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(54) **Titre : EXPRESSION DE PROTEINES RECOMBINEES DANS DES CHRYSALIDES DE TRICHOPLUSIA NI**
(54) **Title: EXPRESSION OF RECOMBINANT PROTEINS IN TRICHOPLUSIA NI PUPAE**

(57) **Abrégé/Abstract:**

The present invention covers means and methods to increase the efficiency of recombinant protein expression, in particular to optimize the industrial production of recombinant proteins in insect pupae, particularly in *Trichoplusia ni* (T. ni) pupae. Moreover, the present invention is also directed to the pupae itself comprising baculovirus, pupae infected, transformed, transduced or transfected with baculoviruses or bacmids, as well as devices suitable for performing the methods of the present invention.

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(57) Abstract: The present invention covers means and methods to increase the efficiency of recombinant protein expression, in particular to optimize the industrial production of recombinant proteins in insect pupae, particularly in *Trichoplusia ni* (T. ni) pupae. Moreover, the present invention is also directed to the pupae itself comprising baculovirus, pupae infected, transformed, transduced or transfected with baculoviruses or bacmids, as well as devices suitable for performing the methods of the present invention.



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EXPRESSION OF RECOMBINANT PROTEINS IN *TRICHOPLUSIA NI* PUPAE**FIELD OF THE INVENTION**

The present invention may be included in the field of biotechnology and it covers means and methods to increase the efficiency of recombinant protein expression, in particular for the industrial production of recombinant proteins in insect pupae, particularly in *Trichoplusia ni* (*T. ni*) pupae, including its optimization. Moreover, the present invention is also directed to the pupae itself comprising a baculovirus, pupae infected, transformed, transduced or transfected with baculoviruses or bacmids.

STATE OF THE ART

The baculovirus expression vector system (BEVS) is a well-established method for the production of recombinant proteins, for example proteins to be used as vaccines, therapeutic molecules or diagnostic reagents. With its potential for over-expression and rapid speed of development, the BEVS is one of the most attractive choices for producing recombinant proteins for any purpose. The most employed baculovirus vector used in industry for recombinant protein expression is based on *Autographa californica* multinuclear polyhedrosis virus (AcMNPV) with *Spodoptera frugiperda* 9 (Sf9) or 21 (Sf21) insect cells as suitable expression hosts (Nettleship, J.E., Assenberg, R., Diprose, J.M., Rahman-Huq, N., Owens, R.J. Recent advances in the production of proteins in insect and mammalian cells for structural biology. *J. Struct. Biol.* 2010, 172, 55–65), as well as *Trichoplusia ni* (*T. ni*) insect larvae as living biofactories (Gomez-Casado E, Gomez-Sebastian S, Núñez MC, Lasa-Covarrubias R, Martínez-Pulgarín S, Escribano JM. Insect larvae biofactories as a platform for influenza vaccine production. *Protein Expr Purif.* 79: 35-43. 2011). Since the BEVS was developed in the 80's (Smith, G.E., M.D. Summers, and M.J. Fraser. 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* 3: 2156-21 65), hundreds of recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins, have been successfully produced in baculovirus-infected insect cells.

Recently, new baculovirus vectors have been described. For instance, WO 2012/168493 and WO 2012168492 describe recombinant DNA elements for the expression of recombinant proteins in insects and insect cells.

Worldwide, about 70,000 tons of silk are produced annually in a process that converts a low-value substrate, the leaves of the mulberry tree, to a high-value protein-based product: silk. Insects are highly efficacious protein producers because of their accelerated metabolism.

Lepidoptera such as *Bombyx mori* (*B. mori*, silkworm) or *T.ni* (cabbage looper), are two of the most used insects in biotechnology. They grow in size about 5000 times in less than 2 weeks and produce more than a kilometer of silk per *B. mori* insect. While a cell from a silk gland may produce about 80 µg protein/cell/day, the best mammalian cell culture systems produces only about 50 pg protein/cell/day.

Accordingly, insects as living biofactories constitute a promising alternative to insect cells, conventional fermentation technologies and also to plant-derived proteins because of the production versatility, scalability, automation possibilities, efficiency and speed of development. For example, insects as living biofactories avoid the necessity of bioreactors for the expression of proteins in, e.g., insect cells. Bioreactors are a technological and economical barrier to produce new and existing recombinant proteins, since they are inefficient, expensive, technologically complex (it takes several years to be built, are hard to validate, need highly qualified personal to their manipulation, they are prone to contaminations and are not reliable). In addition, they face the problem of limited scalability.

Larvae of *B. mori* have been widely used as living biofactories for the expression of recombinant proteins using the baculovirus expression vector system (Wang, DN; Liu, JW; Yang, GZ; Zhang, WJ; Wu, XF. 2002. Cloning of anti-LPS factor cDNA from *Tachypleus tridentatus*, expression in *Bombyx mori* larvae and its biological activity in vitro. *Molecular Biotechnology*, 21(1), 1-7; Wu, XF; Kamei, K; Sato, H; Sato, S; Takano, R; Ichida, M; Mori, H; Hara, S. 2001. High-level expression of human acidic fibroblast growth factor and basic fibroblast growth factor in silkworm (*Bombyx mori* L.) using recombinant baculovirus. *Protein Expression And Purification*, 21(1), 192-200; Kulakosky, PC; Hughes, PR; Wood, HA. 1998. N-linked glycosylation of a baculovirus-expressed recombinant glycoprotein in insect larvae and tissue culture cells. *Glycobiology*, 8(7), 741-745; Suzuki, T; Kanaya, T; Okazaki, H; Ogawa, K; Usami, A; Watanabe, H; KadonoOkuda, K; Yamakawa, M; Sato, H; Mori, H; Takahashi, S; Oda, K. 1997. Efficient protein production using a *Bombyx mori* nuclear polyhedrosis virus lacking the cysteine proteinase gene. *Journal Of General Virology*, 78, 3073-3080; Sumathy, S; Palhan, VB; Gopinathan, KP. 1996. Expression of human growth hormone in silkworm larvae through recombinant *Bombyx mori* nuclear polyhedrosis virus. *Protein Expression And Purification*, 7(3), 262-268; U. Reis, B. Blum, B.U. von Specht, H. Domdey, J. Collins, Antibody production in silkworm cells and silkworm larvae infected with a dual recombinant *Bombyx mori* nuclear polyhedrosis virus, *Biotechnology* (NY) 10 (1992) 910-912).

