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Gene expression and molecular phylogenetic analyses of beta-glucosidase in the termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae)

Keisuke Shimada [1], Kiyoto Maekawa *

Graduate School of Science and Engineering, University of Toyama, 3190 Gofuku, Toyama, Toyama 930-8555, Japan

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ABSTRACT

Beta-glucosidase (BG) is known as a multifunctional enzyme for social maintenance in terms of both cellulose digestion and social communication in termites. However, the expression profiles of each BG gene and their evolutionary history are not well understood. First, we cloned two types of BG homologs (RsBGI and RsBGII) from the termite *Reticulitermes speratus* (Kolbe). Gene expression analyses showed that RsBGI expression levels of primary queens and kings from 30 to 100 days after colony foundation were high, but those of reproductives dropped after day 400. Extremely low gene expression levels of RsBGI were observed in eggs, whereas workers had significantly higher expression levels than those of soldiers and other colony members. Consequently, RsBGI gene expression levels changed among each developmental stage, and RsBGI was shown to be involved in cellulose digestion. On the other hand, the RsBGII gene was consistently expressed in all castes and developmental stages examined, and notable expression changes were not observed among them, including in eggs. It was indicated that RsBGII is a main component involved in social communication, for example, the egg-recognition pheromone shown in this species previously. Finally, we obtained partial gene homologs from other termite and cockroach species, including the woodroach (genus *Cryptocercus*), which is the sister group to termites, and performed molecular phylogenetic analyses. The results showed that the origin of the BG gene homologs preceded the divergence of termites and cockroaches, suggesting that the acquisition of multifunctionality of the BG gene also occurred in cockroach lineages.

1. Introduction

In eusocial termites, primary reproductives (alates, queens and kings) and neotenic reproductives (i.e. supplementary queens and kings such as nymphoids and ergatoids) are devoted to reproduction (Korb and Hartfelder, 2008). Workers forage for food and care for their siblings, whereas soldiers defend the colony (Wilson, 1971). Molecular mechanisms to regulate the division of labor among these castes and their sociality have developed during the course of termite evolution, and common mechanisms should be shared by all extant termites (reviewed in Miura and Scharf, 2011). Several common genes responsible for social maintenance and genes showing caste-specific expression have been identified in several termite species (e.g. encoding hexamerines and vitellogenins) (Scharf et al., 2005a, 2007). Endogenous termite cellulase is also a good example of such a gene.

Cellulolytic protists in the gut of lower termites are well-known mutualistic symbionts (Cleveland, 1923), but termites also secrete cellulases by themselves (Watanabe et al., 1998). Endo-beta-1, 4-glucanase (EG; EC 3.2.1.4) belonging to the glycosyl hydrolase family (GHF) 9 and beta-glucosidase (BG; EC 3.2.1.21) affiliated with GHF1 are known as termite-derived cellulases (reviewed in Lo et al., 2011). EGs and BGs are known as common cellulase components in bacteria, fungi, protists, plants and animals. Cellulose chains are hydrolyzed to cellobiose and cellotriose by EGs, and short-chain sugars are converted into glucose by BGs (Ni et al., 2005; Zhang et al., 2009). In termites and their relatives (i.e. cockroaches), EG genes are found in many species (Lo et al., 2000; Shimada and Maekawa, 2008), but BG genes have only be identified in some termite species, including *Neotermes koshunensis* (Tokuda et al., 2002), *Coptotermes formosanus* (Zhang et al., 2010, 2012a), *Reticulitermes flavipes* (Scharf et al., 2010), *Macrotermes barneyi* (Wu et al., 2012) and *Nasutitermes takasagoensis* (Tokuda

* Corresponding author. Tel.: +81 76 445 6629; fax: +81 76 445 6641.
E-mail address: kmaekawa@sci.u-toyama.ac.jp (K. Maekawa).
[1] Present address: Ishikawa Museum of Natural History, Ri-441 Choshi-machi, Kanazawa, Ishikawa 920-1147, Japan.

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et al., 2009). In higher termites, EGs and BGs are mainly produced in the midgut (Tokuda et al., 2004, 2009; Wu et al., 2012). However, salivary glands are the major expression sites of EG and BG genes in lower termites and cockroaches (Tokuda et al., 2004; Shimada and Maekawa, 2008; Scharf et al., 2010; Zhang et al., 2012b).

A previous study showed that the EG gene is highly expressed in workers, but soldiers had low expression levels in *R. flavipes* (Scharf et al., 2005b). Similar EG gene expression patterns were also observed in *Hodotermopsis sjostedti* (Fujita and Miura, 2008). Furthermore, Zhang et al. (2012a) showed that workers had higher expression levels of BG (clone Glu1B beta-glucosidase) than those of soldiers in *C. formosanus*. It was also shown that the activity of BG enzyme is higher in workers than soldiers (Sugio et al., 2006; Fujita and Miura, 2008). These differences in gene expression and enzyme activity of cellulases are thought to reflect donor-recipient relationships in trophallactic interactions. Because the task of workers is wood digestion and brood care, they are recognized as a 'donor'. On the other hand, soldiers are a 'recipient' because their mandibles are too specialized to ingest wood by themselves (Machida et al., 2001). Similar tendencies are also observed in the non-termite insect group. In the subsocial wood-feeding cockroach *Salganea esakii*, EG gene expression levels of first-instar nymphs were extremely low compared with those of adults, whereas first-instar nymphs of the gregarious wood-feeding cockroach *Panesthia angustipennis* had high EG gene expression levels (Shimada and Maekawa, 2008). The adults of *S. esakii* showed parental care via stomodeal trophallaxis to their offspring, but the basic social unit of *P. angustipennis* observed in the field did not appear to be the family (Maekawa et al., 2008a; Nalepa et al., 2008; Shimada and Maekawa, 2011).

