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Sperm-mediated Gene Transfer in the Chinese Honeybee, *Apis cerana cerana* (Hymenoptera: Apidae)

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Abstract Transgenic *Apis cerana cerana* were produced by sperm-mediated gene transfer (SMGT). In the experiment, the foreign DNA was linearized and introduced with sperm during the instrumental insemination of virgin queen. The descendants of the experimental colonies were analyzed. In the green fluorescent-positive offspring, green fluorescence was observed for 1- to 2-day-old larvae, the predicted fragment was isolated by means of PCR amplification of genomic DNA and the expression of transferred genes was confirmed at transcriptional level by reverse transcription-polymerase chain reaction. These results showed that the exogenous gene could be integrated in a fraction of the germ line cells of the queen *Apis cerana cerana* and transmitted to offspring by SMGT.

Key words *Apis cerana cerana*, Sperm, Gene transfer, EGFP

Introduction

Genetic transformation is crucial for understanding the genetic basis of biology. In the past decades, incredible amount of basic knowledge about development, genetics and biochemistry has been accumulated by transgenic analysis of model organisms, such as mice and rice. At the same time, more and more genetic modified organisms have been created for various demands of human being (Amdam *et al.*, 2005). In the field of arthropod, genetic transformation systems have been established in many variety of insects (Rubin *et al.*, 1982; Handler, 2001; Robinson *et al.*, 2004).

The honeybee is a social insect and an established model in systems theory, behavioral ecology, neurobiology and aging (Amdam *et al.*, 2005; Robinson *et al.*, 2005). The honeybee is also a commercially important insect. Genetic manipulation of honeybee genome may herald strategies for controlling disease and increasing productivity. Thus, development of transformation technology for the species would be most valuable.

Currently appropriate sets of transgenic techniques have not yet been fully developed for honeybee (Kunieda and Kubo, 2004). Some methods are urgently developed for the introduction of exogenous genes into honeybee. Generally, Transgenic insects are produced primarily by microinjecting exogenous DNA into embryos. However, in eusocial insects like the honeybee, the embryos and larvae are cared for by nurse worker honeybees. Once injury or abnormality is detected in the larvae, they will be destroyed or removed from the colony and die thereafter. For the reason, it is difficult to rear manipulated honeybee embryos into adults if normal gene manipulation methods like microinjection are used. Therefore it is critical for us to pursue a successful gene manipulation technique that enables direct gene transfer on a natural colony environment. We focus on sperm-mediated method. The concept of exogenous DNA transfer using sperm as mediated vector was first demonstrated by Brackett *et al.* (1971) and rediscovered by Lavitrano *et al.* (1989). Sperm-mediated gene transfer (SMGT) is based on the ability of sperm cells to bind and internalize exogenous DNA and to transfer DNA into eggs at fertilization (Milne CP *et al.*, 1989; Atkinson *et al.*, 1991; Maione *et al.*, 1998). SMGT is an advantageous alternative method in insects in which microinjection is difficult or not possible, for example, for eggs having rigid chorion (Shamila and Mathavan, 2000; Zuo *et al.*, 2006) and for eggs being reintroduced uneasily into the colony (Robinson *et al.*, 2000; Kunieda and Kubo, 2004).

As for honeybee sperm-mediated gene transfer, first, the drone sperm are mixed with foreign DNA, followed by transferring the sperm into the oviduct of the virgin honeybee queen with the technique of artificial instrumental insemination. Then the sperm

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would migrate into the spermatheca where sperm are stored for months to years. By the use of SMGT, Robinson *et al.* (2000) successfully introduced plasmid DNA into the honeybee *Apis mellifera*. To date there has no report for exogenous gene transfer on *Apis cerana* published yet. In view of the fact, we selected *Apis cerana cerana* for experimental object. *Apis cerana cerana* is specially distributed in most parts of China, especially in the mountains areas of south China, where it is adapted to the geographic and climate condition. *Apis cerana cerana* is one of the main subspecies of *Apis cerana* with higher vitality and resistance to adversity as well as better collection ability to dispersed flowers (Yang, 2005). In the study we use enhanced green fluorescent protein (EGFP) as a reporter gene, explore and analyze the feasibility of SMGT technique in *Apis cerana cerana*. Consequently, we successfully introduced the EGFP DNA into the germ line cells of the queen *Apis cerana cerana* and produced a green fluorescence-positive colony. The research is advantageous to provide theoretical and basic data for honeybee genetic engineering breeding and for using honeybee as a biological reactor of production goal protein.