Larvae of *T. ni* have also been used for the expression of recombinant proteins (Perez-Martin, E., Gomez-Sebastian, S., Argilagué, J.M., Sibila, M., Fort, M., Nofrarias, M., Kurtz, S., Escribano, J.M., Segales, J., Rodríguez, F., 2010. Immunity conferred by an experimental vaccine based on the recombinant PCV2 Cap protein expressed in *Trichoplusia ni*-larvae. *Vaccine* 28 (11), 2340–2349); Gomez-Casado E, Gomez-Sebastian S, Núñez MC, Lasacovarrubias R, Martínez-Pulgarín S, Escribano JM. Insect larvae biofactories as a platform for influenza vaccine production, *Protein Expr. Purif.* 2011, 79: 35–43; Medin, JA; Hunt, L; Gathy, K; Evans, RK; Coleman, MS. 1990. Efficient, low-cost protein factories - expression of human adenosine-deaminase in baculovirus-infected insect larvae. *Proceedings of the National Academy of Sciences of the United States of America*, 87(7), 2760-2764; Shafer, AL; Katz, JB; Eernisse, KA. 1998. Development and validation of a competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera. *Avian Diseases*, 42(1), 28-34; Cha, HJ; Pham, MQ; Rao, G; Bentley, WE. 1997. Expression of green fluorescent protein in insect larvae and its application for heterologous protein production. *Biotechnology and Bioengineering*, 56(3), 239-247; Burden, JP; Hails, RS; Windass, JD; Suner, MM; Cory, JS. 2000. Infectivity, speed of kill, and productivity of a baculovirus expressing the itch mite toxin txp-1 in second and fourth instar larvae of *Trichoplusia ni*. *Journal of Invertebrate Pathology*, 75(3), 226-236; Perez-Filgueira, D. M.; Resino-Talavan, P.; Cubillos, C.; Angulo, I.; Barderas, M. G.; Barcena, J.; Escribano, J. M.. 2007. Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. *Virology*, 364(2), 422-430; Perez-Filgueira, D. A.; Gonzalez-Camacho, F.; Gallardo, C.; Resino-Talavan, P.; Blanco, E.; Gomez-Casado, E.; Alonso, C.; Escribano, J. M.. 2006. Optimization and validation of recombinant serological tests for African swine fever diagnosis based on detection of the p30 protein produced in *Trichoplusia ni* larvae. *Journal of Clinical Microbiology*, 44(9), 3114-3121; Hellers, M; Gunne, H; Steiner, H. 1991. Expression and posttranslational processing of preprocecropin-a using a baculovirus vector. *European Journal of Biochemistry*, 199(2), 435-439).

In silkworm, comparative studies demonstrated that, for most proteins, the highest expression yields were obtained in larvae instead pupae (Akihiro Usami *et al* (Akihiro Usami, Seiji Ishiyama, Chiaki Enomoto, Hironobu Okazaki, Keiko Higuchi, Mashahiro Ikeda, Takeshi Yamamoto, Mutsumi Sugai, Yukiko Ishikawa, Yumiko Hosaka, Teruyuki Koyama, Yoneko Tobita, Syoko Ebihara, Toshiko Mochizuki, Yoshimi Asano and Hidekazu Nagaya, Comparison of recombinant protein expression in a baculovirus system in insect cells (Sf9) and silkworm, *J. Biochem.* 2011;149(2):219–227; Chazarra S, Aznar-Cervantes S, Sánchez-del-Campo L, Cabezas-Herrera J, Xiaofeng W, Cenis JL, Rodríguez-López JN, Purification and kinetic properties of human recombinant dihydrofolate reductase produced in *Bombyx*

mori chrysalides, *Appl Biochem Biotechnol.* 2010 Nov;162(7):1834-46). Also, a susceptibility decrease of pupae to baculovirus infection in silkworm related with the age of pupae has been described (*Journal of General Virology* (1992), 73, 3195-320). In addition, in larvae, the infection of the baculovirus is generally performed orally, instead by inoculation (injection) for the scale up production. Pupae cannot be orally infected, so they have to be injected manually, which is tedious and time consuming. In addition, silkworm pupae are covered by a thick cocoon which has to be (manually) removed before the inoculation of the virus takes place, which is generally a tedious process.

Together with the general lower protein expression yield in silkworm pupa and the difficulties in its manipulation, have led to a general preference for the use of larvae for the production of recombinant proteins.

Similar disadvantages can be found in other systems, such as *Hyalophora cecropia* pupae. The expression of certain proteins in *H. cecropia* pupae is lower than in *T. ni* larvae (Hellers, M. and Steiner, H.; *Insect Biochem. Molec. Biol.*, Vol 22, Bo.1, pp. 35-39, 1992). In addition, *Hyalophora cecropia* moths are difficult to rear, are strictly univoltine (they have one generation per year), they have very low quantities of eggs per cycle and they have a high density cocoon (the thick cocoon has to be (manually) removed before the inoculation of the virus takes place, which is generally a tedious process). All these disadvantages make *Hyalophora cecropia* pupae a poor system for the expression of recombinant proteins, in particular a poor efficient, scalable and automatized system for the expression of recombinant proteins.

There is a need of more efficient and easy to automatize (scale up) systems for the expression of recombinant proteins in insects using the BEVS, particularly for the industrial expression of recombinant proteins in insects using the BEVS.

After intensive research, the inventors of the present invention have found a solution to the above problem, namely a protein expression system in pupae belonging to the *Lepidoptera* order, more preferably belonging to the species *Trichoplusia ni*, which is more efficient than the expression in larvae and moreover allows for an almost complete automation (scale up), which increases the efficiency and reduces costs associated to recombinant protein expression, in particular at industrial scale.

The present invention is thus directed to the use of pupae (chrysalises), preferably belonging to the *Lepidoptera* order, more preferably belonging to the species *Trichoplusia ni*, in

combination with baculovirus vectors derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV), to produce recombinant proteins to be used in diagnostic, vaccines and therapeutic treatments. The use of pupae (chrysalises) from the species of *Trichoplusia ni* for recombinant protein expression, in particular the industrial use of pupae (chrysalises) from the species of *Trichoplusia ni* for recombinant protein expression has not yet been reported.

SUMMARY OF THE INVENTION

The present invention provides a pupa comprising a recombinant baculovirus and/or a transfer vector/bacmid derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV).

In addition, the present invention provides a pupa comprising a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

The invention also relates to the use of the pupa of the invention for the expression of recombinant proteins.

Further, the present invention provides a method for producing at least one recombinant protein comprising the steps of:

- (a) Providing a pupa;
- (b) Inoculating the pupa of step (a) with a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV);
- (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the at least one recombinant protein to be expressed;
- (d) Obtaining the pupae comprising the at least one recombinant protein;
- (e) Optionally, harvesting the at least one recombinant protein; and
- (f) Optionally, purifying the at least one recombinant protein.

In addition, provided herein is a method that can be automated to reduce manipulation for producing a silk-free pupa belonging to the order *Lepidoptera* comprising the steps of:

- (a) Providing a pupa contained in a cocoon;
- (b) Treating the pupa contained in a cocoon, preferably by a specially designed device, with a solution of a salt of hypochlorous acid, preferably sodium hypochlorite; and

(c) Obtaining a silk-free (and, optionally, essentially externally disinfected) pupa.

A method for producing a recombinant baculovirus comprising the steps of:

- (a) Providing a pupa;
- (b) Transfecting the pupa of step (a) with a transfer vector/bacmid suitable for producing a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV);
- (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the recombinant baculovirus is produced;
- (d) Obtaining the pupae comprising the recombinant baculovirus;
- (e) Optionally, harvesting the recombinant baculovirus; and
- (f) Optionally, purifying the recombinant baculovirus.

