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Differential Gene Expression in the Hypopharyngeal Glands of Worker Honeybees (*Apis mellifera* L.) Associated with an Age-Dependent Role Change

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Associated with the age-dependent role change of worker honeybees (*Apis mellifera* L.) from nurse bees to foragers, some structural and functional changes occur in the hypopharyngeal glands (HPGs): nurse bee HPGs are well developed and synthesize major royal jelly proteins (MRJPs), while forager HPGs shrink and synthesize α-glucosidase, which converts nectar into honey. To identify candidate genes involved in the structural and functional HPG changes associated with the age-dependent role change of worker honeybees, we searched for genes whose expression in the HPGs depends on the role of workers, by using differential display and quantitative reverse transcription-polymerase chain reaction. Here, we newly identified a *buffy* homolog encoding a Bcl-2-like protein as a gene whose expression, like *MRJP*, is higher in nurse bees than in foragers, and a *matrix metalloproteinase 1* (*MMP1*) homolog as a gene whose expression, like *α-glucosidase*, is higher in foragers than in nurse bees, suggesting that both suppression of inhibition of the caspase cascade by buffy and degradation of the extracellular matrix by MMP1 are involved in the functional and structural changes of the HPGs. Furthermore, although both *buffy* and *MMP1* were highly expressed in various tissues other than the HPGs, *buffy* expression in the other tissues did not differ significantly between nurse bees and foragers, whereas *MMP1* expression in midgut was also significantly higher in foragers than in nurse bees, as in the HPGs. These results suggest that in *buffy* and *MMP1*, expression is regulated in a tissue-preferential manner according to the age-dependent role change of workers.

Key words: honeybee, hypopharyngeal gland, age-dependent role change, matrix metalloproteinase, buffy, tussue-preferential gene expression

INTRODUCTION

The European honeybee, *Apis mellifer*a L., is a eusocial insect, and females differentiate into either queens (the reproductive caste) or workers (the labor caste) (Winston, 1987). Queens devote their life to reproduction, whereas workers engage in various tasks related to colony growth and maintenance, such as brood rearing, comb building, and foraging. In addition, the role of workers changes depending on their age after eclosion (Winston, 1987). The lifespan of a worker is usually 30 to 40 days from spring to autumn (Dayer, 1991); young workers (generally, less than 13 days after eclosion) take care of the brood in the hive by secreting royal jelly (nurse bees), whereas old workers (more than 18 days) collect nectar and pollen outside the hive (foragers) (Lindauer, 1952; Sakagami, 1953; Winston, 1987).

Some physiological changes in certain organs accompany this age-dependent role change in workers. For example, the hypopharyngeal glands (HPGs), a pair of exocrine glands in the worker's head, undergo structural and functional changes associated with this age-dependent role change in workers. In nurse bees, the HPGs are well developed and synthesize major royal jelly proteins (MRJPs) (Kubo et al., 1996; Ohashi et al., 1997), whereas in foragers, they shrink and synthesize carbohydrate-metabolizing enzymes that process nectar into honey, such as α-glucosidase, α-amylase, and glucose oxidase (Kubo et al, 1996; Ohashi et al., 1996; 1997; 1999). We previously reported that the mode of gene expression for MRJP isoforms and the carbohydrate-metabolizing enzymes changes at the level of single secretory cells in the HPG acini associated with the age-dependent role change in workers (Ohashi et al., 1996; Ohashi et al., 1997).

Both the roles and the physiology of workers have plasticity to be modulated concurrently depending on the colony demand. For example, in colonies where brood decreases in number, older workers tend to retain well-developed HPGs, like nurse bees (Hrassnigg and Crailsheim, 1998). Furthermore, in orphan colonies, where no newly emerging workers are supplied and thus older workers need to take care of their brood, the older workers continue to synthesize MRJP in the HPGs and work as nurse bees (Ohashi et al., 2000). These findings strongly suggest a close link between

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the physiology of the HPGs and the role of workers. Therefore, honeybee HPGs could be a model organ whose structure and function change at the cellular level associated with the animal's behavior. The molecular mechanisms underlying the structural and functional changes of the HPGs associated with the role change of workers, however, remain unknown.

In the present study, to identify candidate genes involved in the structural and functional changes of the HPGs, we searched for genes whose expression in the HPGs differs between nurse bees and foragers, using differential display and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). We newly identified a *buffy* homolog, *Ambuffy*, as a nurse bee-selective gene, and a *matrix metalloproteinase 1* homolog, *AmMMP1*, as a forager-selective gene. The findings rescpectively suggest possible involvement of the caspase cascade and extracellular matrix degradation in the structural and functional changes of the worker HPGs.

