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Abstract

The production of an Fab fragment of the catalytic antibody 6D9 in stably transformed lepidopteran insect cells was investigated. On the basis of an expression vector that utilizes the *Bombyx mori* cytoplasmic actin promoter, from which foreign gene expression is stimulated with the *B. mori* nucleopolyhedrovirus (BmNPV) IE-1 transactivator and the BmNPV HR3 enhancer, two plasmid vectors were constructed which contain either a neomycin or a blasticidin resistance gene for use as a selectable marker. The genes encoding the heavy chain (Hc; Fd fragment) and light chain (Lc) of the 6D9 Fab fragment were inserted separately into the expression vectors. After cotransfection with the resulting plasmids to introduce the Hc and Lc genes and the two different antibiotic resistance genes, *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells were cultured in the presence of G418 and blasticidin. Colonies of cells resistant to the antibiotics were obtained around 2 weeks after cotransfection. Western blotting and enzyme-linked immunosorbent assay (ELISA) of the cell culture supernatant suggested that the resistant cells stably secrete an Fab fragment which retains an antigen-binding activity. High yields of over 300 μg/ml of Fab fragment were achieved in simple batch shake-flask culture of transfected insect cells. These results indicate that recombinant insect cells may offer a novel approach for efficient production of antibody molecules.

Keywords: Insect cell culture; Recombinant protein production; Cell transformation; Antibody; Fab fragment; High Five cells

1. Introduction

The baculovirus–insect cell system has been extensively used for the production of a wide variety of biologically active recombinant proteins for use as bioinsecticides, vaccines, and diagnostic and therapeutic proteins [1–3]. In the typical baculovirus–insect cell system, a recombinant nucleopolyhedrovirus (NPV) is generated in which the polyhedrin gene is replaced with the foreign gene of interest. On infection with the recombinant baculovirus, insect cells in culture often express extremely large quantities of foreign proteins through post-translational processing and modifications of higher eukaryotes under the control of the very strong polyhedrin promoter during the very late stage of infection. The major disadvantage of the system, however, is that continuous protein production is virtually impossible because of the lytic nature of the viral infection process. Transient expression in batch culture is therefore commonly used for recombinant protein production by the baculovirus–insect cell system. The lysis of host insect cells following baculovirus infection and the resulting release of intracellular proteins may also result in the need for complicated downstream processing and purification of products and in protein degradations by proteases [4, 5].

Stably transformed insect cell lines have emerged as attractive alternative platforms for the continuous production of complex recombinant proteins [6–8]. In the stably transformed insect cell system, host insect cells are transfected with a plasmid vector into which the foreign gene of interest is cloned under the control of an appropriate promoter. If the introduced vector integrates into the chromosomal DNA of the host cell, the foreign protein can be synthesized continuously [9–12]. In order to identify a small fraction of the stably transformed cells, antibiotic resistance genes are used as

selectable markers and are cotransfected together with the heterologous gene of interest. This system is especially useful for the production of secreted complex proteins as the protein synthesis and processing machinery of the host insect cell is not compromised by baculoviral infection. Under this system, the choice of a promoter to drive the heterologous gene expression is important as the use of weak promoters results in low recombinant protein yields. While there have been reports announcing the generation of stably transformed lepidopteran cells expressing foreign proteins [9, 10], the protein yields have often been considerably lower than those obtained in the baculovirus–insect cell system [9, 13].

Recently, the expression vector pIE1/153A has been developed for continuous high-level expression of secreted proteins by transformed lepidopteran insect cells [11, 14, 15]. pIE1/153A (*ca.* 11,000 bp) utilizes the *Bombyx mori* cytoplasmic actin promoter, from which foreign gene expression is stumulated with the *B. mori* NPV (BmNPV) IE-1 transactivator and the BmNPV HR3 enhancer. The use of the IE-1 transactivator and the HR3 enhancer has been reported to result in an increase of over 1000-fold in the stimulation of foreign gene expression through the actin promoter [16]. In this system, lepidopteran insect cells are first cotransfected with the expression vector containing the heterologous gene and a plasmid carrying a selectable marker, after which antibiotic selection and isolation of highly productive clones is carried out [11, 14, 15]. Stable cell lines expressing secreted proteins including tissue plasminogen activator and secreted alkaline phosphatase have shown higher expression levels than the baculovirus–insect cell system [11, 17, 18].

In the present study, the production of an Fab fragment of the catalytic antibody 6D9 [19] in stably transformed lepidopteran insect cells was investigated. When the above-

mentioned expression vector utilizing the BmNPV IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* actin promoter is employed for the expression of an antibody Fab fragment, host insect cells need to be cotransfected with three plasmid vectors in total: two sets of expression vectors that separately contain the genes encoding the heavy chain (Hc; Fd fragment) and light chain (Lc) of the Fab fragment; and a plasmid carrying a selectable marker. Cotransfection with three plasmid vectors inevitably results in low efficiency in obtaining stably transformed cells. Taking as our basis the expression vector utilizing the IE-1 transactivator, the HR3 enhancer, and the actin promoter, we constructed two plasmid vectors that contain either a neomycin or a blasticidin resistance gene for use as a selectable marker. After transfection with these plasmid vectors, into which the Hc and Lc genes of the 6D9 Fab fragment were separately inserted, *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells secreting a high concentration of Fab fragment were efficiently generated by incubation in the presence of G418 and blasticidin. High yields of over 300 μg/ml of Fab fragment were produced in shake-flask culture of the recombinant insect cells.

