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In vivo gene transfer into the adult honeybee brain by using electroporation

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Abstract

The honeybee, *Apis mellifera* L., is a social insect and they show wide variety of exquisite social behaviors to maintain colony activity. To enable the elucidation of those social behaviors at a molecular level and gene function in the nervous system, we developed an in vivo method to perform gene transfer in the adult brain of living honeybee by electroporation. When green fluorescent protein-expressing plasmid was transferred to the brain with this system, green fluorescence was observed near the anode location. The expression of transfected genes was confirmed at both transcriptional and translational levels by reverse transcription-polymerase chain reaction and immunoblot analyses. This system will facilitate the analysis of gene function and the regulatory mechanisms of gene networks in the nervous system and provide clues to clarify the relation between those genes and the complex behaviors of the honeybee.

Keywords: Honeybee; Brain; Electroporation; Gene transfer; Behavior; Eusocial insect

The honeybee (*Apis mellifera* L.) is a eusocial insect with a colony that is composed of three types of adults: the queen, workers, and drones. While queens and drones are specialized for a reproductive role, workers are engaged in various social labors, such as nursing the brood, guarding the colony from natural enemies, and foraging for nectar and pollen in an age-dependent manner [1]. Another prominent ability of the honeybee is their use of the dance language. The workers can communicate the location of a food source by the waggle-dance, known as dance language [1,2]. This behavior is observed only in the honeybee species, suggesting that during evolution they acquired a unique brain function to use the dance language. The molecular basis underlying the highly advanced social behaviors of the honeybee, however, remains unclear.

Recently, many candidate genes involved in honeybee social behaviors were identified using differential display and/or cDNA microarray technology, and their expression was demonstrated to be behavior-associated [3–6], age-dependent [7], or brain region-selective [8–12]. The biochemical properties were also characterized for some of the genes [13,14]. The in vivo functions of these genes, however, were analyzed in only a few cases and in an indirect manner using chemical compounds [6]. Quantitative trait loci analyses have identified some genetic loci associated with some of the honeybee social behaviors [15–18]. The genes responsible for these social behaviors, however, have not been identified.

To gain a better understanding of the honeybee social behaviors at the molecular level, technology is needed that allows for the introduction of exogenous genes into living individuals. Few gene manipulation methods, however, are available for the honeybee [19]. In eusocial insects like the honeybee, the embryos and larvae are cared for by adult workers. Once injury or abnormality is detected in the larvae, they are destroyed and/or removed from the colony and die thereafter. Thus, avoidance of this rejection is critical for successful gene manipulation in the honeybee and it would be advantageous to develop a technique that enables direct gene transfer into the adult honeybee. We focused on electroporation, as it is applicable for differentiated adult post-mitotic cells and is easily adapted to non-model animals, like the honeybee. Electroporation has been

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successfully used to introduce DNA into cultured cells, organs in vitro [20], and recently into embryos, brains, or other tissues in various species in vivo [21–25].

Here, we report that in vivo electroporation was successfully applied to the adult brain of living honeybees with green fluorescent protein (GFP)-expressing plasmids driven by the cytomegalovirus (CMV) promoter. This method could enhance the analyses of gene function, gene regulatory mechanisms, and interrelation of genes in the honeybee brain and provide a clue to the molecular understanding of the complex social behaviors of the honeybee.

Materials and methods

Animals. Honeybee *A. mellifera* L. colonies maintained at the University of Tokyo were used throughout the experiments. A few honeybee colonies were also purchased from the Kumagaya Bee Keeping Company (Saitama, Japan). Electroporated honeybees were separated from the colony and reared in a plastic cage supplied with honey and water.

Plasmids. We used phGFP-S65T (Clontech), which expresses a GFP variant under the control of the CMV promoter for fluorescence detection and RT-PCR analyses. For protein analysis, CMV-3×HA-GFP-S65T was constructed as follows. The tet promoter of pTRE2 (Clontech) was replaced with the CMV promoter of pTet-On at the *Xho*I–*Sac*II sites and termed pCMV2, and used as a vector only control. Three-time repeats of the hemagglutinin (HA) tag were amplified by PCR and inserted into the *Bam*HI–*Sal*I sites of pCMV2, and the GFP-S65T fragment excised from phGFP-S65T (*Nco*I–*Xba*I, *Nco*I was blunted) was inserted into blunted *Sal*I and *Xba*I. Plasmids were purified using a Qiagen Plasmid Midi Kit (Qiagen).