MATERIALS AND METHODS

Animals and tissues

European honeybee (*Apis mellifera* L.) colonies were purchased from the Kumagaya bee farm (Saitama, Japan) and maintained at the University of Tokyo (Tokyo, Japan). Nurse bees were collected when they were feeding brood, and foragers were collected when they returned to the colony after foraging pollen and honey (Kubo et al., 1996). After the workers were anesthetized on ice, the heads were removed and the HPGs were dissected from them with fine tweezers and a surgical knife under a binocular microscope. Nurse bees with well-developed HPGs and foragers with shrunken HPGs were used in experiments, as described previously (Ohashi et al. 1999). Tissues to be used for extraction of RNA were stored frozen at –80°C until use.

Differential display

Total RNA was extracted from HPGs using TRIZol Reagent (Invitrogen, Carlsbad, CA), treated with DNase I, and reverse-transcribed by using SuperScript III (Invitrogen) with rhodamine-labeled downstream primer 1 (TaKaRa, Tokyo, Japan). Differential display was performed using a Fluorescence Differential Display Kit, Rhodamine Version (TaKaRa) and *LA Taq* (TaKaRa) with a combination of 24 primers, as described previously (Ito et al., 1994; Kamikouchi et al., 1998). PCR conditions were (94°C × 2 min + 40°C × 5 min + 72°C × 5 min) × 1 cycle + (94°C × 30 sec + 40°C × 2 min + 72°C × 1 min) × 34 cycles) + 72°C × 5 min. To ensure the reproducibility of the differential display profiles, duplicate reactions with two lots of RNA obtained from two honeybee colonies were performed.

Bands whose signal intensities differed between nurse bees and foragers in the two lots of RNA samples were selected as candidate bands. Bands whose intensities differed between nurse bees and foragers were excised from gels, and the DNA in the bands was reamplified by PCR. The resulting PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI) and transfected into *Escherichia coli* DH5α competent cells (TaKaRa). The cDNA sequences were determined using an ABI PRISM 3100 Genetic Analyzer with the BigDye Terminator v3.1 Cycle Sequencing Kit. Similarity searches of cDNA sequences were performed by using NCBI Honey Bee Genome Resources.

Cloning of full-length open reading frame sequences

RT-PCR was performed using *Ex Taq* (TaKaRa), with cDNA derived from the HPG as the template. Primers (*Ambuffy*, 5'-GAAATGAATCGTTCGTTGTAG-3' and 5'-CAAGATGAAATATTTA-ATGAATCATTTATGGAGG-3'; *AmMMP1*, 5'-GACGATCTACGG-GAACAC-3' and 5'-GATGACACAATATTGCATGCGAC-3') were designed on the basis of sequences in the 5' and 3' untranslated regions, obtained from the NCBI Honey Bee Genome Resources. Cloning and sequencing were performed as described above. Domain and motifs in deduced amino acid sequences were predicted by using the Pfam database (GenomeNet Database Resource: http://motif.genome.jp/), SignalP (The Center for Biological Sequence Analysis at the Technical University of Denmark: http://www.cbs.dtu.dk/services/SignalP/), and information previously reported by Llano et al. (2000) and Igaki and Miura (2004). The C-terminal hydrophobic membrane anchor (MA) was predicted by using TMHMM (The Center for Biological Sequence Analysis at the Technical University of Denmark: http://www.cbs.dtu.dk/services/TMHMM/). The amino acid sequences of *Am*buffy and *Am*MMP1 were aligned by using ClustalX (Thompson et al., 1997).

Quantitative RT-PCR

To investigate tissue-preferential gene expression, we simply dissected worker bodies into HPGs, the head except for the HPGs, thorax, midgut, and abdomen except for the midgut. We dissected the midgut from the abdomen, because the midgut contained much food pollen, which might have disturbed the RNA extraction procedure. We washed the pollen from the midgut and used the remaining midgut also for RNA extraction. Total RNA was extracted from these tissues with TRIZol Reagent (Invitrogen), treated with DNase I, and reverse-transcribed by using SuperScriptIII (Invitrogen) with the oligo dT primer. Real-time PCR was performed with SYBR *premix Ex Taq* II (TaKaRa) and LightCycler (Roche, Basel, Switzerland) according to manufacturers' protocols, using gene-specific primers. The amount of transcript was normalized with that of *elongation factor 1α-F2* (*EF1α-F2*; Danforth and Ji, 1998) or *ribosomal protein 49* (*rp49)* (Ben-Shahar et al., 2003). We also examined the expression of *mrjp2* and *α-glucosidase* as reference genes that are expressed preferentially in nurse bee and forager HPGs, respectively, which we previously showed by Northern blotting analysis (Ohashi et al., 1997).

