 26th chromosome (Fig. 1B) of *Bombyx mori*.

Protein expression, purification, and polyclonal antiserum production

Expression of the V-ATPase B subunit fusion protein was analyzed in *E. coli* strain BL21 (DE3) (Fig. 2), and a high level expression fusion protein of about 60 kDa was obtained, which was consistent with the expected molecular mass of the fusion protein of the V-ATPase B subunit/pET30a. The specificity of the antiserum for the V-ATPase B subunit was tested by Western blot analysis. The result showed that the antiserum can specifically identify the *E. coli*-expressed fusion protein (Fig. 2).

Quantitative real-time PCR analysis

The specificity of quantitative real-time PCR results was assessed by dissociation curve analysis. The melting curves showed two specific products (data not shown). The result means that the V-ATPase B subunit real-time PCR assay was gene-specific and that the results were not confounded by non-specific amplification or primer dimer. The relative expressions of the V-ATPase B subunit were analyzed by the $2^{-\Delta\Delta Ct}$ method. Results from these analyses are shown in Fig. 3A. The expression of the V-ATPase B subunit was very high 24 h p.i. in both the resistant strain NB and the susceptible strain 306, then decreased rapidly to a very low level. At 24 h p.i. the expression level of the V-ATPase B subunit in the resistant strain was much higher than in the susceptible strain ($P < 0.05$).

RT-PCR analysis of the V-ATPase B subunit expression

RT-PCR using cDNA was prepared from RNAs which were extracted from *B. mori* in different tissues of 5th instar larvae, namely midgut, fat bodies, silk gland, testis, ovary, malpighian tube, egg, and hemocyte. Fig. 3B indicates that the V-ATPase is ubiquitous; all tissues which were used for mRNA extraction contained V-ATPase. There was a low level of V-ATPase expression in hemocyte, silk gland and testis, and a higher in the midgut, fat bodies, ovary and egg. The highest expression was found in the malpighian tube.

Immunocytochemistry of the V-ATPase in the midgut cell

V-ATPase was localized immunocytochemically in the midgut cells at the light microscope level. Fig. 4 shows highly specific staining of the *B. mori* midgut goblet cell apical membrane (Figs. 4A, C). Goblet cells have a cavity, which was formed by invagination of the apical membrane; so the antibody also specifically labeled the goblet cell cavities (Fig. 4B). We observed a non-specific immunoreaction in the control experiment (Fig. 4D).

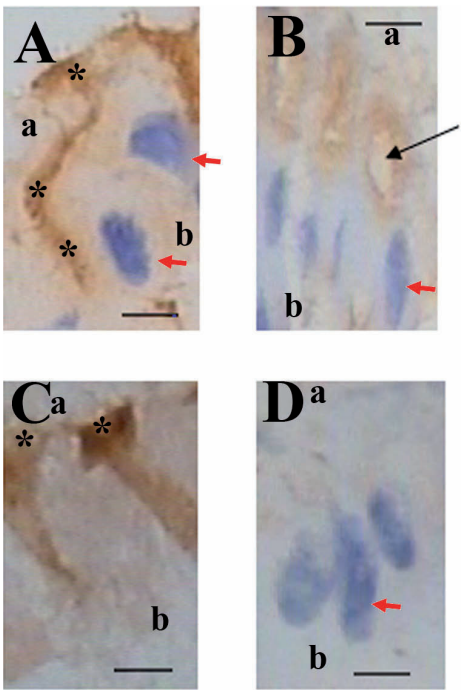

Fig. 4. Immununohistochemical staining. (A) *B. mori* middle midgut, labeled with the purified antibody, showing staining in the goblet cell apical membranes. (B) The positive signal in goblet cell cavity. (C) The positive signal in goblet cell apical membranes. (D) Negative controls of the same tissues incubated with 5% BSA. Scale bars, 25 μm; a, apical; b, basal; asterisks indicate goblet cell apical membranes; red arrows point to goblet cell nuclei; black arrows point to goblet cell cavity.

Discussion

The V-ATPase is composed of two functional domains, a peripheral V_1 domain and an integral V_0 domain. The V_1 domain is responsible for ATP hydrolysis, the catalytic site comprising three copies each of the A subunit and B subunit. Traditionally, the A subunit is described as catalytic, whereas the B subunit is considered as regulatory, although in reality the active sites for nucleotide binding and proton flux may lie in the interfaces between the neighbouring A and B subunits (Liu *et al.*, 1996). In the *Drosophila melanogaster* V-ATPase B subunit, the PPVNVLPSLS motif is essential for the ATPase function (Davies *et al.*, 1996). This motif was also found in the B subunit of *B. mori* and other insects. According to the NCBI database, the SAAGLPHN motif is a degenerated motif, based on similarity to F1 ATPases, and the ILTD motif coordinates the Mg^{2+} transport. The V-ATPase plays major roles in endomembrane and plasma membrane proton transport in eukaryotes; an inactivated V-ATPase B subunit caused a larval lethal phenotype in *Drosophila melanogaster* (Davies *et al.*, 1996).