In vivo electroporation. Micropipettes were prepared from 10-μl microcapillary tubes using a microputler equipped with a heating element (PN-3, Narishige, Japan). The micropipette was connected to a 1-ml disposable syringe with a silicone tube and the injection was performed by hand. Adult worker honeybees were caught from the hive and anesthetized by incubating on ice. For injection and electroporation, honeybees were put into a 0.5-ml tube and the neck was immobilized by inserting two U-shaped plastic sheets at right angles. An access window to the brain was opened by removing the upper part of the head cuticle and pushing aside the hypopharyngeal glands. Parallel needle electrodes (CUY567, NEPA GENE) were inserted until they touched with the target region of the brain. Plasmid solution (1 μl; 1 μg/μl DNA, 0.01% Fast Green FCF) was injected through a glass capillary controlled by a micromanipulator (MN-153, Narishige, Tokyo, Japan). Immediately after the injection, five square electrical pulses of defined voltages, 50 ms, each at intervals of 950 ms were delivered. We used an electroporation apparatus (CUY-21EDIT, NEPA GENE) to generate precise square pulses. After electroporation, the window was covered by replacing the head cuticle back to the original position. Honeybees were then released from the tube and reared in a plastic cage. One day later, the whole brain was dissected, analyzed by fluorescence microscopy, and subjected to RNA/protein analyses.

RT-PCR analysis. Total cellular RNA was extracted from whole brain of electroporated honeybee using TRIzol LS reagent (Gibco-BRL). Extracted RNA was treated with 1.0 U/μg RNA RNase-free DNaseI (Gibco-BRL) and reverse-transcribed using SuperScriptII (Gibco-BRL) with random hexamers. Control samples without reverse-transcription were produced by using water in place of reverse-transcriptase. A 1/100 volume of RT products served as the PCR template. PCR amplification was performed with primers specific for GFP: 5′ CGT AAA CGG CCA CAA GTT CA 3′ and 5′ TTC TTC TGC TTG TCG GCC AT 3′, which produced a 414-bp amplified fragment, for 30 cycles. PCR products were subjected to agarose gel electrophoresis. Primers derived from honeybee actin cDNA (5′ GAA ATG GCA ACT GCT GCA TC 3′ for sense and 5′ TCC ACA TCT GTT GGA AGG TG 3′ for antisense) were used as a control.

Immunoblot analysis. Immunoblot analysis was performed as described previously [26]. The whole brain was dissected from electroporated honeybee and lysed in 20 μl 2× of sodium dodecyl sulfate (SDS)–sample buffer (150 mM Tris–HCl buffer, pH 6.8, containing 1.2% SDS, 30% glycerol, and 15% of 2-mercaptoethanol). The lysate (8 μl) was separated by SDS–polyacrylamide gel electrophoresis (12% gel) and transferred onto a polyvinylidene fluoride membrane. Blots were blocked in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dried milk. The blots were then washed with TBST three times at 5-min intervals and incubated overnight at 4 °C with 5% nonfat dried milk/TBST containing anti-HA rat monoclonal antibody 3F10 (1:1000) (Roche, Switzerland). The blots were then washed three times for 15-min each and incubated with 5% nonfat dried milk/TBST containing horseradish peroxidase-conjugated secondary antibody (1:5000) (Sigma A5795; Sigma Chemical, St. Louis, MO) for 1.5 h at room temperature. After washing the membrane three times for 15-min with TBST, the blots were developed by chemiluminescence, LumiGLO (Cell Signaling Tech., MA).