2. Materials and methods

2.1. Insect cells and media

The Sf9 and *T. ni* BTI-TN-5B1-4 (High Five) insect cells used in the present study were obtained from Invitrogen (Carlsbad, CA, USA). The Sf9 cells were derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda*, while the High

Five cells originated from the ovarian cells of the cabbage looper, *T. ni*. Cell density was determined by microscopically counting the number of cells with a Bürker-Türk hemocytometer, while cell viability was judged by trypan blue dye exclusion [20]. The cells were maintained at 27°C in T-flasks in a non-humidified incubator. The culture medium used for routine maintenance of Sf9 cells was TNM-FH, consisting of 51.19 g/l TNM-FH (Sigma Chemical, St. Louis, MO, USA) and 0.35 g/l $NaHCO_3$, with addition of 10 mg/l gentamicin sulfate (Invitrogen) and 1 g/l Pluronic F-68 [block co-polymer glycol of poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene), Sigma], supplemented with 10% fetal bovine serum (FBS) (BioWest, Nuaillé, France) [20]. The serum-free medium EX-CELL 420 (SAFC Biosciences, Lenexa, KS, USA) was used when Sf9 cells were transfected with plasmid DNA. For High Five cells, the serum-free medium Express Five (Invitrogen) supplemented with 0.24 g/l L-glutamine and 10 mg/l gentamicin was used throughout.

2.2. Plasmid construction for transient expression

The catalytic antibody 6D9, which was generated by immunization of mice with a transition state analog, catalyzes the hydrolysis of a non-bioactive chloramphenicol monoester derivative to produce chloramphenicol [19, 21]. For the transient expression of the Fab fragment of this antibody, plasmids were constructed by inserting the DNA fragment encoding the *Drosophila* BiP secretion signal peptide sequence and the Hc (Fd fragment) or Lc gene of the Fab fragment into pXINSECT-DEST38 (12,419 bp; Invitrogen) that contains the BmNPV IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* cytoplasmic actin promoter and pIB/V5-His (3521 bp;

Invitrogen) that contains the OpIE2 promoter from *Orgyia pseudotsugata* NPV.

The DNA encoding the Hc gene of the Fab fragment of 6D9 was amplified from the plasmid pARA7-6D9Fab [19] by polymerase chain reaction (PCR) using the forward primer 1 (Table 1) containing the *Drosophila* BiP secretion signal peptide sequence and the reverse primer 2. The amplified fragment was digested with *Aor*51H I and *Xba* I and ligated into the *Eco*47 III (*Aor*51H I)-*Xba* I site of the pXINSECT-DEST38 vector. The resultant plasmid was designated as pXINSECT-6D9Hc. The DNA fragment encoding the Lc gene of the Fab fragment of 6D9 was amplified from the plasmid pARA7-6D9Fab using the forward primer 3 including the *Drosophila* BiP signal sequence and the reverse primer 4. The PCR product was digested with *Bam*H I and *Xba* I and cloned into pXINSECT-DEST38 with the *Bam*H I-*Xba* I site. The resulting plasmid was designated as pXINSECT-6D9Lc.

Table 1 ➔

The DNA fragment encoding the *Drosophila* BiP signal sequence and the Hc gene of the 6D9 Fab fragment was amplified from the plasmid pXINSECT-6D9Hc by PCR using the forward primer 5 and the reverse primer 2. The amplified fragment was digested with *Kpn* I and *Xba* I and ligated into the *Kpn* I-*Xba* I site of the plasmid pIB/V5-His. The resultant plasmid was designated as pIB-6D9Hc. The plasmid pXINSECT-6D9Lc was digested with *Bam*H I and *Xba* I and the DNA fragment encoding the *Drosophila* BiP signal sequence and the Lc gene of the 6D9 Fab fragment was ligated into the plasmid pIB/V5-His with the *Bam*H I-*Xba* I site. The resulting plasmid was designated as pIB-6D9Lc.

2.3. Plasmid construction for stable transformation

For the stable expression of the Fab fragment of 6D9, two plasmid vectors were constructed which contain either a blasticidin or a neomycin resistance gene for use as a selectable marker. In the first (Fig. 1a), after digestion of pIB/V5-His with *Bsp*H I and *Hin*d III, the protruding ends of the DNA fragment containing the blasticidin resistance gene were converted to blunt ends using a DNA-blunting kit (Takara Bio, Otsu, Japan) and the resulting blunt-ended DNA was self-ligated. The resultant plasmid and pXINSECT-6D9Lc were digested with *Kpn* I and *Bam*H I and the DNA fragment encoding the BmNPV IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* cytoplasmic actin promoter was inserted into the former plasmid with the *Kpn* I-*Bam*H I site. The resultant plasmid was designated as pIHAbla (9623 bp). pIHAbla and pXINSECT-6D9Hc were digested with *Aor*51H I and *Xba* I and the DNA fragment encoding the *Drosophila* BiP signal sequence and the Hc gene of the Fab fragment of 6D9 were ligated into the site *Aor*51H I-*Xba* I of pIHAbla. The resulting plasmid was designated as pIHAbla-6D9Hc. pIHAbla and pXINSECT-6D9Lc were digested with *Bam*H I and *Xba* I and the DNA fragment encoding the *Drosophila* BiP signal sequence and the Lc gene of the 6D9 Fab fragment were ligated into pIHAbla with the *Bam*H I-*Xba* I site. The resultant plasmid was designated as pIHAbla-6D9Lc.