Gene-specific primers (*Ambuffy*, 5'-CATGGCACTTCTCATC-CTTTTC-3' and 5'-GAGAACGGTTTCAGCATCAATC-3'; *AmMMP1*, 5'-GCTTCCCGATAATCTTGATG-3' and 5'-CATCCGAACCACCAG-TAAG-3'; *MRJP2*, 5'-AAATGGTCGCTCAAAATGACAGA-3', and 5'-ATTCATCCTTTACAGGTTTGTTGC-3'; *α-glucosidase*, 5'-TACCTG-GCTTCGTGTCAAC-3' and 5'-ATCTTCGGTTTCCCTAGAGAATG-3'; *EF1α-F2*, 5'-CATCAAAAACATGATTACTGGTACCTC-3' and 5'-CAGAATACGGTGGTTCAGTGG-3'; and *rp49*, 5'-AGAAACTGGCG-TAAACCTAAAG-3' and 5'-GTTCCTTGACATTATGTACCAAAAC-3') were derived from cDNA sequences for each gene and information from Honey Bee Genome Resources. PCR conditions were: (95°C × 30 s) + (95°C × 5 sec + 60°C × 15 s + 72°C × 20 sec) × 45 cycles.

RESULTS

Screening of nurse bee and forager HPG-preferential genes

To search for genes whose expression in the HPGs differs between nurse bees and foragers, we used the differential display method. By screening approximately 900 bands, 48 candidate bands were obtained (23 nurse bee-selective and 25 forager-selective bands). Among these, 14 bands were selected based on the extent of differences in intensity between nurse bees and foragers in the differential display images. Sequence analysis assigned these bands to seven genes: *MRJP1*, *3* (Ohashi et al, 1997), and *7* (Stefan and Jaroslav, 2004)*, α-glucosidase* (Ohashi et al, 1996), and three other clones (1 to 3). In the present study, we focused on clones 1 and 2, as we could not detect significant expression of clone 3 (data not shown).

A database search suggested that clone 1 corresponded to a part of a predicted gene, *GB18455*, which is located in linkage group 3 of the honeybee genome and encodes a Bcl-2-like protein (Fig. 1A). To identify full-length open reading frame sequences, we performed RT-PCR using primers binding within the 5' and 3' untranslated regions of each gene, designed based on information obtained from the NCBI Honey Bee Genome Resources, and obtained a 1233-bp sequence for clone 1 that contained initiation and stop codons. The cDNA identified for clone 1 encoded a protein consisting of 283 amino acid residues. The deduced amino acid sequence contained putative Bcl-2 homology domains (BH domains), including the BH3, BH1, and BH2, and a putative C-terminal hydrophobic membrane anchor (MA), which are typical of Bcl-2 proteins (Fig. 1B, C). A database search using protein BLAST indicated that the BH3, BH1, and BH2 domains of the identified protein had 50%, 57%, and 50% sequence identity with buffy, which is a Bcl-2 protein in *Drosophila melanogaster* (Fig. 1B). In *Drosophila*, there are two Bcl-2 family genes, *Drob-1*/*Debcl*/*dBorg-1*/*dBok* and *Buffy*/*dBorg-2* (Igaki et al., 2000; Cloussi et al., 2000; Brachmann et al., 2000; Zhang et al., 2000). In contrast, no other bcl-2 family genes apart from clone 1 were found in the honeybee genome, indicating that the gene corresponding to clone 1 is the honeybee *buffy* homolog (*Ambuffy*).

Clone 2 corresponded to part of a predicted gene, *GB19151*, located in linkage group 10 and encoding a matrix metalloproteinase (MMP) homolog (Fig. 2A). We obtained a 2531-bp sequence for clone 2 that contained initiation and stop codons. The cDNA identified for clone 2 encoded a protein consisting of 608 amino acid residues. The deduced amino acid sequence showed all the structural features typical of the MMP family (Fig. 2B). The open reading frame sequence contained the sequence PRCGVXD, which is a conserved motif in the prodomain of MMP, as well as a putative catalytic domain including the consensus motif HEXGHXXGXXHS, and a putative hemopexin domain (Fig. 2C). The hemopexin domain has sequence similarities to the serum protein hemopexin, a heme-binding protein that transports heme to the liver (Gomis-Ruth et al., 1996). The hemopexin domain binds to several proteins such as cell surface proteins and tissue inhibitor of matrix metalloproteinases (TIMPs). Thus this domain is thought to be involved in cellular protein-protein interactions and the inhibition of MMP (Page-McCaw et al., 2003). A protein BLAST search indicated that these three domains had 57%, 75%, and 72% sequence identity with MMP1 of *Drosophila melanogaster* (Fig. 2B). The database search revealed another predicted MMP gene, *GB16274*, in the honeybee genome. *Drosophila melanogaster* MMP1 (*Dm1*-MMP) was more similar in amino acid sequence to clone 2 than to GB16274 (data not shown), indicating that the gene corresponding to clone 2 is the honeybee *MMP1* homolog (*AmMMP1*). *AmMMP1* contained at the N terminus a putative signal peptide for secretion, suggesting the product could work in the extracellular matrix (Fig. 2B, C).