Results

In vivo electroporation delivers gene into the brain of adult honeybee

To introduce an exogenous gene to the brain of adult honeybees, we first examined whether exogenous DNA was successfully delivered into the brain of adult honeybee by in vivo electroporation. The brain was exposed by removing the head capsule and electrodes were positioned to directly touch the brain as shown in Fig. 1 to avoid the high resistance of the insect cuticle. Circular plasmid DNA harboring GFP under the control of the CMV promoter, which is reported to work equally well in honeybee and fly [19,27], was injected between the electrodes and spread over the upper half of the brain. To determine the optimum conditions for electroporation, electrical square pulses of various voltages were applied to brain of a honeybee whose cavity was injected with a plasmid vector containing the GFP gene as a marker. After five electric square pulses were applied, the bees were maintained in a free-walking condition for 1 day and the brain was then dissected and observed under a fluorescence microscope (Fig. 2). The rate of survival and number of fluorescent positive bees 1 day after the transfection are shown in Fig. 3. Surviving bees walk, fly, and are otherwise apparently normal. Green fluorescence was observed in the restricted area pulsed at 10–90 V, whereas brains injected with naked DNA alone and no electroporation had no fluorescence. The gene transfer rate (No. of fluorescence positive/No. of surviving bees) increased as the voltage increased. As higher voltages were applied, the survival rate decreased and the gene transfer rate increased. At 50 V, the survival

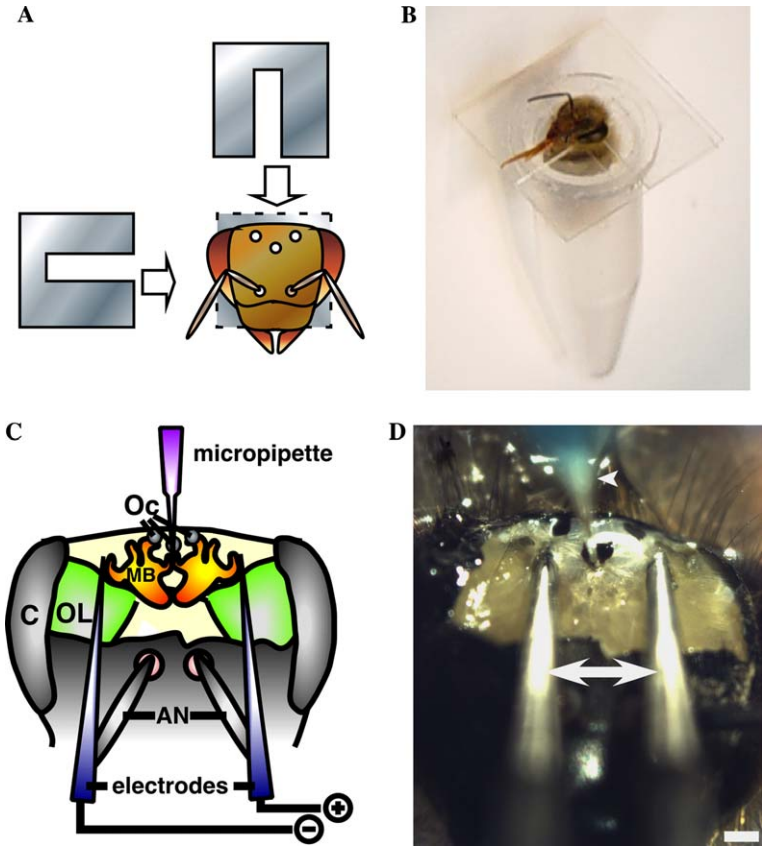


Fig. 1. In vivo electroporation. (A) The necks of honeybees that had settled in the tube were fixed in place by inserting two U-shaped plastic sheets for surgical ease and later release. (B) A honeybee settled in the tube with her neck fixed prior to the operation. (C) Schematic representation of the micropipette and electrodes placed in the honeybee brain. Purple indicates micropipette filled with DNA solution, blue indicates electrodes. AN, antennae; C, compound eyes; MB, mushroom bodies; Oc, ocelli; OL, optic lobes. (D) DNA injection and electroporation with needle electrodes. Arrows and arrowhead indicate electrodes and micropipette for DNA injection, respectively. Scale bar, 300 μm.