The present invention also relates to a device comprising a precision pump, a mobile mechanic arm and a (removable) needle/s suitable for injecting a fluid into a pupa belonging to the order *Lepidoptera*, preferably to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, more preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*, even more preferably to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia cynthia*.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Massive single-use *T. ni* insect rearing module. Fifth instar stage larvae growing in the insect rearing module.

Figure 2: Comparison of cocoons (left part of the image) and pupae free of silk (right part of the image) formed by *Bombyx mori* and *Trichoplusia ni* Lepidoptera.

Figure 3: Semi-automatic device for silk removal from the *T. ni* cocoons. Schematic representation of the machine containing two containers and a mechanic arm in which the single-use rearing module is allocated. The first container contains hypochlorous acid and a system to project the liquid through the rearing modules containing the cocoons (it helps to dissolve more efficiently the silk surrounding the pupa). The second container is to wash the pupae, and it sprays water over the chrysalises. On the top of this container there is a system that dispenses air to dry the pupae. At the end of the process, the pupae are free of silk and ready to be used in infection or to be stored refrigerated until use.

Figure 4: Baculovirus expression cassettes used to produce the Green fluorescent protein (GFP). A) Conventional baculovirus expression cassette using the polyhedrin promoter (e.g., SEQ ID NO.: 23). B) TB expression cassette comprising the *Ac-ie-01* cDNA encoding for the transactivators IE1 and IE0 expressed under the control of the polyhedrin promoter; the enhancer sequence *hr1* and the chimeric promoter *p6.9-p10* driving the expression of the GFP (e.g., SEQ ID NO.:17 and a nucleic acid sequence encoding GFP, e.g., SEQ ID NO.: 38).

Figure 5: Automatic inoculation of *T. ni* pupae by a robot injecting the recombinant baculovirus. A) Schematic representation of the inoculation robot. B) Schematic representation of a matrix of alveolus where the insect pupae are allocated. These alveoli containing the pupae have a perforated top and are stackable facilitating the transport of the pupae to the production laboratory and are compatible with the inoculation robot. In the same panel a real photograph image of the alveolus containing the pupae and with the top. C) Schematic representation of the pupae inoculation with a needle connected with the robotic arm.

Figure 6: Comparative analysis of the expression yields of the GFP protein in infected pupae by using a conventional baculovirus TB(-) (polyhedrin promoter, e.g., SEQ ID NO.: 23) or a TB-modified baculovirus TB(+). A) Coomassie blue staining of a SDS-PAGE resolving protein extracts from infected pupae with the TB(-) or TB(+) baculoviruses. (-) corresponds to an extract from an uninfected control pupa. B) Quantification of GFP production yields obtained in pupae infected by every baculovirus analyzed expressed in mg per g of pupae biomass.

Figure 7: Baculovirus expression cassettes used to produce the capsid protein from porcine circovirus type 2 (Cap) (e.g., SEQ ID NO.: 26) or the hemagglutinin (HA) from influenza virus (e.g., SEQ ID NO.: 30). A) Conventional baculovirus expression cassette using the polyhedrin promoter. B) TB expression cassette comprising the *Ac-ie-01* cDNA encoding for the transactivators IE1 and IE0 expressed under the control of the polyhedrin promoter; the enhancer sequence *hr1* and the chimeric promoter *p6.9-p10* driving the expression of the above mentioned proteins.

Figure 8: Comparative analysis of the expression yields of the Cap protein (e.g., SEQ ID NO.: 26) in infected pupae by using a conventional baculovirus TB(-) (polyhedrin promoter, SEQ ID NO.: 10, SEQ ID NO.: 28) or a TB-modified baculovirus TB(+) (e.g., SEQ ID NO.: 27 or 29). A) Coomassie blue staining of a SDS-PAGE resolving protein extracts from infected pupae with the TB(-) or TB(+) baculoviruses. (-) corresponds to an extract from an uninfected control pupa. B) Quantification of Cap production yields obtained in pupae infected by every baculovirus analyzed expressed in mg per g of pupae biomass.

Figure 9: Comparative analysis of the expression yields of the HA protein (SEQ ID NO.: 30) in infected pupae by using a conventional baculovirus TB(-) (polyhedrin promoter, SEQ ID

NO.: 10) or a TB-modified baculovirus TB(+) (e.g., SEQ ID NO.: 31). A) Coomassie blue staining of a SDS-PAGE resolving protein extracts from infected pupae with the TB(-) or TB(+) baculoviruses. (-) corresponds to an extract from an uninfected control pupa. B) Quantification of HA production yields obtained in pupae infected by every baculovirus analyzed expressed in mg per g of pupae biomass.

Figure 10: Baculovirus expression cassette used to produce the GFP, Cap (SEQ ID NO.: 26), HA (SEQ ID NO.: 30) and the VP60 protein from rabbit haemorrhagic disease virus (RHDV, SEQ ID NO.:32 or 33) in *T. ni* larvae and pupae. Schematic representation of the TB expression cassette comprising the *Ac-*ie-01** cDNA encoding for the transactivators IE1 and IE0 expressed under the control of the polyhedrin promoter; the enhancer sequence *hr1* and the chimeric promoter *p6.9-p10* driving the expression of the above mentioned proteins.

Figure 11: Comparative analysis of the expression yields of the GFP protein in infected pupae and larvae. A) Coomassie blue staining of a SDS-PAGE resolving protein extracts from infected pupae (P) or infected larvae (L) with the TB(+) baculovirus. (-) corresponds to an extract from an uninfected control pupa. B) Quantification of the GFP production yields obtained in infected pupae or larvae and expressed in mg per g of insect biomass.

Figure 12: Comparative analysis of the expression yields of the Cap protein in infected pupae and larvae. A) Coomassie blue staining of a SDS-PAGE resolving protein extracts from infected pupae (P) or infected larvae (L) with the TB(+) baculovirus. (-) corresponds to an extract from a uninfected control pupa. B) Quantification of the Cap production yields obtained in infected pupae or larvae and expressed in mg per g of insect biomass.

Figure 13: Comparative analysis of the expression yields of the HA protein in infected pupae and larvae. A) Coomassie blue staining of a SDS-PAGE resolving protein extracts from infected pupae (P) or infected larvae (L) with the TB(+) baculovirus. (-) corresponds to an extract from an uninfected control pupa. B) Quantification of the HA production yields obtained in infected pupae or larvae and expressed in mg per g of insect biomass.

Figure 14: Comparative analysis of the expression yields of the RHDV capsid VP60 proteins (G1 and RHDVb) in infected pupae and larvae. A) Coomassie blue staining of a SDS-PAGE resolving protein extracts from infected pupae (P) or infected larvae (L) with the TB(+) baculovirus. (-) corresponds to an extract from an uninfected control pupa. B) Quantification of the RHDV capsid proteins production yields obtained in infected pupae or larvae and expressed in mg per g of insect biomasses (L) with the TB(+) baculovirus. (-) corresponds to an extract from an uninfected control pupa. B) Quantification of the RHDV capsid proteins production yields obtained in infected pupae or larvae and expressed in mg per g of insect biomass.