We could demonstrate that our antibodies recognized the desired V-ATPase subunits using our polyclonal antiserum. The antibody revealed a specific band at approx. 60 kDa in Western blot analysis of proteins from the *E. coli*-expressed V-ATPase B subunit fusion protein. The results of the immunocytochemical staining clearly showed that the expression of the V-ATPase subunit B is strictly limited to the midgut epithelium cells (Figs. 4A, B and C). Immunocytochemistry showed the immunolocalization of the subunit B in the goblet cells apical membranes of the midgut of *B. mori* larvae and no positive signal in the basal membranes. These labeling patterns in tissue sections of *B. mori* are similar to those shown in *Manduca sexta* midgut and *Heliothis virescens* midgut (Klein *et al.*, 1991; Pietrantonio and Gill, 1995).

In most insects, the pH value of the midgut is 8 to 12. In order to maintain such a high pH value, a great amount of V-ATPase was distributed among the midgut. The study of *Aedes aegypti* (Zhuang *et al*., 1999) found that a basal localization would be expected intuitively for lumen alkalization, because a basal ATPase would pump protons out of cells and away from the lumen, but lumen alkalinization by the apically located V-ATPase in *Manduca sexta* midgut goblet cells is different; protons are pumped towards the lumen, hyperpolarizing the apical membrane to –240 mV, which drives K^+ to the lumen *via* an apical, electrophoretic $K^+/2H^+$ antiporter *(*Wieczorek *et al*., 1991; Azuma *et al*., 1995) and/or an apical amino-acid-gated OH channel. Our results suggest that the midgut of *B. mori* larvae resembles the midgut of *Manduca sexta*, whose lumen is alkalified by the apically located V-ATPase.

Midgut-based resistance to baculovirus was reported previously (Kirkpatrick *et al*., 1998; Washburn *et al*., 1998; Hoover *et al*., 2000). The midgut plays very important roles in the resistance against baculovirus, but the resistance mechanism is not very clear. The peritrophic membrane along the midgut could prevent the virus from entering into the midgut epithelial cells; so the peritrophic membrane was considered as one of the resistance barriers to *Ag*MNPV in *Anticarsia gemmatalis* (Levy *et al*., 2007). After infected by *Ac*MNPV, a great sloughing rate of infected midgut epithelial cells results in strong developmental resistance to fatal infection (Washburn *et al*., 1998). In gypsy moths, the larval susceptibility to NPV was significantly reduced when larvae consumed viruses on highly acidic foliage and acidic artificial diets. Diet pH value may affect the virus activity by changing the midgut pH value and altering the rate and location of virion release in the midgut lumen (Keating *et al*., 1989, 1990). Similar results were obtained in *Neodiprion sertifer* (Neuvonen *et al*., 1990). These evidences indicated that the pH value of midgut may play a role in the resistance against NPV. In *Manduca sexta* midgut, the V-ATPase generates a voltage of approx. –240 mV across the apical plasma membrane of goblet cells, which drives H^+ from lumen back into the cells in exchange for K^+, resulting in K^+ secretion into the lumen. This activity raises the lumen to a very high pH value, but the goblet cells to a very low pH value (Wieczorek *et al*., 2000). From Fig. 3A, the expression level of the V-ATPase in the midgut was very high 24 h p.i., but at 48 h and 72 h p.i. the expression level decreased in both strains, suggesting that the physiology of midgut may change and cause the expression level of the V-ATPase decrease. 24 h p.i. the expression level of the V-ATPase in the resistant strain was about 3 times higher than in the susceptible strain. We can hypothesize that the high expression level of the V-ATPase may raise the lumen pH value to a higher value and that of the epithelium cells to a lower one. When the larvae ingest the polyhedra, this higher pH value of the lumen not only could led to consumption the polyhedra and release of virions, but also could kill the virions. If the virions could penetrate the peritrophic membrane, the lower pH value of the epithelium cells is not suitable for them to survive; this needs more investigation.

Acknowledgements

This project was supported by the National Basic Research Program of China (No. 2005CB121000) and High Tech Research and Development (863) Project, China (No. 2008AA10Z145).

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