Materials and Methods

Bees

Apis cerana cerana colonies bred at the experimental apiary of honeybee research institute of Jiangxi agricultural university of China. Virgin queens used in the inseminations were reared in queenless colonies and maintained in queen cages inside those queenless colonies until insemination. Mature drones were available from a large number of colonies.

Reagent

The plasmid pEGFP-N1 and *E. coli* DH5α came from Model Organism Research Center in Quanzhou Normal University. RevertAid^{TM} first stand cDNA synthesis kit, Fermentas Taq DNA polymerase, Proteinase K, *Eco0109I* enzyme and Gene Ruler 100bp Marker were purchased from Jingmei Biotech Co. Ltd. TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 was purchased from TaKaRa Biotechnology (Dalian). CASpure Plasmid Isolation kit was purchased from CASarray Co. Ltd. Primers was synthesized from Shanghai GeneCore BioTechnologies Co. Ltd. Trizol and other biochemical reagents were purchased from Shanghai Sangon Biological Engineering Technology & Services.

Vector of Exogenous Gene

We used pEGFP-N1 as a plasmid vector of exogenous gene, which could express a EGFP variant under the control of the CMV promoter for florescence detection and was reported to work equally well in honeybee and fly (Sinclair, 1987; Robinson *et al.*, 2000; Kunieda and Kubo, 2004). The plasmid pEGFP-N1 was transformed into *E. coli* DH5α and amplified selectively in the Luria-Bertani culture medium containing 50 ug/ml Kanamycin. The plasmid DNA was isolated and purified via the CASpure Plasmid Isolation kit. The plasmid DNA was then digested with *Eco0109I* enzyme. Digested DNA was separated by electrophoresis. The linearized DNA was reclaimed, purified and resuspended in sterile saline (0.75%NaCl) via TaKaRa Agarose Gel DNA Purification Kit. The final concentrations were 262 ng/μl.

Foreign genes transfer

Firstly we collected sperm from mature drones artificially. Semen was collected from eight to fifteen drones for instrumental insemination of each virgin queen. Subsequently 5 μl of freshly collected semen was incubated with 3 μl linearized DNA plasmid for 15 min in a tube, during the time we gently mixed the solution for several times. Then the 8 μl solution was injected into the oviducts of a virgin honeybee queen via an artificial insemination instrument (Apiculture Science Institute of Jilin Province, P.R. China). In the control group, a virgin queen was inseminated with 5 μl of untreated fresh semen and 3 μl sterile saline (0.75% NaCl). Each queen was anaesthetized with CO_2 during the procedure. The whole manipulation was done at room temperature (23-28℃). After insemination, wings of queens were clipped, and we restored the queens in the original queen cages. After queens recovered from anesthesia, every queen was introduced into a queenless colony with several nurse worker bees in a wire cage. The quantity of bees in each queenless colony for the experimental group was the same as the control group. After the queens were accepted, those wire cages were discarded.

Fluorescence microscopy

EGFP fluorescence was monitored using a fluorescence inverted microscope (Axiovert 40; Carl Zeiss, Germany) equipped with a filter set (Excitation 450-490 nm, Emission 515-565 nm) suitable for the detection of green fluorescence protein. Larvae and pupae from the experimental or control group were collected, washed carefully with sterile water and placed on a microscope slide. Then, they were observed under the fluorescence microscope.

DNA extraction and PCR Amplification

Genomic DNA was isolated from the larvae and pupae according to Guo *et al.* (2005). For DNA PCR amplification, we designed the following two primers based on available sequences from EGFP: 5'-CACAAGTTC AGCGTGTCCG-3' for sense and 5'-AGTTCACCTT GATGCCGTTC-3' for antisense, which produced a 421- bp amplified fragment. PCR amplification conditions were 4 min at 94℃, followed by 35 cycles of 35 s at 94℃, 35 s at 54℃, 1 min at 72℃ and final extension for 10 min at 72℃.