Figure 15: VLPs formed after infection of *T. ni* pupae a TB (+) baculovirus expressing the VP60 protein from the RHDV G1 and RHDVb. Extracts from infected pupae at the optimal

production times with each baculovirus were processed for VLP purification. Samples were observed by Electron microscopy using negative staining. The figure shows the VLPs at two magnifications. VLPs obtained with the two baculoviruses presented identical sizes and shapes. The micrographs are representative of the fields analyzed.

Figure 16: Schematic example of procedure to obtain a virus inoculum from infected pupae in absence of insect cell cultures.

Figure 17: Schematic example of upstream and downstream processing procedure to obtain a purified recombinant protein from infected pupae in three steps. A) Production of *T. ni* chrysalises, their manipulation and optionally shipping to the final destination (e.g., pharmaceutical company) for recombinant protein production. B) Pupae storage, robotic inoculation with recombinant baculovirus using the device of the present invention, incubation and frozen insect biomass, which can be stored for months before processing *in situ*, or which can be easily shipped to other locations for proceeding with the downstream processing. C) Downstream processing by conventional means of frozen biomass, including homogenization, tangential flow filtration and protein purification.

Figure 18: Comparison of Porcine circovirus Cap protein expression yields in insect cells by using a conventional baculovirus and a baculovirus modified by TopBac in insect cells and in insect pupae.

Figure 19: Schematic example of procedure to obtain a virus inoculum from infected pupae.

Figure 20: Schematic example of downstream processing procedure to obtain a purified recombinant protein from infected pupae.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, “*pupa*” refers to the life stage of some insects undergoing transformation. The pupal stage is found in insects that undergo a complete metamorphosis (holometabolous insects). These insects go through four life stages: embryo, larva, pupa and imago. The pupa of butterflies is also called chrysalis. Insects may protect the pupa covering them with a cocoon, which is a casing spun of silk which protects the pupa of many insects.

As used herein, “*baculovirus*” refers to a family of infectious viruses for invertebrates, mainly infecting insects and arthropods. A “*recombinant baculovirus*” has further introduced recombinant DNA through, for example, homologous recombination or transposition. The recombinant baculovirus may originate from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV).

As used herein, an “*expression cassette*” comprises recombinant DNA elements that are involved in the expression of a certain gene, such as the gene itself and/or elements that control the expression of this gene (e.g. the promoter). For example, an expression cassette useful in the present invention comprises the following recombinant DNA elements:

1. a nucleic acid sequence that allows expression of a recombinant protein, such as the recombinant proteins described below in the present specification, and preferably nucleic acid sequences controlling its expression (at least a promoter); and
2. a nucleic acid sequence that allows expression of baculovirus transcriptional regulators, such as IE-1 and IE-0, above the normal, i.e. endogenous, levels of said regulators that are obtained during baculovirus infection of an insect cell or an insect.

In some embodiments, the expression cassette further comprises an enhancer homologous region (*hr*), such as *hrl*, operably linked to the promoter of said sequence encoding the recombinant protein. The recombinant DNA elements forming part of the expression cassette of the invention may be present in a single nucleic acid molecule. The recombinant DNA elements forming part of the expression cassette may be present in distinct nucleic acid molecules. Preferably, the distinct nucleic acid molecules are present within the same cell.

As used herein, “*recombinant DNA*” refers to a form of artificial DNA that is engineered through the combination or insertion of one or more DNA strands, thereby combining DNA that would normally not occur together.

As used herein, “*recombinant DNA element*” refers to a functional element within recombinant DNA, such as a promoter, enhancer or a gene.

As used herein, “*transcriptional regulator*” refers to a regulatory protein that has the ability to modulate the transcription of specific genes by, for example, binding to enhancer or repressor regions and/or recruiting further proteins that are involved in transcription. IE-1 and its splice variant IE-0 are transcriptional regulators that are endogenously expressed during baculovirus infection. The expression level of the proteins IE-1, IE-0 and/or fragments thereof may be determined at both the mRNA and at the protein level with methods conventionally known to the person skilled in the art, such as quantitative PCR and Western Blot analysis.

According to the present invention, IE-1, IE-0 and/or fragments thereof may be recombinantly expressed to increase the total level of these proteins above endogenous levels during baculovirus infection. This can be achieved through, for example, introducing further

copies of the endogenous gene or manipulating the expression of the promoter of the endogenous gene. Further copies of the endogenous genes can be introduced as transgenes under the control of a suitable promoter such as *polh* or *pB29*.

IE-1, IE-0 and fragments thereof may be encoded by the nucleic acids of SEQ ID NO: 1 (also referred to as *Ac-ie-01*) to SEQ ID NO: 5. SEQ ID NO: 1 is the *Ac-ie-01* cDNA that encodes both IE-1 and IE-0, SEQ ID NO: 2 is the coding sequence (CDS) of IE-1 and SEQ ID NO: 3 is the CDS of IE-0. SEQ ID NO: 4 and 5 are the CDSs of the N-terminal domains of IE-1 and IE-0 respectively that retain the catalytic transcriptional regulator activity. The proteins that are encoded by SEQ ID NOs: 2-5 are represented by SEQ ID NOs: 6-9 respectively.

Nucleic and amino acid sequences referred to in the present invention shall be distinguished from other nucleic and amino acid sequences by their degree of sequence identity or similarity respectively as determined using EMBOSS Needle with the default parameters. Methods for the generation of such variants include random or site directed mutagenesis, site-saturation mutagenesis, PCR-based fragment assembly, DNA shuffling, homologous recombination in vitro or in vivo, and methods of gene synthesis.

As used herein, "*variants*" are nucleic or amino acids whose nucleic or amino acid sequence differs in one or more positions from the parental nucleic or amino acid sequence, whereby differences might be additions, deletions and/or substitutions of nucleic acids or amino acid residues.

As used herein, "*homologous regions*", (*hr*), are comprised of repeated units of about 70-bp with an imperfect 30-bp palindrome near their center. For instance, homologous regions are repeated at **eight** locations in the *AcMNPV* genome with 2 to 8 repeats at each side. Homologous regions have been implicated as both transcriptional enhancers and origins of baculovirus DNA replication.

As used herein, "*enhancer region*" refers to a control sequence, whose binding by transcriptional regulators increases the level of transcription of associated genes.

As used herein, "*recombinant protein*" refers to a protein that originates from recombinant DNA. Such proteins can be used for the benefit of humans and animals and may have industrial, commercial or therapeutic application.

As used herein, “*being operably linked*” refers to two nucleic acid sequences that are connected in a way that one influences the other in terms of, for example, transcriptional regulation.

As used herein, “*promoter*” refers to a DNA sequence to which RNA polymerase can bind to initiate transcription. The sequence may further contain binding sites for various proteins that regulate transcription, such as transcription factors. The promoter sequence may be composed of different promoter fragments (either different or the same fragments) that are localized closely in the DNA sequence and may be separated by linkers or spacer. Such promoters are referred to as chimeric promoters.