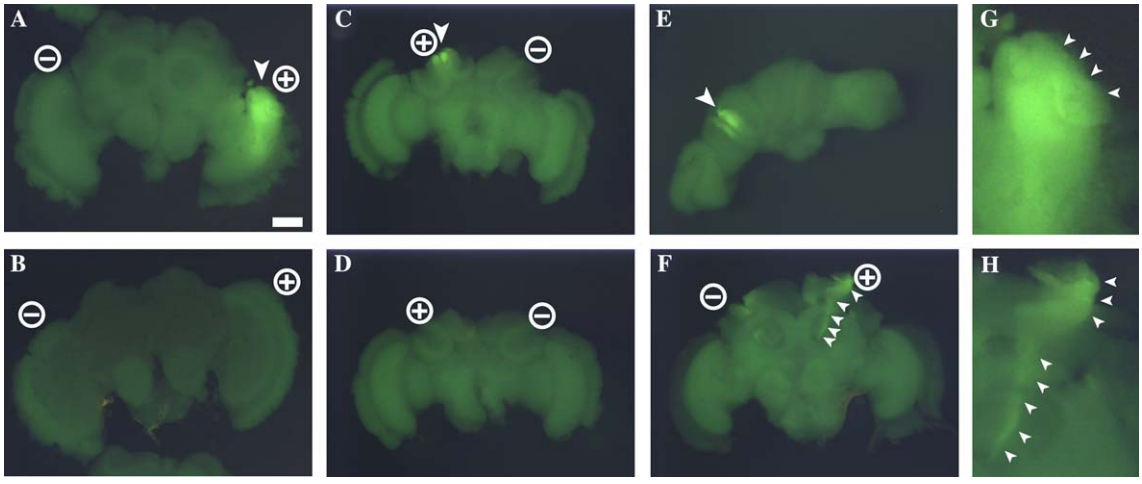
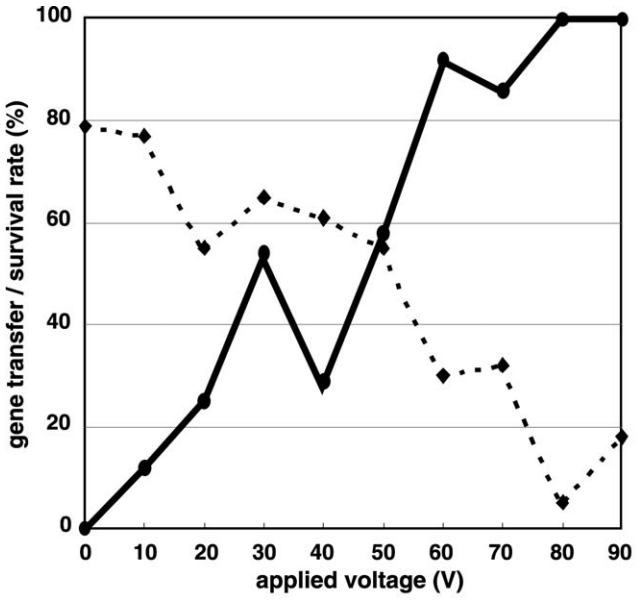


Fig. 2. Fluorescent images of electroporated honeybee brain at 50 V, targeting the optic lobe (A,B) and mushroom bodies (C–F). Fluorescent signals were detected around the anode position in the brain electroporated with GFP-expressing plasmid driven by the CMV promoter (A,C,E,F) and not in those electroporated without plasmid (B,D). (G,H) Magnified views of GFP-expressing areas shown in (A) and (F), respectively. Positions of electrodes are indicated by circles containing + or −. Arrowheads indicate the regions in which fluorescence was detected (A,C,E,G) and neural projections from mushroom body cells (F,H). Frontal view (A,B,F,G,H), rear view (C,D), and top view (E). Panels (C) and (E) show different views of the same brain.



voltage (V)	0	10	20	30	40	50	60	70	80	90
n	14	22	22	20	23	22	44	22	22	22
number of surviver	11	17	12	13	14	12	13	7	1	4
number of GFP positive	0	2	3	7	4	7	12	6	1	4

Fig. 3. Effects of voltage on survival and gene transfer rates of brains by electroporation. Five pulses were delivered for 50 ms each with interval of 950 ms. Survival and gene transfer rates are indicated by dashed and solid lines, respectively.

rate was 55% and the gene transfer rate was 58%. At 60 V, the survival rate was 30% and the gene transfer rate was 92%. Although suitable voltage depends on the purpose of the experiment, 50–60 V can be used for most cases.