RNA extraction and RT-PCR to confirm EGFP gene expression

Total RNA from collected larvae or pupae was extracted using Trizol reagent (Invitrogen) according to the protocol recommended by the supplier. Extracted RNA was reverse-transcribed using RevertAid^{TM} first stand cDNA synthesis kit (Fermentas).

The resulting cDNA served as the PCR template. PCR amplification was performed with primer specific for EGFP as described previously. The honeybee housekeeping gene *Actin* (XM_393368) was used as a control. Amplification of the *Actin* gene was performed using the following primer pair: 5'-TCCTGCTATGT ATGTCGC-3' for sense and 5'-AGTTGCCATTTCC TGTT-3' for antisense. Amplified DNA fragments was 301-bp. Amplification conditions were 3 min at 94℃, followed by 30 cycles of 30 s at 94℃, 30 s at 50℃, 35 s at 72℃ and final extension for 10 min at 72℃.

Results

State of inseminated queens

In the experimental group, ten virgin queens were inseminated with semen which had been mixed with linear DNA plasmids. Five of those queens produced eggs and larvae, but the age of larva or pupae on the same frame of brood was discontinuous. The longevities of all five queens were less than one year. Three of the remaining five queens in the experimental group died, the other two produced eggs and larvae on later 7-8 days, however, all the eggs and larvae of the two colonies developed for drones, so the two queens were rejected. In the control group, ten virgin queens were also inseminated, and eight of those produced normal eggs and larvae, the other two queens were rejected by worker honeybee.

Detection of the EGFP fluorescence in the honeybees

From the colonies of the control group, larvae were observed with some faint background autofluorescence (Fig. 1A and 1C.), but the signal was always limited to the gut. The results from four of five producing queens of the experimental group were alike. However, another colony of the experimental group was different, bright fluorescence was detected in other tissues of many larvae from it (Fig. 1B and 1D.). Strong green fluorescence was showed outside the guts of larvae of the colony which was called positive colony. During detectable procedure, we found that the special signal was observed on 1- to 2-day-old larvae in the positive colony, especially from 1 to 2 weeks after initial egg-laying, about two positive larvae were detected from a hundred of 1 to 2-day-old larvae. But it was not found on 3- or 4-day-old larvae or pupae of the same positive colony.

PCR Amplification

Samples were collected from the positive colony of the experimental group. DNA was isolated respectively from 1- to 2-day-old larvae, 3- to 4-day-old larvae and pupae. Mixed larvae were tested from a random colony of the control group. Examples from PCR amplification of EGFP fragment from isolated DNA of progeny are displayed in Fig. 2A. Seen from Fig. 2A, the presence of EGFP DNA in the green fluorescence-positive offspring was reconfirmed by the PCR analysis, while it failed from those of the control colony.

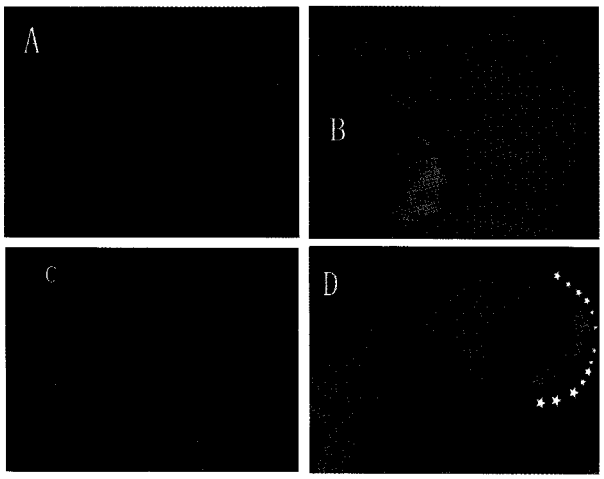

Fig. 1. Fluorescent imaging of larvae. Fluorescent signal were detected in larvae of the positive colony (B, D), and not in larvae of a control colony (A, C)