Expression analysis of *Ambuffy* and *AmMMP1* in HPGs

We performed quantitative RT-PCR to confirm the differ-

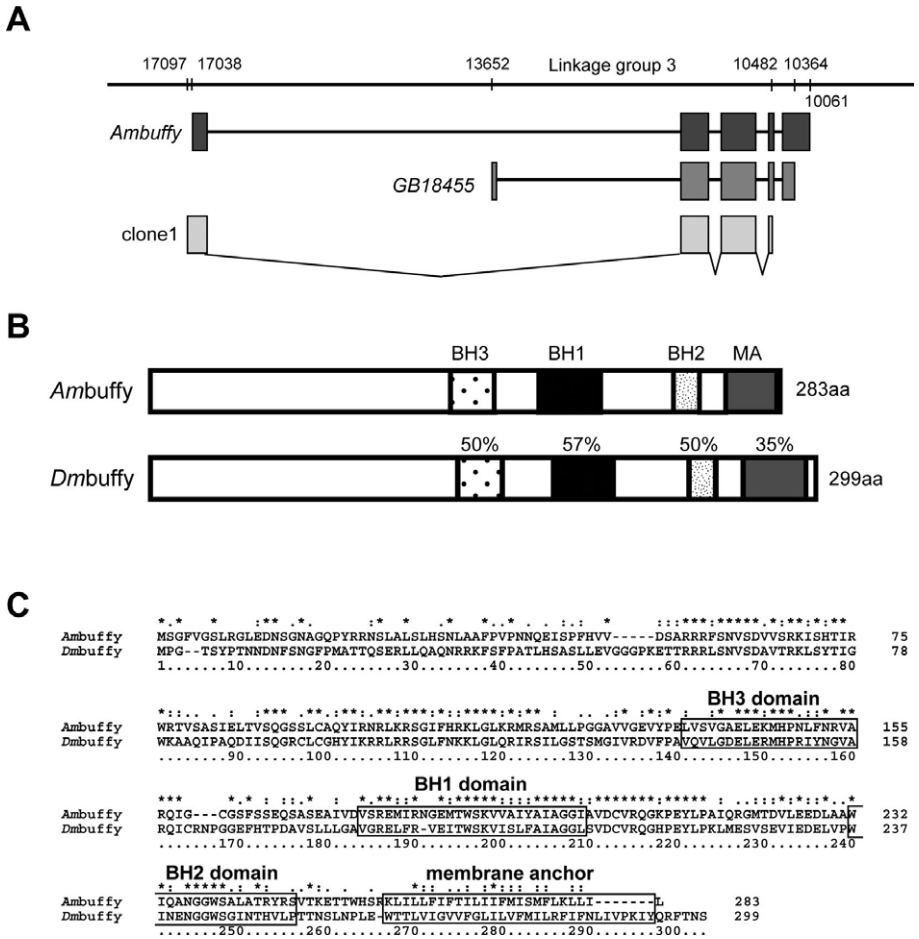

Fig. 1. Identification of clone 1, obtained by differential display, as *Ambuffy*. **(A)** Genomic organization of the gene for clone 1. Exons (filled boxes) and introns (lines) of the newly identified gene, the gene predicted by NCBI Honey Bee Genome Resources, and the cDNA structure of clone 1 obtained by differential display are indicated below the corresponding linkage group. **(B)** Comparison of the domain structures of *Am*buffy and *Dm*buffy. Homologous domains (the Bcl-2 homology domains [BH3, BH1, BH2] and the C-terminal hydrophobic membrane anchor [MA]) are labeled. The number above each domain in *Dm*buffy indicates the amino acid sequence identity of that domain between *Am*buffy and *Dm*buffy. **(C)** Alignment of the predicted protein sequences of *Am*buffy and *Dm*buffy. The predicted BH3, BH1, BH2, and MA domains are boxed. The BH domains were predicted with the Pfam database (Bateman et al., 2002) and information reported by Igaki and Miura (2004). The MA was predicted by using TMHMM (Sonnhammer et al., 1998).