Localized delivery of gene in the brain

Localized fluorescence was observed in a restricted area of the optic lobe that touched the anode when the electric pulses were applied (Figs. 2A and G). DNA molecules are negatively charged and move towards the anode when an electric field is generated. In this system, the DNA solution was not retained around the injected area and spread throughout the brain because there are no ventricles in bee brain. The area around the anode seems somehow the most suitable for a strong electric field. The localized signal led us to try to regulate the region of incorporation by changing the position of the electrodes. Next, we positioned the electrodes to touch the mushroom bodies and the same procedure was performed. In this case, localized fluorescence was detected in the mushroom bodies, which were next to the anode (Figs. 2C and E). Thus, the transfer region can be controlled by the position of the anode to enable region-selective gene delivery. In some cases, projections of mushroom body cells were also visualized by fluorescence (Figs. 2F and H).

Expression of transfected gene

To confirm that exogenous GFP was expressed, we examined the presence of the GFP transcript in the electroporated brain by RT-PCR. In GFP-electroporated brains, 8 of 8 bees show the signal in a reverse-transcriptase-dependent manner, while there was no signal detected in any control brains (Fig. 4). Thus, CMV-GFP exogenous DNA was incorporated and expressed in the electroporated cells. A weak band for the GFP gene was detected in the RT− lane for sample #5, suggesting that a small quantity of the expression vector contaminated the RNA sample, whereas there was no band for the actin gene in the RT− lane, which indicates that the honeybee genome was effectively removed. This might be because a small DNA like expression vector could contaminate the RNA preparation much more easily than a huge genome. The rate of gene transfer was much higher than that determined by fluorescence observation. This was possibly due to the detection of fluorescence, which requires a significant amount of GFP molecules and RT-PCR has much higher sensitivity.

Furthermore, we examined whether the transfected gene successfully produced its encoding protein. To do this, we constructed HA-tagged GFP-expressing plasmid driven by the CMV promoter and the production of protein was determined by immunoblot analysis using anti-HA antibody. Strong signals at the predicted size were detected in brains electroporated with CMV-3×HA-GFP (Fig. 5). Brains electroporated with control CMV (−) vector showed no significant signal. Taken together, these results indicate that gene transfected by electroporation was successfully transcribed and translated in the brain of living honeybee.

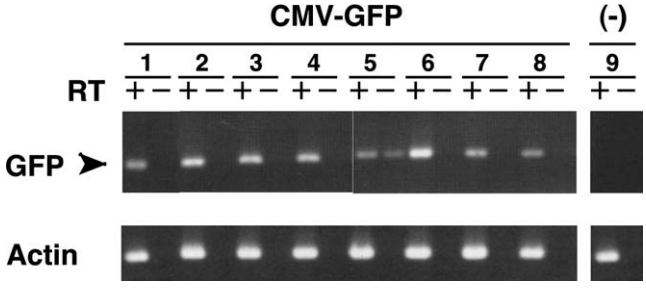


Fig. 4. RT-PCR analysis of GFP expression in the electroporated brains. Total RNA was extracted from the brains electroporated with GFP-expressing plasmid (lanes 1–8) or vector backbone alone (lane 9). With GFP primers, a single band was amplified of the predicted size only in CMV-GFP electroporated brains. Actin was amplified from every sample as a loading control. RT− indicates the negative control without reverse transcription.

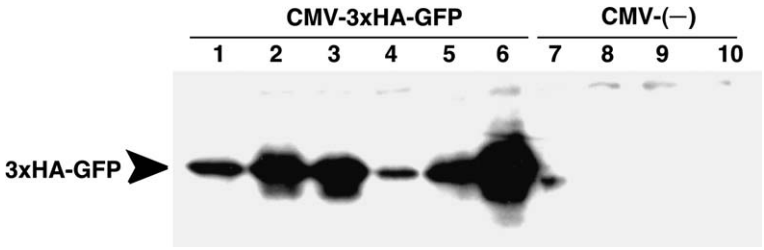


Fig. 5. Immunoblot analysis of electroporated brains. Proteins were extracted with SDS sample buffer, separated on a 12% SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, probed with anti-HA monoclonal antibody, and visualized by chemiluminescent technology. Intense signals were detected at the predicted size in samples electroporated with CMV-3×HA-GFP (lanes 1–6), while no significant signal was observed in the case with the CMV-promoter alone (lanes 7–10).