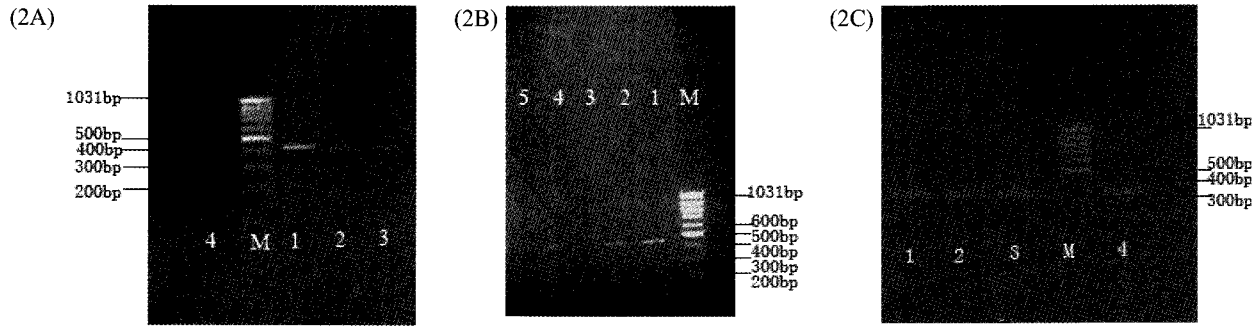

Fig. 2A. PCR Amplification of Genomic DNA. Note: M: Gene Ruler 100 bp Marker, Lanes 1,2 and 3 represent young larvae (1-2 days), older larvae (3-4 days) and pupae of the green fluorescence-positive colony, Lane 4: negative control.
Fig. 2B. RT-PCR analysis of EGFP Expression. Note: M: Gene Ruler 100 bp Marker; 1-4 samples from the green fluorescence-positive colony: Lane 1: 1- to 2-day-old larvae, Lane 2: 3- to 4-day-old larvae, Lane 3 and 4: pupae; Lane5: larvae of control.
Fig. 2C. *Actin* was amplified as a loading control. Note: M: Gene Ruler 100 bp marker; 1-3 samples from the green fluorescence-positive colony: Lane 1: 1- to 2-day-old larvae. Lane 2: 3- to 4-day-old larvae. Lane 3: pupae; Lane 4: larvae of control.

Detection of the EGFP mRNA in the honeybees

In order to confirm whether foreign EGFP was expressed, we examined the presence of the EGFP transcription in offspring by RT-PCR. Total RNA was extracted respectively from 1- to 2-day-old larvae, 3- to 4-day-old larvae and pupae of the positive colony and from 1- to 2-day-old larvae of the control colony. Evaluation by RT-PCR analysis showed EGFP expression in Fig. 2B. For the positive colony, 1- to 2-day-old larvae and 3- to 4-day-old larvae showed the signal in a reverse-transcriptase-dependent manner. RT-lane for 1- to 2-day-old larvae was the brightest, which suggested that the level of the EGFP expression was high in 1- to 2-day-old larvae. RT-lane for 3- to 4-day-old larvae was weaker. The signal was seldom seen for pupae like RT-lane3 of Fig. 2B, the faint band was occasionally found for pupae like RT-lane4 of Fig. 2B. Whereas no EGFP message was detected in any control larvae (RT-lane5). Amplification imaging of an *Actin* fragment was displayed in Fig. 2C. The map indicated that the *Actin* fragment could be amplified from every sample as a loading control.

Discussion

In the present study, green fluorescence was observed under a fluorescence microscope on larvae and we were able to detect the plasmid DNA fragment in the green fluorescence-positive progeny by means of PCR amplification. Furthermore, we confirmed the expression of transferred genes by RT-PCR analysis. These results show that foreign DNA can be introduced to the honeybee genome by mixing linearized plasmid DNA with semen used to instrumentally inseminate virgin queens. The approach takes advantage of the in vivo colony rearing conditions for transgenic progeny, which is important for success. The technique may be used to manipulate the genome of honeybee *Apis cerana ceran* and might contribute to a better understanding of the complex social behaviors of honeybee at the molecular level. The development of SMGT for honeybee will increase the ability of entomologists to find, isolate and analyze genes.