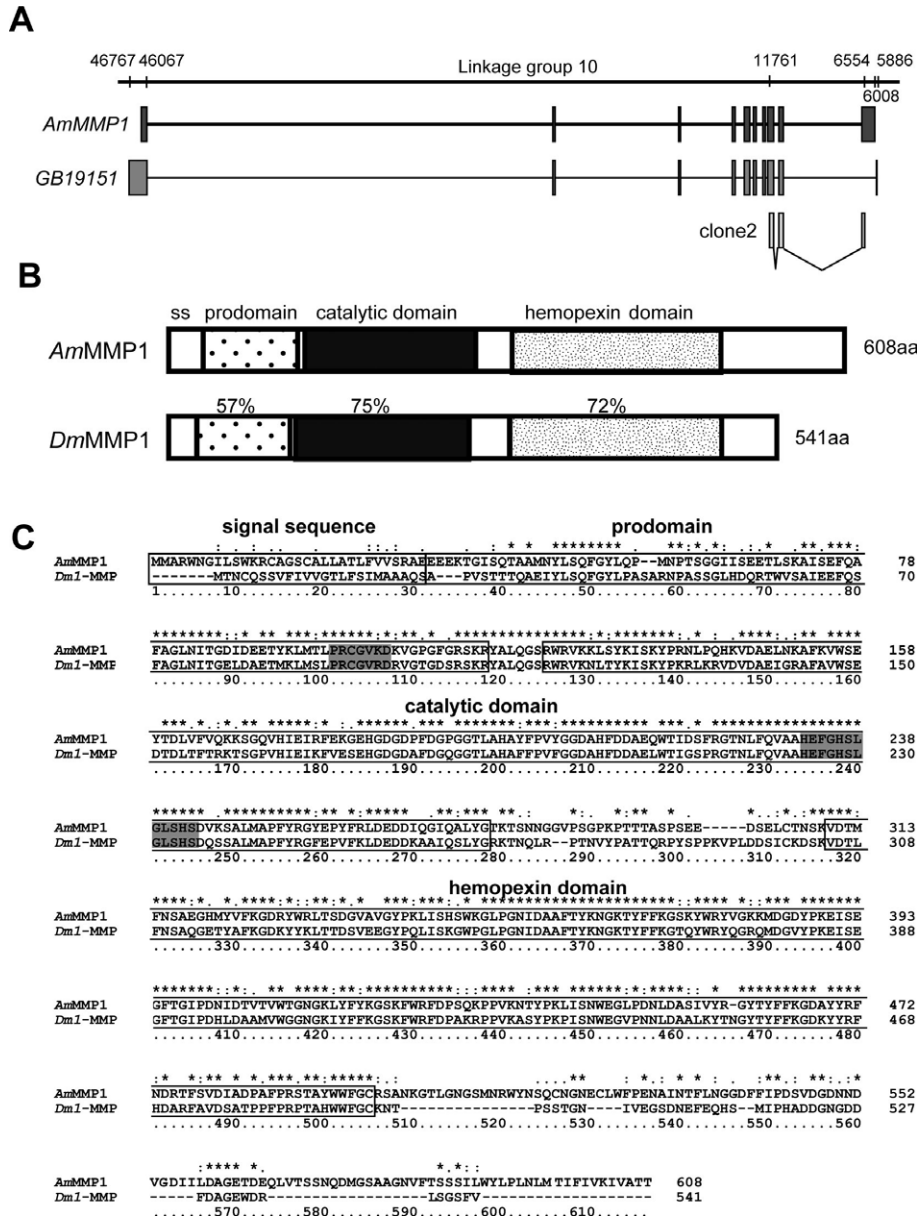

Fig. 2. Identification of clone 2, obtained by differential display, as *AmMMP1*. **(A)** Genomic organization of the gene for clone 2. Exons (filled boxes) and introns (lines) of the newly identified gene, the gene predicted by NCBI Honey Bee Genome Resources, and the cDNA structure of clone 2 obtained by differential display are indicated below the corresponding linkage group. **(B)** Comparison of the domain structures of *Am*MMP1 and *Dm1*-MMP. Homologous domains (the pro-, catalytic, and hemopexin domains) are labeled. The number above each domain in *Dm1*-MMP indicates the amino acid sequence identity of that domain between *Am*MMP1 and *Dm1*-MMP. **(C)** Alignment of the predicted protein sequences of *Am*MMP1 and *Dm1*-MMP. The predicted pro-, catalytic, and hemopexin domains are boxed. Amino acid residues corresponding to the conserved PRCGVXD motif in the prodomain and the consensus motif HEXGHXXGXXHS in the catalytic domain are shaded. Domains and motifs were predicted by the Pfam database, SignalP (Bendtsen et al., 2004), and information reported by Llano et al. (2000).