Fig. 1 ➔

In the other vector (Fig. 1b), the DNA fragment encoding the neomycin resistance gene was amplified from the plasmid pBmA:neo (Invitrogen) by PCR using the forward primer 6 and the reverse primer 7 (Table 1). The PCR product was digested with *Nco* I and *Sma* I to obtain the *Nco* I-*Nco* I fragment (*ca.* 560 bp) and the *Nco* I-*Sma* I fragment (*ca.* 240 bp). pIHAbla was digested with *Nco* I and *Sma* I to remove the blasticidin resistance gene. The resulting DNA fragment (*ca.* 9100 bp) and the *Nco* I-*Sma* I fragment were ligated with the *Nco* I-*Sma* I site. The resultant plasmid was digested

with *Nco* I and ligated with the *Nco* I-*Nco* I fragment, resulting in the plasmid pIHAneo (10,022 bp). pIHAneo and pXINSECT-6D9Hc were digested with *Aor*51H I and *Xba* I and the DNA fragment encoding the *Drosophila* BiP signal sequence and the Hc gene of the 6D9 Fab fragment were ligated into the *Aor*51H I-*Xba* I site of pIHAneo. The resultant plasmid was designated as pIHAneo-6D9Hc. pIHAneo and pXINSECT-6D9Lc were digested with *Bam*H I and *Xba* I and the DNA fragment encoding the *Drosophila* BiP signal sequence and the Lc gene of 6D9 were ligated into pIHAneo with the *Bam*H I-*Xba* I site. The resulting plasmid was designated as pIHAneo-6D9Lc.

2.4. Transient expression

Sf9 cells and High Five cells were inoculated into 35-mm plastic culture dishes with 2 ml of serum-free media at a cell density of 2×10^5 cells/cm^3 24 h before transfection. The cells were transfected with 1 μg of plasmids for the Hc and Lc genes of the Fab fragment of 6D9 at a ratio (w/w) of 1:1 using 3 μl of FuGENE 6 transfection reagent (Roche Diagnostics, Tokyo, Japan). Three days after transfection, culture supernatant was removed to analyze the production of the 6D9 Fab fragment.

2.5. Stable transformation and cell culture

High Five cells were inoculated into 35-mm dishes with 2 ml of Express Five at a cell density of 2×10^5 cells/cm^3 24 h before transfection. The cells were transfected with 1 μg of pIHAneo-6D9Hc and pIHAbla-6D9Lc mixed at different ratios using 3 μl of FuGENE 6. Two days after transfection, the cells were removed from the dish and

inoculated into a 100-mm plastic culture dish with 10 ml of serum-free medium. After 24-h incubation, the medium was replaced with fresh Express Five containing 1.0 mg/ml G418 (Invitrogen) and 80 μg/ml blasticidin (Invitrogen) to select stable expression cells. The selective medium was replaced every 4 d until colonies of neomycin- and blasticidin-resistant cells were formed. Cells were isolated from each colony into a 96-well plate with 100 μl of medium without antibiotics. After the cells had grown to confluence in the presence of G418 and blasticidin, culture supernatant was removed to assay the Fab fragment of 6D9. Cells with a high yield of the Fab fragment of 6D9 were expanded in T-flasks with medium containing 0.5 mg/ml G418 and 40 μg/ml blasticidin.

Cells in the exponential growth phase were collected and suspended at a density of 2×10^5 cells/cm^3 in fresh medium containing 0.5 mg/ml G418 and 40 μg/ml blasticidin. Five milliliter of cell suspension was transferred into T25-flasks and 15 ml of cell suspension into 100-ml screw-capped Erlenmeyer flasks. The cells in the T-flasks were statically cultivated and one flask removed every day to measure cell density and production of the 6D9 Fab fragment. The cells in the Erlenmeyer flasks were cultivated at 27°C on a reciprocal shaker (90 oscillations/min; amplitude 25 mm) and aliquots of the cell suspension sampled every day.

2.6. Western blot analysis

Culture supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gel under non-reducing conditions and transferred to a poly(vinylidene difluoride) (PVDF) membrane. Western blot analysis

was carried out using a goat alkaline phosphatase-conjugated anti-mouse IgG (H+L) (Promega, Madison, WI, USA) and immunoreactive bands were detected using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Promega).