SMGT uses spermatozoa as the vector for introducing exogenous genes into the zygote. The method is simple, convenient and inexpensive. It overcomes some disadvantages of microinjection for producing transgenic insects. At present it is reported that the technique has been used successfully to transform foreign gene on many insects such as *Drosophila* (Davis *et al.*, 1995), *Bombyx mori* (Shamila and Mathavan, 2000; Zuo *et al.*, 2006), mosquito (Jasinskiene *et al.*,1998) and so on. For honeybee, queens mate with several to dozens of drones and store viable sperm for months to years in the spermatheca. During fertilization, one to several sperm enter the egg through the micropyle (Kerkut and Gilbert, 1985; Robinson *et al.*, 2000). While DNA sticks tightly to sperm, it is likely that the DNA is carried as the sperm enter the egg. Subsequently the DNA may be capable of inheriting to offspring of queens.

In the experiment, we found that PCR-positive in larvae or pupae of the positive colony was tested only within 4 weeks after the queen initially laid eggs. After that, we rarely found positive results. Especially during the fluorescence detection of progeny, green fluorescence was observed easily on 1- to 2-day-old larvae only within 1 to 2 weeks, afterwards few larvae were tested with green fluorescence. It is possible that exogenous DNA would naturally degrade overtime as the sperm is stored in the queen's spermatheca. Moreover

for 3- to 4-day-old larvae or pupae, no green fluorescence was observed under the fluorescence microscope while RT-PCR analysis demonstrated gene transfer on them. This is likely due to the detection of fluorescence, which requires a significant amount of EGFP molecules and RT-PCR has much higher sensitivity. This also may be partly due to interference of rich protein and fat body of old larvae or pupae on observation.

As a reporter gene, the enhanced green fluorescent protein (EGFP) can be directly visualized in living organisms. EGFP transformations have been successfully demonstrated in a variety of insects by SMGT, for example, *Apis mellifera* (Robinson *et al.*,2000), *Drosophila* (Davis *et al.*, 1995), *Bombyx mori* (Zuo *et al.*, 2006). EGFP has been used as a general reporter gene. But it is worth our attention that EGFP may be partially detrimental to the survivorship of progeny. In the study, for example, we observed that colonies of the experimental group produced 'spotty' brood patterns, which were an intermixing of different ages of progeny on a frame of brood. Under normal conditions queens lay eggs in clusters on a comb so that the brood in any given patch will be of a similar age. This phenomenon is similar as *Apis mellifera* (Robinson *et al.*, 2000) indicates that the larvae and/or eggs either have died or have been removed by worker bees. Furthermore, the longevity of inseminated queens with treated sperm was shorter than that of inseminated queens with untreated sperm, and the successful ratio of inseminated honeybee queens with DNA-treated sperm also are by far lower than that with untreated sperm. It is very possible for all of those that EGFP is influential to sperm, queens or eggs. Another shortcoming for fluorescence protein marker is that the things from insect gut are easily mistaken for *egfp* expression. In fact those things are usual insect foods. But EGFP may still be usable for honeybee transgenic techniques as a marker in spite of its potential detrimental effect. For example, by using plasmid vectors containing the EGFP gene as a marker, Robinson *et al.* (2000) made sperm-mediated transformation of *Apis mellifera*, Kunieda and Kubo (2004) conducted gene transfer into the adult honeybee brain of *Apis mellifera* by electroporation. Therefore EGFP gene is also chosen wisely for *Apis cerana cerana* as a marker in transgenic study.

In this study, we did not test whether DNA dose and sperm vigor affected gene transfer efficiency in honeybee *Apis cerana cerana* when SMGT was performed. Some questions remain unexplained, one is why the other four colonies within the experimental groups did not produce positive offspring but produced normal worker larvae, the other is when, for how long and in what quantity exogenous DNA must be added to sperm in order that the exogenous DNA is taken up by the majority of sperm. We do not have a clear answer how DNA is integrated in the germ line cells by. Thus, it is evident that we need improving our understanding of the process of sperm-mediated DNA transfer to eggs and enhance transgenic efficiency in honeybee.