ential expression of *Ambuffy* and *AmMMP1* between nurse bees and foragers. For this, 9 to 14 nurse bees and foragers were collected at the same time from a single colony, and gene expression was compared using four batches of samples derived from four different colonies. The nurse bees and foragers were collected based on behavior as well as on HPG development (Ohashi et al., 1999). Quantitative RT-PCR showed that the expression of *Ambuffy* in the HPGs was approximately 12-fold higher in nurse bees than in foragers (Fig. 3A). We also analyzed the expression of *MRJP2* and *α-glucosidase* as reference genes, because our previous Northern blotting analysis showed that differential expression depended on the role change (Ohashi et al., 1997). Expression of *MRJP2*, which showed the most prominent differential expression between nurse bee and forager HPGs among MRJPs 1 to 3 (Ohashi et al., 1997), was approximately 10-fold higher in nurse bees than in foragers (Fig. 3C), indicating that the extent of role-dependent change in *Ambuffy* expression in the HPGs was almost comparable to that in *MRJP2* expression.

In contrast, the expression of *AmMMP1* in the HPGs was approximately 4.5-fold higher in foragers than in nurse bees (Fig. 3B). Quantitative analysis of the differential expression of *α-glucosidase* between nurse bee and forager HPGs indicated that its expression in the HPGs was approximately 360-fold higher in foragers than in nurse bees (Fig. 3D). Although the extent of differential expression of *AmMMP1* in the HPGs between nurse bees and foragers was smaller than that of *α-glucosidase*, there was a reproducible and significant difference in *AmMMP1* expression level between nurse bee and forager HPGs.

Expression analysis of *Ambuffy* and *AmMMP1* in various tissues

We investigated whether tissues other than HPGs also express *buffy* and *MMP1*, and whether expression in those tissues also changes in association with the role change of workers. The amounts of transcripts of these genes in various body parts (the HPGs, the head without the HPGs, thorax, midgut, and abdomen without the midgut) were examined by quantitative RT-PCR. Each sample was prepared by using two or three nurse bees or foragers per batch, and eight batches of samples were prepared from three colonies and analyzed. In the above experiments to analyze *Ambuffy* and *AmMMP1* expression in the nurse bee and forager HPGs

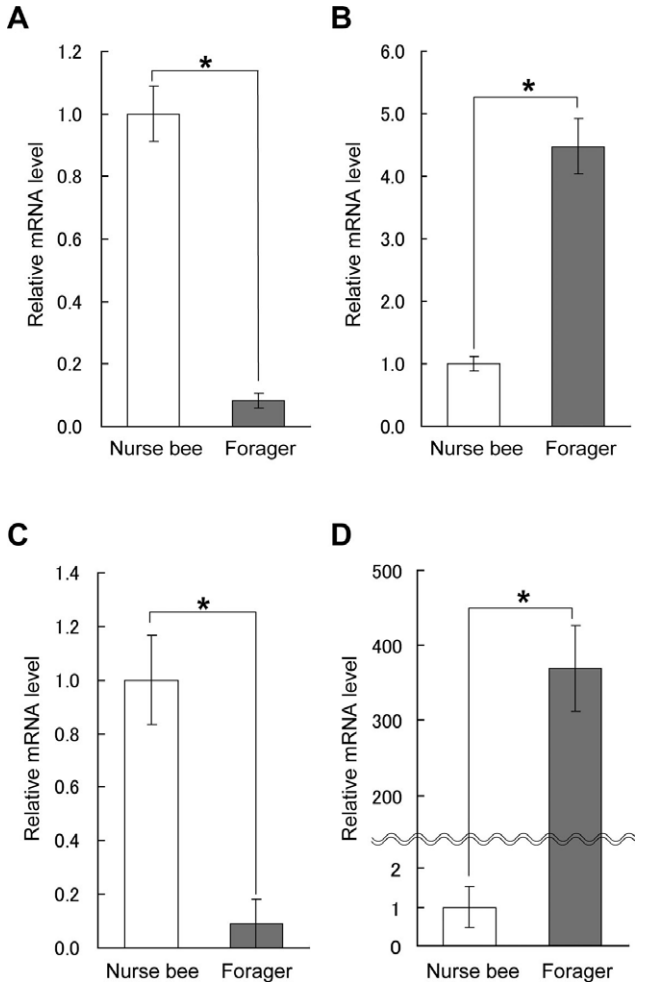

Fig. 3. Quantitative analysis of *Ambuffy* and *AmMMP1* transcripts in the HPGs. Nurse bees or foragers (n=9–14/group) were collected as one batch, and four batches prepared from four different colonies were subjected to real-time RT-PCR. Bars indicate mean relative mRNA levels with the standard deviation for **(A)** *Ambuffy*, **(B)** *AmMMP1*, **(C)** *MRJP2*, and **(D)** *α-glucosidase*, with the amount of mRNA in nurse bee HPGs defined as 1. Asterisks indicate significant differences between nurse bees and foragers (* $p<0.01$; Welch's t-test).