2.7. Enzyme-linked immunosorbent assay

The concentration of the 6D9 Fab fragment secreted into the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Stably transformed High Five cells secreting the His-tagged Fab fragment of 6D9, which contained six histidines at the C-terminus of Hc, were generated in a manner similar to the above. The His-tagged Fab fragment of 6D9 was purified with Ni-NTA Agarose resin (Qiagen, Tokyo, Japan) according to the recommended protocol and used as a standard. The culture supernatant and the standard were diluted with washing solution [10-fold-diluted Block Ace (Snow Brand Milk Products, Sapporo, Japan) containing 0.05% Tween 20 (polyoxyethylenesorbitan monolaurate)]. A conjugate (5 μg/ml) of hapten (a transition-state analog of the hydrolysis of a chloramphenicol monoester derivative) and bovine serum albumin (BSA) [21] was allowed to absorb onto 96-well plates (Corning, Inc., Corning, NY, USA) overnight at 4°C. After washing with the washing solution, 4-fold-diluted Block Ace was added to each well for blocking and the plate incubated for 1 h at 37°C. After washing once more, the culture supernatant and standard were added to each well and the plate incubated for 2 h at room temperature. After additional washing, horseradish peroxydase-conjugated goat anti-mouse IgG (κ) (Exalpha Biologicals, Watertown, MA, USA) diluted with the washing solution (5 μg/ml) was added and incubation continued for a further 1 h at room temperature.

After a final washing, freshly prepared substrate solution containing 2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS; Zymed Laboratories, San Francisco, CA, USA) and H_2O_2 was added and the preparation allowed to react in the dark at room temperature. The absorbance for each well was measured at 405 nm using a microplate reader and then converted to the Fab concentration by interpolating the value on a standard curve.

3. Results and discussion

3.1. Transient expression of 6D9 Fab fragment in insect cells

Transient expression of the 6D9 Fab fragment by insect cells was first performed to investigate the expression level using pXINSECT-DEST38 containing the BmNPV IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* actin promoter. Fig. 2 shows western blot analysis of the culture supernatant of Sf9 and High Five cells transfected with the Hc and Lc genes of the 6D9 Fab fragment cloned into pXINSECT-DEST38 and pIB/V5-His containing the OpIE2 promoter. Specific protein bands were detected at electrophoretic mobility of approximately 48 kDa in the culture supernatant of the transfectants. The mobility coincides with the molecular weight of the 6D9 Fab fragment. The electrophoretic mobility of specific protein bands of approximately 24 kDa is consistent with the molecular weight of the Lc of the 6D9 Fab fragment. The result of western blotting also indicates that larger amounts of the 6D9 Fab fragment were produced in High Five cells than Sf9 cells and that higher level expression of the

Fig. 2 ➔

Fab fragment was obtained with pXINSECT-DEST38 than pIB/V5-His. The use of the BmNPV IE-1 transactivator and the BmNPV HR3 enhancer with the *B. mori* actin promoter appears to result in high yields of the Fab fragment, as of other recombinant proteins including juvenile hormone esterase [14], human tissue plasminogen activator [11], and granulocyte-macrophage colony-stimulating factor [15]. High Five cells have been demonstrated to be an excellent host for the production of recombinant secreted proteins [11, 15]. High Five cells and pXINSECT-DEST38 were therefore used as the host insect cells and expression vector in subsequent investigations.

Culture supernatant of transfected High Five cells was also analyzed by ELISA against hapten-BSA (Fig. 3). When the cells were transfected with both the Hc and Lc genes of the 6D9 Fab fragment, a relatively strong signal was observed. On the other hand, culture supernatant of cells transfected with either the Hc or Lc gene alone showed almost no ELISA signal. These results suggest that the Fab fragment capable of binding the corresponding antigen was secreted into the culture medium by the transfected High Five cells. It was also confirmed in hydrolytic activity assay [19] that culture supernatant of cells transfected with the Hc and Lc genes showed the catalytic activity to hydrolyze a chloramphenicol monoester derivative and produce chloramphenicol (data not shown).

Fig. 3 ➔

3.2. Transient expression using expression vectors containing antibiotic resistance genes

For the stable expression of the Fab fragment of 6D9, and taking as the basis of the expression vector utilizing the actin promoter, the IE-1 transactivator, and the HR3

enhancer, we constructed two plasmid vectors, pIHAbla and pIHAneo, which respectively contain a blasticidin and a neomycin resistance gene for use as a selectable marker (Fig. 1). Transient expression of the 6D9 Fab fragment was first carried out to compare the expression levels using pIHAbla, pIHAneo, and pXINSECT-DEST38 (Fig. 4). Western blot analysis suggested that expression levels of the Fab fragment with the constructs using pIHAbla and pIHAneo were comparable to those obtained using original pXINSECT-DEST38. pIHAbla and pIHAneo were therefore employed to generate stably transformed insect cells in subsequent investigations.

Fig. 4 ➔

3.3. Production of 6D9 Fab fragment by stably transformed cells

High Five cells were cotransfected with pIHAneo-6D9Hc and pIHAbla-6D9Lc at different ratios and incubated with G418 and blasticidin. After incubation for 13 d in the presence of antibiotics, formation of colonies was observed under the respective transfection conditions. Cells isolated from the colonies were expanded in medium containing antibiotics. Fig. 5 shows a typical result obtained in western blot analysis of the culture supernatant of the transfected cells after incubation with antibiotics for 30 d. After incubation for 30 d following cotransfection, High Five cells secreted large amounts of the 6D9 Fab fragment into the culture medium, indicating that stably transformed cells were efficiently obtained by incubation in the presence of G418 and blasticidin following cotransfection with pIHAneo-6D9Hc and pIHAbla-6D9Lc.