As used herein, a “*transfer vector*” is a vector (namely a DNA molecule used as a vehicle to carry genetic material) that permits the insertion of genetic information into a baculovirus genome.

As used herein, a “*bacmid*” refers to a plasmid construct which contains the DNA sequence sufficient for generating a baculovirus when transfected into a cell or insect.

As used herein, a “*cloning vector*” refers to any vector that is suitable for cloning, which generally involves the presence of restriction sites, an origin of replication for bacterial propagation and a selectable marker.

The cloning vector which may be used in the context of the present invention preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. For example, the cloning vector may comprise a nucleic acid sequence encoding a recombinant protein (also referred to as a “*donor vector*”, namely a cloning vector comprising an expression cassette). Alternatively, the cloning vector lacks such sequence.

As used herein, a “*vaccine*” may be defined as a biological preparation, preferably comprising a recombinant protein that provides active acquired immunity to a particular disease.

As used herein, the term “*about*” means the indicated value $\pm 1\%$ of its value, or the term “*about*” means the indicated value $\pm 2\%$ of its value, or the term “*about*” means the indicated value $\pm 5\%$ of its value, the term “*about*” means the indicated value $\pm 10\%$ of its value, or the term “*about*” means the indicated value $\pm 20\%$ of its value, or the term “*about*” means the

indicated value $\pm$ 30% of its value; preferably the term “*about*” means exactly the indicated value ($\pm$ 0%).

Detailed description

The present invention provides a pupa comprising a recombinant baculovirus and/or a transfer vector/bacmid. The present invention surprisingly shows that introduction into insect pupae recombinant baculoviruses, and particularly of sequences that cause the expression of baculovirus transcriptional regulators above endogenous levels and optionally the introduction of an enhancer homologous region (*hr*) sequence, a promoter or a combination of promoters, is able to increase the production of a recombinant protein to unprecedented levels. This indicates the usefulness of this system for the expression of recombinant proteins *in vivo*, in particular for the industrial production of recombinant proteins *in vivo*.

Pupa of the present invention

The present invention provides a pupa comprising a recombinant baculovirus and/or a transfer vector and/or bacmid. The recombinant baculovirus and/or transfer vector and/or bacmid is preferably derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV). Preferably, the pupa belongs to the order *Lepidoptera*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*, more preferably to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia cynthia*. Even more preferably, the pupa belongs to the species *Trichoplusia ni*. In a preferred embodiment, the pupa of the present invention does not belong to the species *Bombyx mori*. In a preferred embodiment, the pupa of the present invention does not belong to the species *Hyalophora cecropia*.

In a preferred embodiment, the pupa of the invention is a pupa belonging to the genera *Trichoplusia*, preferably to the species *Trichoplusia ni*, which comprises a recombinant baculovirus and/or a transfer vector/bacmid derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV).

The pupae of the invention, and in particular the pupae of *T. ni*, offer several advantages for the expression of recombinant proteins, in particular in automatized and scalable processes. For example, a moth couple of *T. ni* may have around 1.000 eggs per cycle. In addition, the cocoon produced by *T. ni* pupae is not as thick as the cocoon which covers the pupae of other species (such as, for example, *Bombyx mori* or *Hyalophora cecropia*), which makes the pupae

of *T. ni* especially suited for their use in automatized and scalable production processes (industrial production of recombinant proteins). Accordingly, pupae of *T. ni* infected with a recombinant baculovirus and/or a transfer vector and/or a bacmid preferably derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV) provides for an efficient, scalable and easily automatized system for recombinant protein production. Further advantages of this system are its high productivity (up to 20 times more productive than bioreactors), the fact that it is technically simple, easy to implement and validate, its reduced costs (> 90% of reduction in fixed investments with respect to the use of bioreactors), the lower cost of goods, short development times (baculovirus system), its high efficiency with proteins difficult to produce, and the high quality and safety of the produced products.

The inventors of the present application surprisingly found that the expression of recombinant proteins in pupae, in particular in pupae belonging to the order *Lepidoptera*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*, more preferably to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia Cynthia*, is comparable to and even higher than the expression in larvae.

The patent application published as WO 2012/168492 discloses recombinant baculovirus and transfer vectors and bacmids that may be comprised in the pupa of the present invention.

The recombinant baculovirus and/or transfer vector and/or bacmid comprised in the pupa according to the present invention may preferably comprise a recombinant DNA. For example, the recombinant baculovirus and/or transfer vector and/or bacmid comprised in the pupa according to the present invention comprises a nucleic acid sequence encoding a recombinant protein, wherein the recombinant protein is preferably selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and the green fluorescent protein (GFP), and/or wherein the recombinant protein is preferably not a protein which is endogenously produced by pupae, as it will be described below. For example, the recombinant baculovirus and/or transfer vector and/or bacmid comprised in the pupa according to the present invention comprises a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection (for example, the expression above

endogenous levels may be obtained by the presence, in the recombinant baculovirus, of an additional copy of the nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof). Preferably, the recombinant baculovirus and/or transfer vector and/or bacmid comprised in the pupa according to the present invention further comprises a recombinant homologous region (*hr*) operably linked to any promoter suitable for driving the expression of a recombinant protein.

The transfer vector which may be comprised in the pupa of the present invention preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. In one preferred aspect, the transfer vector comprises a nucleic acid sequence encoding said recombinant protein, whereas in another preferred embodiment the transfer vector lacks such sequence. In a preferred embodiment, the transfer vector is a bacmid.

The transfer vector and/or bacmid may be derived from any of the commercially available baculovirus expression systems “Bac-to-Bac®” (invitrogen™), “BacPAK™” (Clontech™), “FlashBAC™” (Oxford Expression Technologies™), “BacuVance™” (GenScript™), “Bac-N-Blue DNA™” (invitrogen™), “BaculoDirect™” (invitrogen™), “BacVector®” 1000, 2000, 3000 (Novagen®), “DiamondBac™” (Sigma-Aldrich®) or “BaculoGold™” (BD biosciences™).

The pupa of the present invention (which preferably belongs to the order *Lepidoptera*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*, more preferably to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia cynthia*, or any other *Lepidoptera* susceptible to AcMNPV infection, even more preferably to the genus *Trichoplusia* and to the species *Trichoplusia ni*) may comprise a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection. The above described recombinant DNA elements are preferably introduced into the pupa by a recombinant baculovirus.

The nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained

during baculovirus infection according to the present invention is preferably selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NOs: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NOs: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NOs: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NOs: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

The sequence of the variants of SEQ ID NOs: 1-5 is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% identical to the sequences of SEQ ID NOs: 1-5.

The sequence of the variants of SEQ ID NOs: 6-9 is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% similar to the sequences of SEQ ID NOs: 6-9.

The pupa of the present invention may further comprise a nucleic acid sequence and/or recombinant baculovirus and/or a transfer vector and/or bacmid which further comprises a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

The recombinant homologous region (*hr*) is preferably the sequence indicated in SEQ ID NO: 21 (*hrI*).

The promoter that drives the expression of said recombinant protein is preferably selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NOs: 10-14, preferably indicated in any of SEQ ID NOs: 11-13; and

- (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NOs: 10-14, preferably indicated in any of SEQ ID NOs: 11-13.