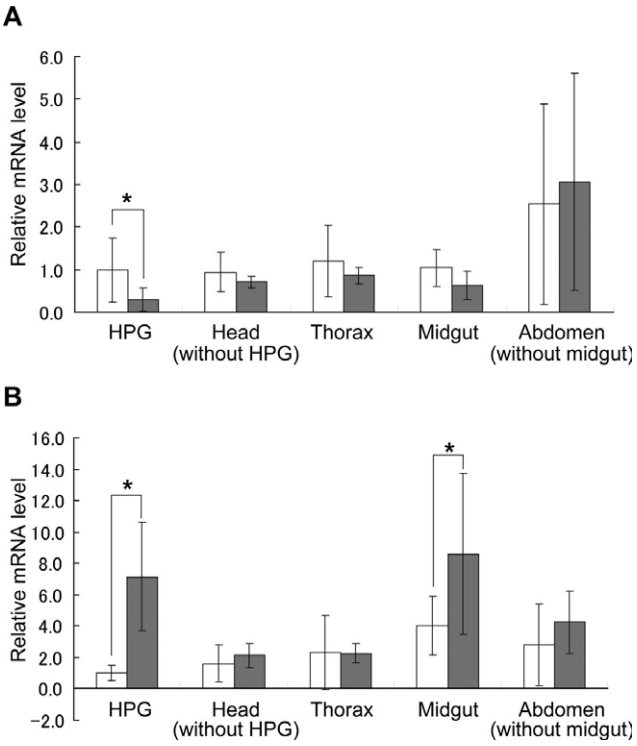

Fig. 4. Quantitative analysis of *Ambuffy* and *AmMMP1* transcripts in various body parts and tissues. Samples were collected from two or three nurse bees or foragers for each batch, and eight batches of samples were prepared from three colonies and subjected to real-time RT-PCR. HPGs of foragers were collected from only seven batches due to a failure in sample preparation. Bars indicate the mean relative mRNA levels with the standard deviation for **(A)** *Ambuffy* and **(B)** *AmMMP1* in various body parts and tissues, with the amount of mRNA in nurse bee HPGs defined as 1. Asterisks indicate significant differences between nurse bees and foragers (* $p< 0.05$; Welch's t-test).

(Fig. 3), the amount of *Ambuffy* transcript was normalized to that of *elongation factor 1α-F2* (*EF1α-F2*) (Danforth and Ji, 1998), which was expressed almost equally in both nurse bee and forager HPGs (data not shown). However, we used *rp49* (Ben-Shahar et al., 2003) as a reference to normalize *Ambuffy* and *AmMMP1* expression in various tissues, because we could not detect any significant *EF1α-F2* expression in the other tissues, and quantitative RT-PCR revealed that there were almost equivalent levels of *rp49* expression in all these tissues (data not shown).

Ambuffy expression was high not only in the HPGs but also in the other body parts and tissues examined. However, interestingly, although the differential *Ambuffy* expression in the HPGs between nurse bees and foragers was again confirmed in this experiment, the expression level of *Ambuffy* in nurse bee HPGs was largely comparable to that in the other body parts and tissues of nurse bees, and the *Ambuffy* expression in other body parts or tissues other than the HPGs did not differ significantly between nurse bees and foragers (Fig. 4A).

Similarly, all body parts or tissues examined showed high *AmMMP1* expression, and the differential *AmMMP1* expression in the HPGs between nurse bees and foragers was again confirmed in this experiment (Fig. 4B). *AmMMP1* expression varied to a larger extent among body parts and tissues than *Ambuffy* expression. *AmMMP1* expression was distinct from *Ambuffy* expression in that it was significantly higher in foragers than in nurse bees, not only in the HPGs but also in the midgut, indicating that *Ambuffy* and *AmMMP1* expression differed between nurse bees and foragers in a tissue-preferential manner.