Fig. 5 ➔

Highly productive cells established by cotransfection with pIHAneo-6D9Hc and pIHAbla-6D9Lc at the ratio (w/w) of 1:1 were incubated in static and shake-flask cultures. Fig. 6 shows the time course of change in the density of the stably

Fig. 6 ➔

transformed cells and the concentration of the 6D9 Fab fragment in the culture supernatant. In static culture, the viable cell density reached 2.8×10^6 cells/cm^3 after exponential growth (0–3 d) and 180 μg/ml of the 6D9 Fab fragment was produced by day 7. The maximum cell density achieved however was 6.1×10^6 cells/cm^3 on day 5 in the shake-flask culture, twice as high as in the static culture. On day 6 in the shake-flask culture, the yield of the 6D9 Fab fragment reached 350 μg/ml, a figure approximately twice that obtained in the static culture. The increased cell density and Fab fragment yield are probably due to better oxygen supply in the shake-flask culture. The specific Fab production rates in the shake-flask culture were almost comparable to those obtained in the static culture (data not shown). The shake-flask culture result was obtained under suboptimal conditions, and more efficient protein production could be attained in a bioreactor system. Immobilization techniques that result in a larger size of small and fragile cells facilitate the separation of cells from culture medium. Immobilization of the recombinant insect cells should therefore allow not only continuous but also efficient production of biologicals including antibody molecules by maintaining favorable conditions in the culture environment [22, 23].

Successful production of functional IgG antibody has been reported in stably transformed insect cells [12, 24], but the IgG yields in the culture supernatant are rather low (≤ 1 μg/ml after 3 d incubation), presumably due to the use of weak promoters such as the IE1 or OpIE2 promoters. The production level of whole IgG antibody should now be investigated in the system demonstrated in the present study. In the meantime, comparative studies of the production level with the baculovirus–insect cell system are in progress.

4. Conclusion

In the present study, we constructed two plasmid vectors that contain the baculoviral IE-1 transactivator, the baculoviral HR3 enhancer, and the *B. mori* actin promoter, together with either a neomycin or a blasticidin resistance gene for use as a selectable marker. After cotransfection with the Hc and Lc genes of the 6D9 Fab fragment cloned into these plasmid vectors, High Five cells stably secreting a high concentration of the functional Fab fragment were efficiently generated by incubation in the presence of G418 and blasticidin. High yields of over 300 μg/ml of Fab fragment were achieved in simple batch shake-flask culture of the transfected insect cells. More efficient production of Fab fragment could be attained in a bioreactor system and an immobilized cell culture. The results obtained in the present study indicate that recombinant insect cells may offer a novel approach for efficient antibody production. The strategy demonstrated here will also provide a simple and convenient way of producing hetero-oligomeric proteins.

Acknowledgement

This work was financially supported by a Grant-in-Aid for Scientific Research (C) (17560690) from the Japan Society for the Promotion of Science.

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Table 1

List of oligonucleotides synthesized for plasmid vector construction

Primer	Sequence [a]
1	5’-GGGAGCGCTATGAAGTTATGCATATTACTGGCCGTCGTGGC *Aor*51H I CTTTGTTGGCCTCTCGCTCGGGCTCGAGTCTGGGGGAGGCTT AGTGAAG-3’
2	5’-TGCTCTAGATTAACTAGTACAATCCCTGGGCACAATTTTC-3’ *Xba* I
3	5’-GGGGGATCCATGAAGTTATGCATATTACTGGCCGTCGTGGC *Bam*H I CTTTGTTGGCCTCTCGCTCGGGGAGCTCGTGATGACCCAGAC TCCACTC-3’
4	5’-CCCTCTAGAATTAACACTCATTCCTGTTGAAGCTCT-3’ *Xba* I
5	5’-CGGGGTACCATGAAGTTATGCATATTACTG-3’ *Kpn* I
6	5’-CATGCCATGGAGATTGAACAAGATGGATTG-3’ *Nco* I
7	5’-TCCCCCGGGACGTGTCAGTCCTGCTCCTCGGCCACGAAGT *Sma* I GCTCAGAAGAACTCGTCAAGAAG-3’

[a] Restriction sites and the *Drosophila* BiP secretion signal peptide sequence are underlined.

Figure captions

Fig. 1. Outline of construction of expression vectors pIHAbla (a) and pIHAneo (b).

Fig. 2. Western blot analysis of culture supernatant of Sf9 (Lanes 1–5) and High Five (Lanes 6–10) cells in transient expression of antibody 6D9 Fab fragment. Insect cells were transfected with pIB-6D9Hc and pXINSECT-6D9Lc (Lanes 2 and 7), pXINSECT-6D9Hc and pIB-6D9Lc (Lanes 3 and 8), pIB-6D9Hc and pIB-6D9Lc (Lanes 4 and 9), and pXINSECT-6D9Hc and pXINSECT-6D9Lc (Lanes 5 and 10). Lanes 1 and 6: untransfected cells.