In a preferred embodiment, the nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions (the nucleic acid sequences that are comprised in the pupa of the present invention) are selected from the group consisting of SEQ ID NOs: 15-20.

The recombinant promoters, sequences encoding transcriptional regulators and enhancer regions of the present invention do not need to form part of a single molecule, instead these sequences may form part of distinct molecules as long as they are operably linked, i.e. contained within the same cells within the pupa.

The pupa of the present invention and/or the recombinant baculovirus and/or transfer vector and/or bacmid may further comprise a nucleic acid sequence encoding a recombinant protein. This nucleic acid sequence is preferably operably linked to the nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof, and optionally to a homologous region (*hr*), these sequences having being described above.

Preferably, the recombinant protein is selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and green fluorescent protein (GFP).

In a preferred embodiment, the recombinant protein is a virus-like particle protein, which is preferably selected from the group consisting of:

- (a) Porcine circovirus capsid protein, preferably from porcine circovirus type 2 (e.g., SEQ ID NO.: 26),
- (b) Foot and mouth disease virus VP1, VP3 or VP0 protein,
- (c) Canine parvovirus VP1 and VP2 proteins,
- (d) Porcine parvovirus VP1 and VP2 proteins,
- (e) Human norovirus (genogroup I or II) capsid protein,

- (f) Calicivirus capsid protein,
- (g) Human papillomavirus L1 protein, preferably from human papillomavirus 16,
- (h) Hepatitis E protein E2,
- (i) Infectious bursal disease virus VP1, VP2 and VP3 proteins,
- (j) Astrovirus ORF2-encoded proteins,
- (k) Influenza virus HA (e.g., SEQ ID NO.: 30), NA and M1 proteins,
- (l) Hepatitis B core and surface antigens,
- (m) Rabbit calicivirus VP60 protein, preferably from rabbit haemorrhagic disease viruses RHDVb and RHDVG1 (e.g., SEQ ID NOs.: 32 and 33).
- (n) Human parvovirus VP1 and VP2 proteins

For instance, the recombinant protein may be:

- Porcine circovirus capsid protein, preferably from porcine circovirus type 2, which is, for example, represented by the amino acid sequence of SEQ ID NO: 26 or encoded by the nucleic acid sequence of SEQ ID NO: 25.
- Foot and mouth disease virus (FMDV) VP1, VP3 and VP0 protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - FMDV serotype O complete genome: GenBank JX570650.1
 - FMDV serotype A complete genome: GenBank HQ832592.1
 - FMDV serotype C complete genome: GenBank AY593810.1
 - FMDV serotype SAT 1 complete genome: GenBank AY593846.1
 - FMDV serotype SAT 2 complete genome: GenBank JX014256.1
 - FMDV serotype ASIA 1 complete genome: GenBank HQ631363.1.
- Canine parvovirus VP1 and VP2 protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Canine parvovirus VP1 gene for capsid protein VP1, partial cds, strain: 1887/f/3. GenBank: AB437434.1.
 - Canine parvovirus VP1 gene for capsid protein VP1, partial cds, strain: 1887/M/2. GenBank: AB437433.1.
 - Canine parvovirus VP2 gene, complete cds, strain: HNI-2-13. GenBank: AB120724.1.
 - Canine parvovirus VP2 gene, complete cds, strain: HNI-3-4. GenBank: AB120725.1.
 - Canine parvovirus VP2 gene, complete cds, strain: HNI-3-11. GenBank: AB120726.1.

- Canine parvovirus VP2 gene, complete cds, strain: HNI-4-1. GenBank: AB120727.1.
- Canine parvovirus VP2 gene, complete cds, strain: HNI-1-18. GenBank: AB120728.1.
- Canine parvovirus VP2 protein (VP2) gene, complete cds. GenBank: DQ354068.1.
- Canine parvovirus VP2 gene, complete cds, strain: HCM-6. GenBank: AB120720.1.
- Canine parvovirus isolate Taichung VP2 gene, complete cds. GenBank: AY869724.1.
- Canine parvovirus VP2 gene, complete cds, strain: HCM-8. GenBank: AB120721.1.
- Canine parvovirus type 1 proteins VP1 and VP2: GenBank AB518883.1
- Canine parvovirus type 2a VP1 and VP2. GenBank: M24003.1
- Canine parvovirus type 2b VP2: GenBank FJ005265.1
- Canine parvovirus Type 2C VP2: GenBank FJ005248.1
- Porcine parvovirus VP1 and VP2 protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Porcine parvovirus strain 693a. GenBank: JN400519.1
 - Porcine parvovirus strain 8a. GenBank: JN400517.1
- Human parvovirus VP1 and VP2 protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Human parvovirus B19 VP1 complete cds. GenBank: AF264149.1
 - Human parvovirus B19 isolate Vn115 NS1 (NS1), 7.5 kDa protein (NS1), VP1 (VP1), 9.5 kDa protein (VP1), and VP2 (VP2) genes, complete cds. GenBank: DQ357065.1
 - B19 virus isolate FoBe VP1 (VP1) and VP2 (VP2) genes, complete cds. GenBank: AY768535.1
 - B19 virus isolate Br543 NS1 (NS1), VP1 (VP1), and VP2 (VP2) genes, complete cds. GenBank: AY647977.1
 - Human parvovirus 4 isolate VES065CSF NS1, VP1, and VP2 genes, complete cds. GenBank: HQ593532.1
 - Human parvovirus 4 isolate VES085CSF NS1 gene, partial cds; and VP1 and VP2 genes, complete cds. GenBank: HQ593531.1
 - Human parvovirus B19 strain BB-2 NS1, VP1, and VP2 genes, complete cds. GenBank: KF724387.1

- Human norovirus (genogroup I or II) capsid protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Norwalk virus: GenBank M87661, NP056821
 - Southampton virus: GenBank L07418
 - Mexico virus: GenBank U22498
 - Seto virus: GenBank AB031013
 - Chiba virus: GenBank AB042808
 - Lordsdale virus: GenBank X86557
 - Snow Mountain virus: GenBank U70059
 - Hawaii virus: GenBank U07611
- Rabbit haemorrhagic disease virus VP60 protein, the sequence of which is indicated or can be derived, for example, from the following sequence:
 - RHDV AST/89 complete genome: GenBank: Z49271.2
 - RHDV N11 complete genome: GenBank: KM878681.1
 - RHDV CBVal16 complete genome; GenBank: KM979445.1
 - SEQ ID NO.: 32
 - SEQ ID NO.: 33
- Human papillomavirus L1 protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - HPV 6: GenBank: JN252323.1
 - HPV 11: GenBank : JQ773411.1
 - HPV 16: GenBank DQ155283.1
 - HPV 18: GenBank FJ528600.1
- Hepatitis E virus E2 protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Hepatitis E virus, complete genome NCBI Reference Sequence: NC_001434.1
 - Swine hepatitis E virus isolate ITFAE11 capsid protein gene. GenBank: JN861806.1
- Infectious bursal disease virus VP1, VP2 and VP3 proteins, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Infectious bursal disease virus VP1 (VP1) gene, complete cds. GenBank: AY099457.1
 - Infectious bursal disease virus isolate PT VP1 gene, complete cds. GenBank: DQ679814.1