EG gene expression levels were also shown to vary among different developmental stages in the same caste of termites. Shimada and Maekawa (2010) showed that EG gene expression levels were up-regulated in primary queens and kings of *R. speratus* between 30 and 100 days after colony foundation, when there were low numbers of workers in the colony. On the other hand, EG gene expression levels of primary reproductives decreased at 400 days after colony foundation (when more than one hundred workers were present), and neotenic reproductives (nymphoids) obtained from a mature field colony had extremely low EG gene expression levels. These results suggested that endogenous cellulose metabolic pathways are differentially regulated among each caste, and those in reproductives are regulated during the course of colony development. Expression level changes of other termite-derived cellulases (i.e. BG) would also be related to those of EG. However, BG is known to be a multifunctional enzyme, and the gene expression patterns among caste and developmental stages are not well understood.

Recently, some studies reported that BG and related proteins have a role other than wood digestion. Matsuura et al. (2009) indicated that BG was one of the main components of egg-recognition pheromone in *R. speratus*. In the drywood termite *Cryptotermes secundus*, genes specifically expressed in female neotenic reproductives were identified, and *Neofem2* similar to BG (belonging to GHF1) was shown to have a function in reproductive suppression (Weil et al., 2007, 2009; Korb et al., 2009). Moreover, male-specific beta-glycosidase protein Lma-p72 (affiliated with GHF1) putatively involved in pheromonal communication was also identified from Madeira cockroach *Rhyparobia* (=*Leucophaea*) *maderae* (Cornette et al., 2003). It would be interesting to understand the evolutionary history of multifunctional BG genes in cockroaches and termites, but comprehensive analyses have not yet been performed.

In this study, we cloned two BG gene homologs from *R. speratus* (Kolbe), and performed expression analyses among castes and developmental stages, especially focusing on the reproductives at different colony stages. Then, we obtained orthologous genes from other termite and cockroach species, including the woodroach (genus *Cryptocercus*), which is a sister group to termites, and performed molecular phylogenetic analyses. Based on the results obtained, we discuss the evolution of the multifunctional roles of BG genes.

2. Materials and methods

2.1. Insects

Several mature colonies of *R. speratus* were collected from rotten wood in laurel forests in Toyama and Ishikawa Prefectures, Japan, in 2008–2010. Pieces of nest wood were brought back to the laboratory and kept in plastic cases in constant darkness. Primary kings, nymphoids (neotenic reproductives differentiated from nymphs) with functional reproductive organs, workers (6th or 7th instars; Maekawa et al., 2008b), soldiers, late-stage nymphs, larvae and eggs were collected from mature field colonies. Ergatoids (neotenic reproductives differentiated from workers) with functional reproductive organs were obtained from other colonies maintained under laboratory conditions.

Old instar larvae (pseudoergates) of *Zootermopsis nevadensis*, adults of subsocial cockroach *S. esakii* and gregarious cockroach *P. angustipennis* were collected from rotten wood in laurel forests in Japan in 2006 and 2007 (Hyogo, Nagasaki and Ishikawa Pref., respectively). We maintained the live insects in the dark at room temperature prior to use. The adults of subsocial cockroach *Cryptocercus punctulatus* were provided by Dr. Nalepa (North Carolina State University).

2.2. Termite colony foundation and sample collection

Incipient colonies of *R. speratus* were set up as described by Maekawa et al. (2010), and primary queens and kings were sampled at 30, 50, 100, and 400 days after colony foundation. The details of colony members of each period are shown in Shimada et al. (2013). For RNA extraction (see below), abdomens of reproductives including symbiotic protozoa and bacteria were removed, then heads and thoraxes were immersed in liquid nitrogen immediately. They were stored at −80 °C until use.

2.3. cDNA preparation

Total RNA was extracted from heads and thoraxes (including salivary glands) of termites and salivary glands of cockroaches stored at −80 °C using a FastPure RNA kit (Takara Bio, Shiga, Japan). For the analyses of primary queens and kings, different individuals (2–4 individuals) were used for each RNA sample and at least three different RNA samples were prepared for each stage. More than 10 individuals of each caste (workers, soldiers, nymphs and ergaoids), 60 larvae and 100 eggs were used for each RNA sample for the comparison among other colony members. After DNase (Takara) treatment, the quality and quantity of extracted RNA were determined by spectroscopic measurements at 230, 260, and 280 nm using a NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Tokyo, Japan). For single-strand cDNA synthesis, DNase-treated mRNA (60 ng) was transcribed using SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, USA) as instructed by the manufacturer.

2.4. Cloning and sequencing of BG genes

Specific cDNAs were amplified by PCR using a thermal cycler GeneAmp 2400 (Applied Biosystems, USA) or MJ-Mini (Bio-Rad,