Fig. 3. Enzyme-linked immunosorbent assay (ELISA) of cell culture supernatant in transient expression. High Five cells were transfected with pXINSECT-6D9Hc (1), pXINSECT-6D9Lc (2), and pXINSECT-6D9Hc and pXINSECT-6D9Lc (3). A_{405}: absorbance at 405 nm in ELISA. Bars represent the means ± S.D. of five different determinations.

Fig. 4. Western blot analysis of cell culture supernatant in transient expression using newly constructed vectors. High Five cells were transfected with pIHAbla-6D9Hc and pIHAbla-6D9Lc (Lane 2), pIHAbla-6D9Hc and pIHAneo-6D9Lc (Lane 3), pIHAneo-6D9Hc and pIHAneo-6D9Lc (Lane 4), pIHAneo-6D9Hc and pIHAbla-6D9Lc (Lane 5), and pXINSECT-6D9Hc and pXINSECT-6D9Lc (Lane 6). Lane 1: untransfected cells.

Fig. 5. Western blot analysis of culture supernatant of stably transformed cells. After

transfection with pIHAneo-6D9Hc and pIHAbla-6D9Lc at ratios (w/w) of 1:1 (Lane 6), 10:1 (Lanes 1–3), and 50:1 (Lanes 4 and 5), High Five cells were incubated with G418 and blasticidin for 30 d.

Fig. 6. Growth of recombinant High Five cells and production of 6D9 Fab fragment. (a) Density of viable cells; (b) concentration of 6D9 Fab fragment in the culture medium. Recombinant cells were incubated in static (open symbols) and shake-flask (closed symbols) cultures.

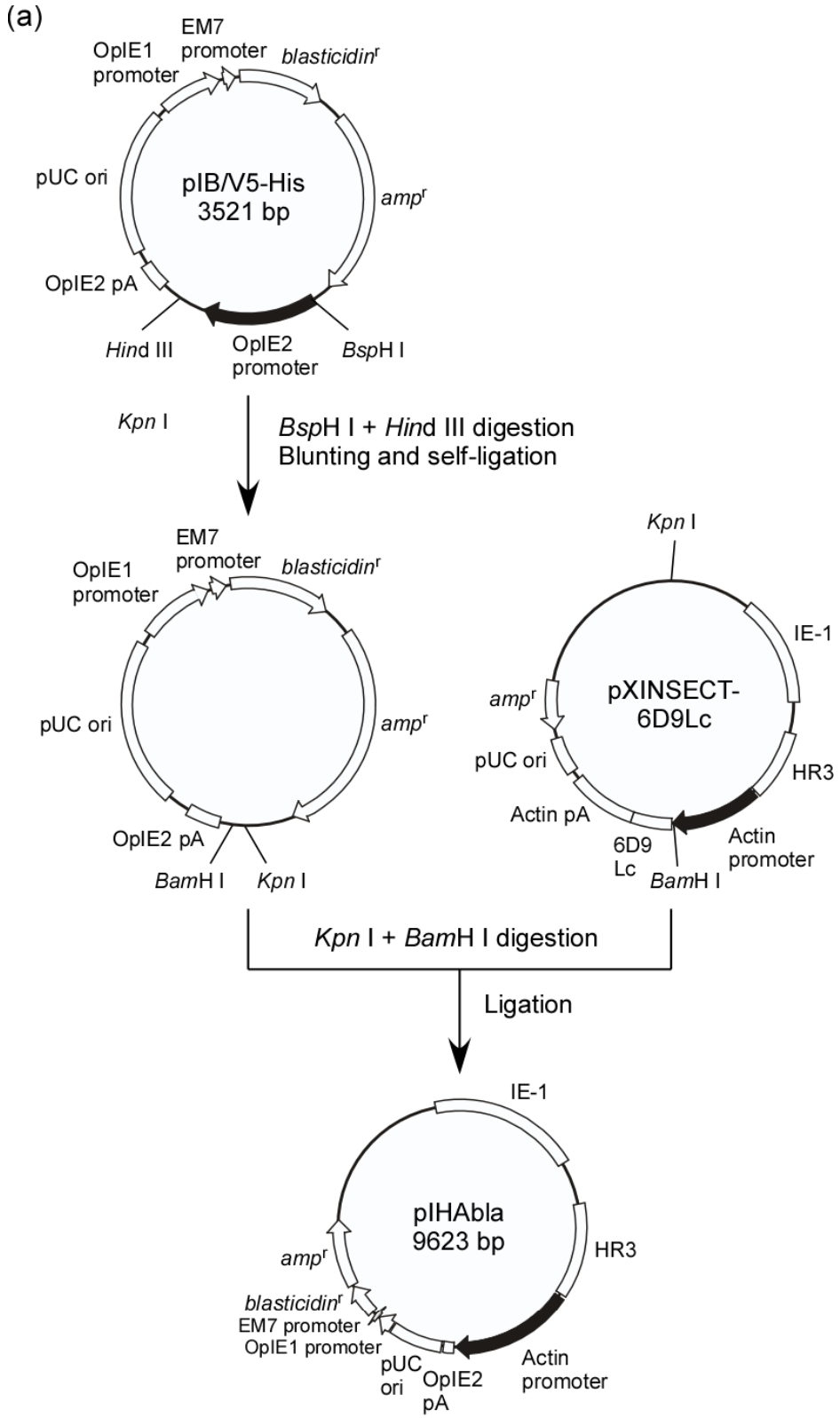

Fig. 1 (a). Yamaji et al.