Discussion

In the present study, we developed a powerful in vivo electroporation system to introduce DNA into the adult brain of living honeybee and confirmed the expression of transfected genes by both RT-PCR and immunoblotting. One major advantage of this system is its ease and simplicity. In particular, the use of U-shaped plastic sheets greatly helped to reduce the time required for restricting the movement, while allowing for the release of honeybee in the tube. This system could be utilized for easy and rapid inspection of several technical aspects; for example, confirmation of the activity of inducible promoters such as a tetracycline-responsive element [28–30], heat shock promoter, or steroid-inducible promoters; to check the activity of transposable elements such as piggyBac [31–33], Hermes [34–36], Marinar [37,38]; or functionality of marker/detector proteins like fluorescent proteins [39,40] or cameleons [41–43].

With this system, gene delivery region is restricted and can be regulated by changing the anode position. Further confinement can be achieved using promoters or enhancers to mediate the specific expression. This localized delivery is beneficial to characterize gene function by comparing transfected cells with non-transfected cells within the same tissue. Thus, the present study clearly demonstrates that this method will be very useful for the analysis of gene function in the adult honeybee brain.

The amount of molecular data of the honeybee has begun to expand rapidly and the genome project will soon be completed. Application of this in vivo electroporation method to those genes will have a great potential to accelerate analyses of their functions and hopefully also their relation with complex honeybee behavior. Furthermore, this system will facilitate analyses of their interrelation and identification of their *cis*-regulatory elements, which will clarify the regulatory mechanisms of gene networks in the nervous system. Recently, gene silencing by RNA interference was reported in the honeybee [44–46]. The present system offers gain of function analysis by ectopic or over-expression and complements the loss of function analysis. The present system can also be used for the loss of function analysis by introducing dominant negative gene forms.

The present system might be a useful method of gene transfer into the adult brain of living honeybee and contribute to a better understanding of the complex social behaviors of honeybee at the molecular level.

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References

[1] M.L. Winston, The Biology of the Honeybee, Harvard University Press, Cambridge, MA, 1987.
[2] K.v. Frisch, Honeybees: Do they use direction and distance information provided by their dancers?, Science 158 (1967) 1072–1077.
[3] T. Fujiyuki, H. Takeuchi, M. Ono, S. Ohka, T. Sasaki, A. Nomoto, T. Kubo, Novel insect picorna-like virus identified in the brains of aggressive worker honeybees, J. Virol. 78 (2004) 1093–1100.
[4] H. Takeuchi, A. Yasuda, Y. Yasuda-Kamatani, T. Kubo, T. Nakajima, Identification of a tachykinin-related neuropeptide from the honeybee brain using direct MALDI-TOF MS and its gene expression in worker, queen and drone heads, Insect. Mol. Biol. 12 (2003) 291–298.
[5] C.W. Whitfield, A.M. Cziko, G.E. Robinson, Gene expression profiles in the brain predict behavior in individual honey bees, Science 302 (2003) 296–299.
[6] Y. Ben-Shahar, H.T. Leung, W.L. Pak, M.B. Sokolowski, G.E. Robinson, cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor, J. Exp. Biol. 206 (2003) 2507–2515.
[7] G. Bloch, S.M. Solomon, G.E. Robinson, S.E. Fahrbach, Patterns of PERIOD and pigment-dispersing hormone immunoreactivity in the brain of the European honeybee (*Apis mellifera*): age- and time-related plasticity, J. Comp. Neurol. 464 (2003) 269–284.
[8] H. Takeuchi, T. Fujiyuki, K. Shirai, Y. Matsuo, A. Kamikouchi, Y. Fujinawa, A. Kato, A. Tsujimoto, T. Kubo, Identification of genes expressed preferentially in the honeybee mushroom bodies by combination of differential display and cDNA microarray, FEBS Lett. 513 (2002) 230–234.