DISCUSSION

In the present study, we newly identified honeybee homologs of *buffy* (*Ambuffy*), whose expression was higher in nurse bee HPGs than in forager HPGs, and *matrix metalloproteinase 1* (*AmMMP1*), whose expression was higher in forager HPGs than in nurse bee HPGs. This is the first identification of genes that are possibly involved in the regulation of HPG structure and/or function (intracellar sig-

naling and extracellular matrix degradation, respectively), rather than the products of the HPGs as an exosecretory gland, such as MRJPs, which are the components of royal jelly, and carbohydrate-metabolizing enzymes that process nectar into honey (Kubo et al, 1996; Ohashi et al., 1996, 1997, 1999).

In *Drosophila*, buffy (*Dm*buffy) is a Bcl-2-like protein with anti-apoptotic activity (Brachmann et al., 2000; Quinn et al., 2003). Genetic analysis suggests that *Dm*buffy functions upstream of the apical caspase Dronc (Quinn et al., 2003). Based on these proposed functions for *Dm*buffy and the fact that *Ambuffy* expression in the HPGs was higher in nurse bees than in foragers (Figs. 3A, 4A), *Am*buffy may repress the caspase cascade in nurse bee HPGs, and this repression is released in forager HPGs, in which *Ambuffy* expression is decreased. We previously demonstrated that the number of secretory cells was almost the same in individual acini of nurse bee and forager HPGs, suggesting that cell death does not occur markedly during the age-dependent role change of workers, and that apoptotic degeneration might not be involved in the HPG morphological change (Ohashi et al., 1997). Hence, our present findings suggest that part, but not all, of the caspase cascade plays a role in the structural and/or functional changes of the HPGs without inducing cell death. In mammals, caspases are also involved in skeletal muscle cell differentiation and osteogenic differentiation of bone marrow stromal stem cells (Fernando et al., 2002; Miura et al., 2004). Caspases can also act as signal-transducing molecules that are not directly related to cell death (Kroemer and Martin, 2005). Therefore, caspases may function as signal-transducing molecules not leading to apoptosis in forager HPGs. In addition, that *Ambuffy* expression decreased only in the HPGs, but not in other body parts or tissues in foragers (Fig. 3A), suggests that *Ambuffy* transcription is regulated in an HPG-specific manner. The caspase cascade may also be activated specifically in the forager HPGs.

On the other hand, based on similarity in sequence and motif structures (Fig. 1B), *Am*MMP1 is expected to function similarly to *Dm1*-MMP. *Dm1*-MMP degrades fibronectin and type-IV collagen, which are extracellular matrix and basement membrane proteins (Llano et al., 2000). Therefore, it is plausible that *Am*MMP1 functions to degrade the extracellular matrix in the forager HPGs, leading to shrinkage of the gland tissue. That *AmMMP1* expression was higher in foragers than in nurse bees not only in the HPGs but also in the midgut (Fig. 4) suggests that *AmMMP1* expression might be regulated in a tissue-preferential manner (HPG and midgut), and that *Am*MMP1 functions to degrade extracellular matrix not only in the HPGs but also in the midgut in foragers. The food of adult workers changes depending on their role; nurse bees feed mainly on protein-rich food such as pollen for synthesizing and secreting royal jelly (Winston, 1987; Crailsheim et al., 1992; Szolderits and Crailsheim, 1993), whereas foragers feed mainly on carbohydrate-rich food such as nectar and honey for flying (Winston, 1987). Therefore, it might be that the structure of the midgut, as a digestive organ, also changes in association with the role change of workers and that *Am*MMP1 is involved in this process. In the present study, we did not discriminate whether the differential expression was due to the change in the age or in the role of the workers. Nonetheless, our findings further support our previous notion that the physiological change (*Ambuffy* and *AmMMP1* expression, in this study) occurs in association with the behavioral change of the workers (Kubo et al, 1996; Ohashi et al., 1996; 1997; 1999).

We showed that the extent of role-dependent change in *Ambuffy* expression in HPGs was almost comparable to that in *MRJP2* expression (Fig. 3A). Although the extent of differential expression of *AmMMP1* associated with the role change of workers was smaller than that of *α-glucosidase*, the synergestic effects of multiple regulators, each of which shows a small level of differential expression, often have an amplified effect on the expression of final products (Cobb, 1999). Therefore, we propose *Ambuffy* and *AmMMP1* as good candidates for genes involved in structural and functional changes in HPGs. In general, behavior (role) and physiology are correlated in social insects, and both can be plastically modulated in accordance with social interactions. For example, bumblebee (*Bombus terrestris*) workers change enormously in body size, and body size and division of labor are closely correlated (Wilson, 1975). Our future study of functional changes in HPGs might also shed light on the related molecular mechanisms underlying the correlated changes in behavior and physiology observed in social insects.

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