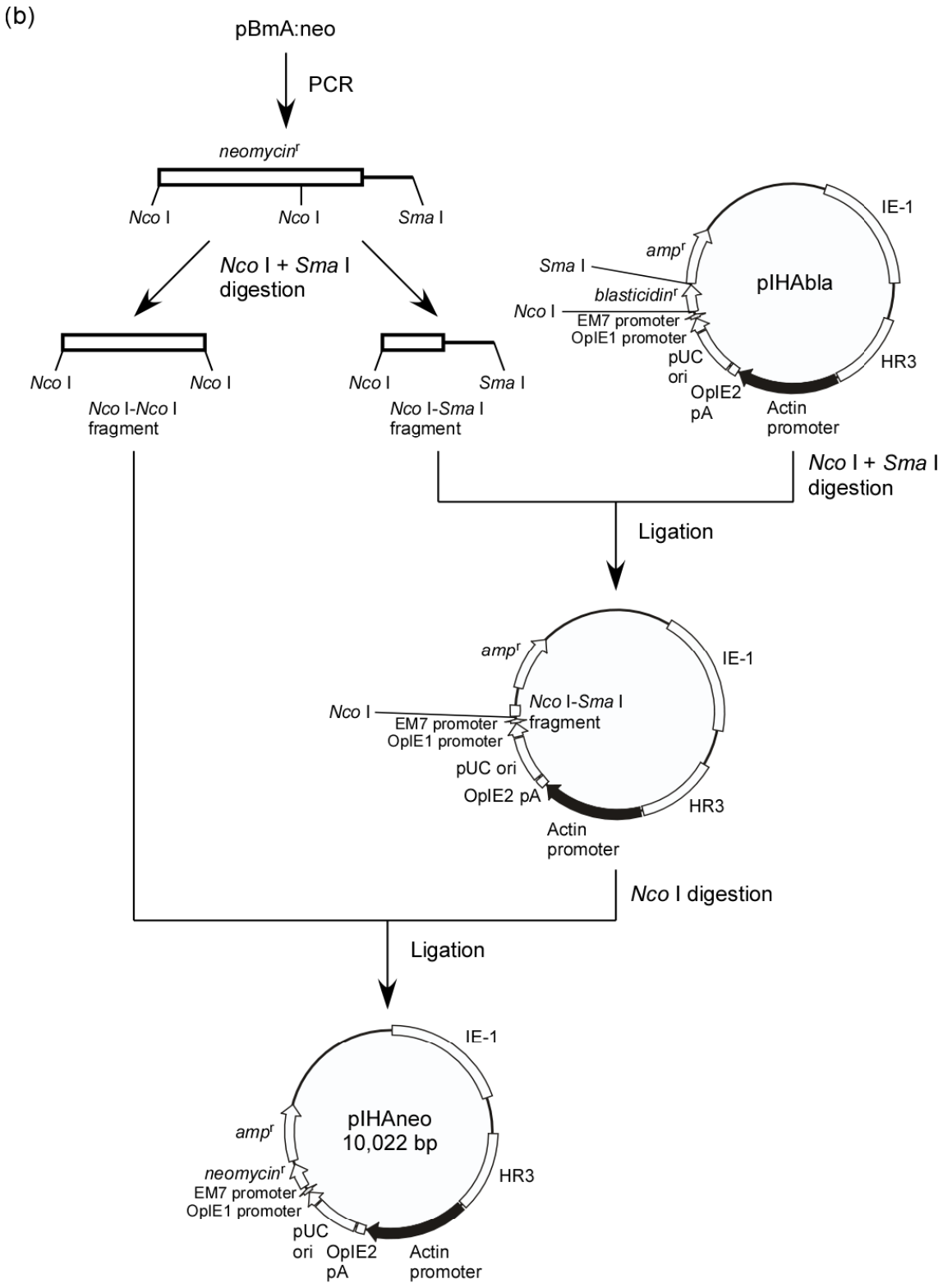

Fig. 1 (b). Yamaji et al.

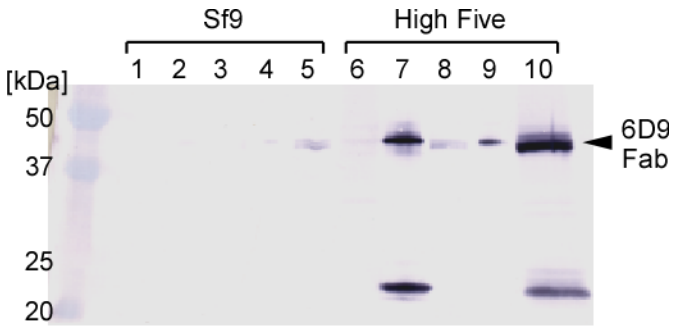

Fig. 2. Yamaji et al.