[9] M. Sawata, D. Yoshino, H. Takeuchi, A. Kamikouchi, K. Ohashi, T. Kubo, Identification and punctate nuclear localization of a novel noncoding RNA, Ks-1, from the honeybee brain, RNA 8 (2002) 772–785.
[10] H. Takeuchi, E. Kage, M. Sawata, A. Kamikouchi, K. Ohashi, M. Ohara, T. Fujiyuki, T. Kunieda, K. Sekimizu, S. Natori, T. Kubo, Identification of a novel gene, Mblk-1, that encodes a putative transcription factor expressed preferentially in the large-type Kenyon cells of the honeybee brain, Insect. Mol. Biol. 10 (2001) 487–494.
[11] A. Kamikouchi, H. Takeuchi, M. Sawata, S. Natori, T. Kubo, Concentrated expression of Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C in the mushroom bodies of the brain of the honeybee *Apis mellifera* L, J. Comp. Neurol. 417 (2000) 501–510.
[12] A. Kamikouchi, H. Takeuchi, M. Sawata, K. Ohashi, S. Natori, T. Kubo, Preferential expression of the gene for a putative inositol 1,4,5-trisphosphate receptor homologue in the mushroom bodies of the brain of the worker honeybee *Apis mellifera* L, Biochem. Biophys. Res. Commun. 242 (1998) 181–186.
[13] J.M. Park, T. Kunieda, T. Kubo, The activity of Mblk-1, a mushroom body-selective transcription factor from the honeybee, is modulated by the ras/MAPK pathway, J. Biol. Chem. 278 (2003) 18689–18694.
[14] J.M. Park, T. Kunieda, H. Takeuchi, T. Kubo, DNA-binding properties of Mblk-1, a putative transcription factor from the honeybee, Biochem. Biophys. Res. Commun. 291 (2002) 23–28.
[15] E. Guzman-Novoa, G.J. Hunt, J.L. Uribe, C. Smith, M.E. Arechavaleta-Velasco, Confirmation of QTL effects and evidence of genetic dominance of honeybee defensive behavior: results of colony and individual behavioral assays, Behav. Genet. 32 (2002) 95–102.
[16] S.B. Chandra, G.J. Hunt, S. Cobey, B.H. Smith, Quantitative trait loci associated with reversal learning and latent inhibition in honeybees (*Apis mellifera*), Behav. Genet. 31 (2001) 275–285.
[17] R.E. Page Jr., M.K. Fondrk, G.J. Hunt, E. Guzman-Novoa, M.A. Humphries, K. Nguyen, A.S. Greene, Genetic dissection of honeybee (*Apis mellifera* L.) foraging behavior, J. Hered. 91 (2000) 474–479.
[18] G.J. Hunt, A.M. Collins, R. Rivera, R.E. Page Jr., E. Guzman-Novoa, Quantitative trait loci influencing honeybee alarm pheromone levels, J. Hered. 90 (1999) 585–589.
[19] K.O. Robinson, H.J. Ferguson, S. Cobey, H. Vaessin, B.H. Smith, Sperm-mediated transformation of the honey bee, *Apis mellifera*, Insect. Mol. Biol. 9 (2000) 625–634.
[20] K. Moto, S.E. Abdel Salam, S. Sakurai, M. Iwami, Gene transfer into insect brain and cell-specific expression of bombyxin gene, Dev. Genes Evol. 209 (1999) 447–450.
[21] T. Saito, N. Nakatsuji, Efficient gene transfer into the embryonic mouse brain using in vivo electroporation, Dev. Biol. 240 (2001) 237–246.
[22] T. Matsuda, C.L. Cepko, Electroporation and RNA interference in the rodent retina in vivo and in vitro, Proc. Natl. Acad. Sci. USA 101 (2004) 16–22.
[23] A. Sano, T. Tagami, T. Harumi, Y. Matsubara, M. Naito, Introduction of exogenous DNA into gonads of chick embryos by lipofection and electroporation of stage X blastoderms in vivo, Br. Poult. Sci. 44 (2003) 36–39.
[24] K. Haas, K. Jensen, W.C. Sin, L. Foa, H.T. Cline, Targeted electroporation in *Xenopus* tadpoles in vivo—from single cells to the entire brain, Differentiation 70 (2002) 148–154.
[25] T. Muramatsu, S. Arakawa, K. Fukazawa, Y. Fujiwara, T. Yoshida, R. Sasaki, S. Masuda, H.M. Park, In vivo gene electroporation in skeletal muscle with special reference to the duration of gene expression, Int. J. Mol. Med. 7 (2001) 37–42.