In conclusion, SMGT is an economic and practical method by which the exogenous EGFP DNA was successfully transferred into *Apis cerana cerana*. The study offers the prospect of a new approach to understand gene function of *Apis cerana cerana*.

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Literature Cited

Amdam, G.V. and R.E. Page. 2005. Intergenerational transfers may have decoupled physiological and chronological age in a eusocial insect. Ageing Research Reviews. 4: 398-408.

Atkinson, P.W., E.R. Hines, S. Beaton, K.I. Matthaei, K.C. Reed and M.P. Bradley. 1991. Associations of exogenous DNA with cattle and insect spermatozoa in vitro. Molecular Reproduction and Development. 29: 1-5.

Brackett, B.G., W. Boranska, W. Sawicki and H. Koprowski. 1971. Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. Proc Natl Acad Sci USA. 68: 353-357.

Davis, I., C.H. Girdham and P.H. O'Farrell. 1995. A nuclear EGFP that marks nuclei in living *Drosophila* embryos: maternal supply overcomes a delay in the appearance of zygotic fluorescence. Developmental Biology. 170: 726-729.

Guo, D.S., L.X. Sun and Q.L. Zhang. 2005. Simple and Rapid Method for Extracting Nucleic Acid of Honeybee. Journal of Quanzhou Normal University. 23: 85-88.

Handler, A.M. 2001. A current perspective on insect gene transformation. Insect Biochemistry and Molecular Biology. 31: 111-128.

Jasinskiene, N., C.J. Coates and M.Q. Benedict. 1998. Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. PNAS: Proc Natl Acad Sci USA. 95: 3743-3747.

Kerkut, G.A. and L.I. Gilbert. 1985. Comprehensive Insect Physiology, Biochemistry, and Pharmacology. New York: Pergamon Press. 11: 647-712.

Kunieda, T. and T. Kubo. 2004. In vivo gene transfer into the adult honeybee brain by using electroporation. Biochemical and Biophysical Research Communications. 318: 25-31.

Lavitrano, M., A. Camaioni, V.M. Fazio, S. Dolci, M.G. Farace and C. Spadafora. 1989. Sperm cells as vectors for introduction of foreign DNA into eggs: genetic transformation of mice. Cell. 57: 717-723.

Maione, B., M. Lavitrano, C. Spadafora and A.A. Kiessling. 1998. Sperm-mediated gene transfer in mice. Molecular Reproduction and Development. 50: 406-409.

Milne, C.P., F.A. Elschen, J.E. Collis and T.L. Jensen. 1989. Preliminary evidence for honeybee sperm mediated DNA transfer. International Symposium on Molecular Insect Science. 120-125.

Robinson, A.S., G. Franz and P.W. Atkinson. 2004. Insect transgenesis and its potential role in agriculture and human health. Insect Biochemistry and Molecular Biology. 34: 113-120.

Robinson, G.E., C.M. Grozinger and C.W. Whitfield. 2005. Sociogenomics: Social life in molecular terms. Nature Reviews: Genetics. 6: 257-270.

Robinson, K.O., H.J. Ferguson and S. Cobey. 2000. Sperm-mediated transformation of the honey bee, *Apis mellifera*. Insect Molecular Biology. 9: 625-634.

Rubin, G.M. and A.C. Spradling. 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science. 218: 348-353.

Shamila, Y. and S. Mathavan. 2000. Sperm/DNA Interaction: DNA Binding Proteins in Sperm Cell of Silkworm *Bombyx mori*. Molecular Reproduction and Development. 56: 289-291.

Sinclair, J.H. 1987. The human cytomegalovirus immediate early gene promoter is a strong promoter in cultured *Drosophila melanogaster* cells. Nucleic acids research. 15: 2392.

Yang, G.H. 2005. Harm of introducing the western honeybee *Apis mellifera* L. to the Chinese honeybee *Apis cerana* F. and its ecological impact. Acta Entomologica Sinica. 3: 401-406.

Zuo, Z.H., C.X. Wu and M.Y. Gui. 2006. Sperm-mediated Repoter Gene *gfp* and *egfp* Transfer in the Silkworm. Journal of Xiamen University. 45: 580-584.