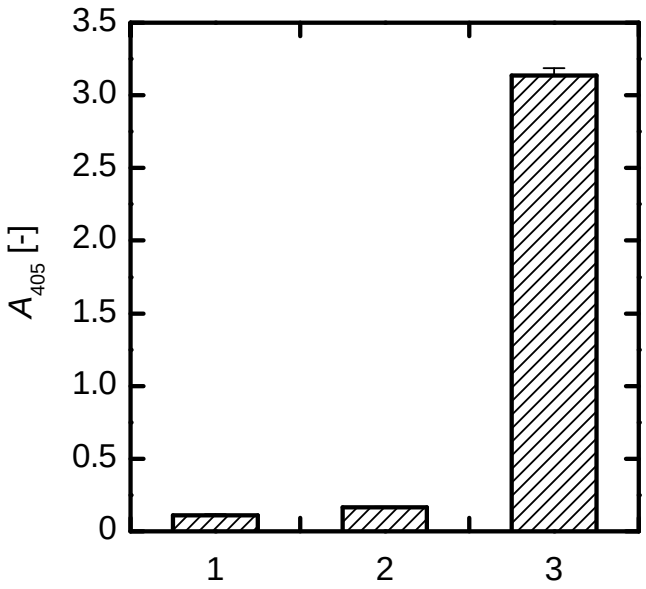

Fig. 3. Yamaji et al.

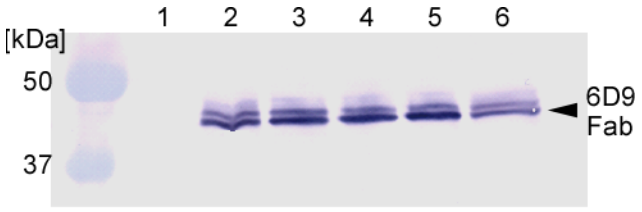

Fig. 4. Yamaji et al.

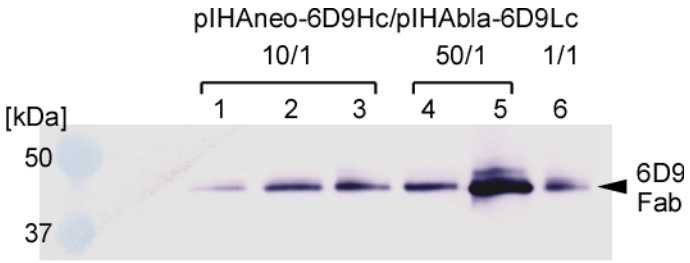

Fig. 5. Yamaji et al.

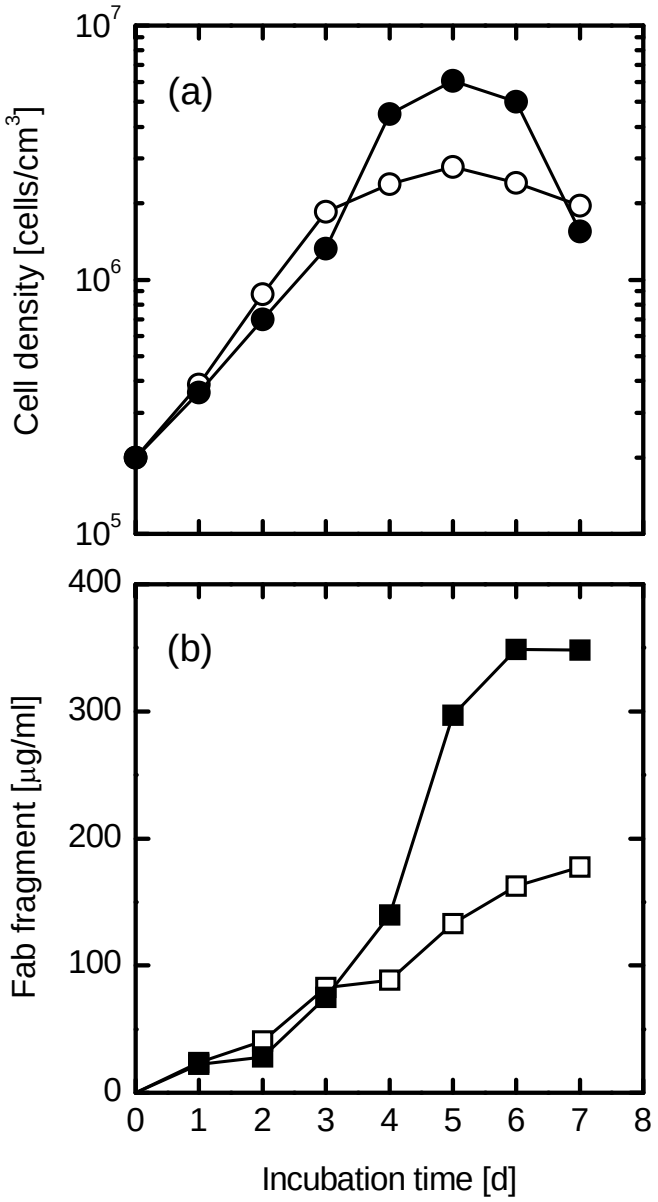

Fig. 6. Yamaji et al.