- Infectious bursal disease virus isolate OE/G2 VP1 gene, complete cds. GenBank: DQ679813.1
- Infectious bursal disease virus isolate OA/G1 VP1 gene, complete cds. GenBank: DQ679812.1
- Infectious bursal disease virus isolate HOL VP1 gene, complete cds. GenBank: DQ679811.1
- Infectious bursal disease virus strain TL2004 VP1 gene, complete cds. GenBank: DQ118374.1
- Infectious bursal disease virus isolate CA-K785 VP1 gene, complete cds. GenBank: JF907705.1
- Infectious bursal disease virus isolate D495 VP1 gene, complete cds. GenBank: JF907704.1
- Infectious bursal disease virus strain A-BH83 VP1 mRNA, complete cds. GenBank: EU544149.1
- Infectious bursal disease virus strain Cro-Pa/98 VP1 gene, complete cds. GenBank: EU184690.1
- Infectious bursal disease virus VP2 mRNA, complete cds. GenBank: AY321509.1
- Infectious bursal disease virus VP2, VP3, VP4 genes, complete cds. GenBank: M97346.1
- Infectious bursal disease virus VP2 gene, complete cds. GenBank: AF508177.1
- Calicivirus capsid protein, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Feline calicivirus capsid protein gene, complete cds. GenBank: L09719.1
 - Feline calicivirus capsid protein gene, complete cds. GenBank: L09718.1
 - Human calicivirus HU/NLV/Wortley/90/UK RNA for capsid protein (ORF2), strain HU/NLV/Wortley/90/UK. GenBank: AJ277618.1
 - Human calicivirus HU/NLV/Thistlehall/90/UK RNA for capsid protein (ORF2), strain HU/NLV/Thistlehall/90/UK. GenBank: AJ277621.1
 - Human calicivirus HU/NLV/Valetta/95/Malta RNA for capsid protein (ORF2), strain HU/NLV/Valetta/95/Malta. GenBank: AJ277616.1
 - Human calicivirus NLV/Stav/95/Nor capsid protein gene, complete cds. GenBank: AF145709.1
 - Bovine enteric calicivirus strain Bo/CV500-OH/2002/US capsid protein gene, complete cds. GenBank: AY549155.1

- Human calicivirus NLV/Mora/97/SE capsid protein gene, complete cds. GenBank: AY081134.1
- Human calicivirus NLV/Potsdam 196/2000/DE capsid protein gene, complete cds. GenBank: AF439267.1
- Human calicivirus NLV/1581-01/SWE capsid protein gene, complete cds. GenBank: AY247442.1
- Human calicivirus Hu/NLV/GII/MD134-10/1987/US capsid protein gene, complete cds. GenBank: AY030313.1
- Astrovirus ORF2-encoded proteins, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Porcine astrovirus 4 ORF1b gene, partial cds; and ORF2 gene, complete cds. GenBank: JX684071.1
 - Astrovirus MLB1 HK05, complete genome. NCBI Reference Sequence: NC_014320.1
 - Astrovirus wild boar/WBAstV-1/2011/HUN, complete genome. NCBI Reference Sequence: NC_016896.1
 - Human astrovirus BF34, complete genome. NCBI Reference Sequence: NC_024472.1
 - Astrovirus MLB1 strain Hu/ITA/2007/PR326/MLB1 RNA-dependent RNA polymerase (ORF1b) gene, partial cds; and capsid protein (ORF2) gene, complete cds. GenBank: KF417713.1
 - Human astrovirus 5 strain Hu/Budapest/HUN5186/2012/HUN nonstructural protein (ORF1a) and nonstructural protein (ORF1b) genes, partial cds; and capsid protein (ORF2) gene, complete cds. GenBank: KF157967.1.
 - Human astrovirus 1 isolate Shanghai capsid protein (ORF2) gene, complete cds. GenBank: FJ792842.1
 - Human astrovirus type 8 orf2 gene for capsid protein. GenBank: Z66541.1
- Influenza virus HA, NA and M1 proteins, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - SEQ ID NO.: 30
 - Influenza A virus (A/duck/Chiba/25-51-14/2013(H7N1)) HA gene for hemagglutinin, complete cds. GenBank: AB813060.1
 - Synthetic construct hemagglutinin (HA) mRNA, complete cds. GenBank: DQ868374.1
 - Influenza virus A (A/swine/Shandong/2/03(H5N1)) hemagglutinin (HA) gene, complete cds. GenBank: AY646424.1

- cDNA encoding HA of influenza type A. GenBank: E01133.1
- Influenza A virus (A/swine/Korea/S452/2004(H9N2)) NA gene, complete cds. GenBank: AY790307.1
- Influenza A virus (A/Thailand/2(SP-33)/2004(H5N1)) neuraminidase (NA) gene, complete cds. GenBank: AY555152.3
- Influenza A virus (A/swine/Binh Dong/02_16/2010(H1N2)) NA gene for neuraminidase, complete cds. GenBank: AB628082.1
- Influenza A virus (A/chicken/Jalgaon/8824/2006(H5N1)) neuraminidase (NA) gene, complete cds. GenBank: DQ887063.1
- Influenza A virus SC35M M2 and M1 genes, complete cds. GenBank: DQ266100.1
- Influenza virus type /Leningrad/134/47/57 (H2N2) M1 and M2 RNA, complete cds's. GenBank: M81582.1
- Influenza A virus SC35M M2 and M1 genes, complete cds. GenBank: DQ266100.1
- Influenza A virus (A/Tochigi/2/2010(H1N1)) M2, M1 genes for matrix protein 2, matrix protein 1, complete cds. GenBank: AB704481.1
- Hepatitis B core and surface antigens, the sequence of which is indicated or can be derived, for example, from the following sequences:
 - Hepatitis B virus strain HBV248 precore protein and core protein genes, complete cds. GenBank: KP857118.1
 - Hepatitis B virus strain HBV401 precore protein and core protein genes, complete cds. GenBank: KP857113.1
 - Hepatitis B virus strain HBV403 precore protein and core protein genes, complete cds. GenBank: KP857068.1
 - Hepatitis B virus S gene for hepatitis B surface antigen, partial cds, isolate: B0503327(PTK). GenBank: AB466596.1

Preferably, the recombinant protein is not a protein which is endogenously produced by pupae. For example, the recombinant protein is not a protein which is endogenously produced by pupae such as pupae of the species *Hyalophora cecropia*. For example, the recombinant protein is not cecropin A or attacin.

The pupa of the present invention may be a silk-free pupa, or may be inside a silk cocoon. If the pupa is not a silk-free pupa, the skilled person is aware of methods of removing the cocoon silk. For instance, the cocoon silk may be dissolved, preferably with a solution of a

salt, preferably a salt of hypochlorous acid (HClO), preferably sodium hypochlorite (NaClO) (also referred to in the present description as “dissolving solution”). This procedure may be automated by a specifically designed device, as shown in **Figure 3**.