[26] W.J. Lee, S.H. Kim, Y.S. Kim, S.J. Han, K.S. Park, J.H. Ryu, M.W. Hur, K.Y. Choi, Inhibition of mitogen-activated protein kinase by a *Drosophila* dual-specific phosphatase, Biochem. J. 349 (Pt. 3) (2000) 821–828.
[27] J.H. Sinclair, The human cytomegalovirus immediate early gene promoter is a strong promoter in cultured *Drosophila melanogaster* cells, Nucleic Acids Res. 15 (1987) 2392.
[28] J.K. Koponen, H. Kankkonen, J. Kannasto, T. Wirth, W. Hillen, H. Bujard, S. Yla-Herttuala, Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2S-M2 shows a tight control of gene expression in vitro and in vivo, Gene Ther. 10 (2003) 459–466.
[29] B. Bello, D. Resendez-Perez, W.J. Gehring, Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system, Development 125 (1998) 2193–2202.
[30] M. Gossen, H. Bujard, Tight control of gene expression in mammalian cells by tetracycline-responsive promoters, Proc. Natl. Acad. Sci. USA 89 (1992) 5547–5551.
[31] T. Tamura, C. Thibert, C. Royer, T. Kanda, E. Abraham, M. Kamba, N. Komoto, J.L. Thomas, B. Mauchamp, G. Chavancy, P. Shirk, M. Fraser, J.C. Prudhomme, P. Couble, T. Toshiki, T. Chantal, R. Corinne, K. Toshio, A. Eappen, K. Mari, K. Natuo, T. Jean-Luc, M. Bernard, C. Gerard, S. Paul, F. Malcolm, P. Jean-Claude, C. Pierre, Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector, Nat. Biotechnol. 18 (2000) 81–84.
[32] A.M. Handler, S.D. McCombs, M.J. Fraser, S.H. Saul, The lepidopteran transposon vector, piggyBac, mediates germ-line transformation in the Mediterranean fruit fly, Proc. Natl. Acad. Sci. USA 95 (1998) 7520–7525.
[33] L.C. Cary, M. Goebel, B.G. Corsaro, H.G. Wang, E. Rosen, M.J. Fraser, Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses, Virology 172 (1989) 156–169.
[34] C. Horn, E.A. Wimmer, A versatile vector set for animal transgenesis, Dev. Genes Evol. 210 (2000) 630–637.
[35] D.A. O'Brochta, W.D. Warren, K.J. Saville, P.W. Atkinson, Hermes, a functional non-Drosophilid insect gene vector from *Musca domestica*, Genetics 142 (1996) 907–914.
[36] W.D. Warren, P.W. Atkinson, D.A. O'Brochta, The Hermes transposable element from the house fly, *Musca domestica*, is a short inverted repeat-type element of the hobo, Ac, and Tam3 (hAT) element family, Genet. Res. 64 (1994) 87–97.
[37] M. Medhora, K. Maruyama, D.L. Hartl, Molecular and functional analysis of the mariner mutator element Mos1 in *Drosophila*, Genetics 128 (1991) 311–318.
[38] G.J. Bryan, D.L. Hartl, Maternally inherited transposon excision in *Drosophila simulans*, Science 240 (1988) 215–217.
[39] H. Mizuno, A. Sawano, P. Eli, H. Hama, A. Miyawaki, Red fluorescent protein from *Discosoma* as a fusion tag and a partner for fluorescence resonance energy transfer, Biochemistry 40 (2001) 2502–2510.
[40] D.C. Prasher, V.K. Eckenrode, W.W. Ward, F.G. Prendergast, M.J. Cormier, Primary structure of the *Aequorea victoria* green-fluorescent protein, Gene 111 (1992) 229–233.
[41] J. Nakai, M. Ohkura, K. Imoto, A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein, Nat. Biotechnol. 19 (2001) 137–141.
[42] G.S. Baird, D.A. Zacharias, R.Y. Tsien, Circular permutation and receptor insertion within green fluorescent proteins, Proc. Natl. Acad. Sci. USA 96 (1999) 11241–11246.
[43] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin, Nature 388 (1997) 882–887.
[44] M. Beye, S. Hartel, A. Hagen, M. Hasselmann, S.W. Omholt, Specific developmental gene silencing in the honey bee using a homeobox motif, Insect. Mol. Biol. 11 (2002) 527–532.

[45] G.V. Amdam, Z.L. Simoes, K.R. Guidugli, K. Norberg, S.W. Omholt, Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA, BMC Biotechnol. 3 (2003) 1.

[46] T. Farooqui, K. Robinson, H. Vaessin, B.H. Smith, Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee, J. Neurosci. 23 (2003) 5370–5380.