Use of the pupa of the present invention for the expression of recombinant proteins

The pupa of the present invention, in any of its variants, may be used for the expression of recombinant proteins. Accordingly, the present invention provides the use of the pupa of the present invention for the expression of recombinant proteins, preferably the recombinant proteins detailed above in this specification.

Methods for producing recombinant proteins of the present invention

The present invention also provides methods for producing recombinant proteins (which, as already described above, are preferably not proteins which are endogenously produced by pupae). For instance, a method for providing at least one recombinant protein according to the present invention comprises, or, alternatively, consists of, the following steps:

- (a) Providing a pupa;
- (b) Inoculating the pupa of step (a) with a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV);
- (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the at least one recombinant protein to be expressed;
- (d) Obtaining the pupae comprising the at least one recombinant protein;
- (e) Optionally, harvesting the at least one recombinant protein; and
- (f) Optionally, purifying the at least one recombinant protein.

The pupa of step (a) of the above method may preferably be the pupa according to the present invention, as described in detail above. The pupa of step (a) preferably belongs to the order *Lepidoptera*. Preferably, the pupa belongs to the order *Lepidoptera*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*, more preferably to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia cynthia*, even more preferably to the species *Trichoplusia ni*. As already described above, the pupa may comprise a nucleic acid sequence that allows for the expression of a recombinant protein. As already described above, the pupa may comprise a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection (see above, “Pupa of the present”).

invention”).

The pupa of step (a) above may evolve from an egg. For example, the pupa of step a) above may evolve from egg to pupa for 15-18 days, e.g., in specially designed single-use or reusable rearing boxes. The pupa of step a) may also be provided already in the form of a pupa.

If the pupa of step (a) above is inside a cocoon, the method for producing recombinant proteins of the present invention may further comprise a step of removing the cocoon silk from the pupa. For instance, the cocoon silk may be dissolved, preferably with a solution of a salt which preferably comprises hypochlorous acid (HClO), preferably sodium hypochlorite (NaClO), for example at concentrations of 0.1% to 5% W/V, such as 0.1%, 0.2%, 0.5%, 1%, 3% or 5% W/V. For example, the pupae may be immersed or sprayed with a solution of sodium hypochlorite at a concentration of from 0.1% to 5% W/V. The dissolution of the cocoon silk may take from several seconds to several minutes. The elimination of the cocoon silk from the pupa of step (a), when the pupa is inside a cocoon, is preferably performed in a semi-automated or automated form (robotized silk elimination). In this regard, the fact that the cocoon of some species such as *Hyalophora cecropia* or *Bombyx mori* is a thick cocoon is a disadvantage over other genera or species (such as genera *Trichoplusia*, in particular the species *Trichoplusia ni*). Pupae of the genera *Trichoplusia*, in particular the species *Trichoplusia ni*, have a cocoon which is less dense in silk, and can be easily dissolved. This represents an advantage, since the whole process can be performed in an automatized or semi-automatized form, increasing the efficiency and reducing the overall costs.

The cocoon may be removed in a semi-automated machine which may comprise a recipient with the dissolving solution to be applied to the cocoon, preferably with pressurized air turbulences, to reduce the time needed for dissolving the silk cocoon. The silk-free pupae may then be washed in a washing container to remove traces of the dissolving solution, and then dried with air.

Accordingly, silk-free pupae are preferred, since then, step (b) is easier to perform. Accordingly, pupae with very dense cocoon are less preferred. For example, pupae of *Bombyx mori* have a very dense, thick and compact cocoon, which cannot be easily removed by dissolution with a salt solution for a few minutes, as described above. The same is true for the pupae of *Hyalophora cecropia*. The cocoon of *Bombyx mori* and/or *Hyalophora cecropia* pupae should be manually removed.

On the contrary, pupae of other species such as *T. ni* comprise a cocoon which is less dense in silk, and can be easily dissolved as described above. These pupae are thus preferred for the method of producing recombinant proteins of the present invention, since their cocoon can be removed by automatic or semiautomatic procedures, facilitating scale-up for obtaining pupae ready to be injected with a recombinant baculovirus (step (b)).

After removing the cocoon silk, the pupae are preferably washed with water, in order to remove traces of the salt solution (e.g., sodium hypochlorite). Silk-free pupae may be subsequently dried and stored at a low temperature (e.g., 4°C) before step (b) is carried out. For instance, silk-free pupae may be storage up to 1 month at low temperature (e.g., 4°C) before step (b) is carried out.

Accordingly, the present invention also provides a method for producing a silk-free pupa (preferably a silk-free pupa belonging to the genus *Lepidoptera*, preferably the pupa of the present invention), comprising the steps of:

- (a) Providing a pupa (preferably the pupa of the present invention as described above) contained in a cocoon;
- (b) Treating the pupa contained in a cocoon with a solution of a salt, preferably a solution of a salt of hypochlorous acid, preferably sodium hypochlorite, as described in detail above; and
- (c) Obtaining a silk-free (and preferably essentially disinfected) pupa.

The present invention thus also provides the pupa of the present invention which is essentially silk-free (namely, without a cocoon). In a preferred embodiment, the essentially silk-free pupa of the present invention does not belong to the species *Bombyx mori*. In a preferred embodiment, the essentially silk-free pupa of the present invention does not belong to the species *Hyalophora cecropia*. In a preferred embodiment, the essentially silk-free pupa of the present invention belongs to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*. In a preferred embodiment, the essentially silk-free pupa of the present invention belongs to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia cynthia*, even more preferably to the species *Trichoplusia ni*.

Step (b) of the method of the present invention is directed to the inoculation of the pupa of step (a) with a recombinant baculovirus derived from *Autographa californica multicapsid*

nucleopolyhedrovirus (AcMNPV). Recombinant baculovirus which may be comprised in the pupa of the present invention have been described in detail above, and an exemplary schema is also shown in Figure 4B. Accordingly, preferably, in step (b), the pupa of the present invention is inoculated with a recombinant baculovirus, in order to provide a pupa according to the present invention, comprising a recombinant baculovirus, as described above. The patent application published as WO2012/168492 discloses recombinant baculovirus that may be inoculated to the pupa according to the present invention in step (b) of the method for producing recombinant proteins of the present invention. The recombinant baculovirus comprises a nucleic acid sequence encoding a recombinant protein, preferably a recombinant protein selected from the group as defined above in this specification (expression cassette).

Silk-free pupae are preferred, since then the inoculation with the recombinant baculovirus is easier, and can be performed automatic or semiautomatic, facilitating the scale-up and reproducibility of the method.

The inoculation of the recombinant baculovirus according to step (b) may be performed by techniques known in the art to the skilled person. As defined herein, “*inoculation*” refers to the introduction of a substance into the body, in this case, the introduction of recombinant baculovirus into pupae. The inoculation may also be referred to as “*injection*”. Since the larvae are inoculated with a baculovirus, this process may also be referred to in the present description as “*infection of the larvae with a baculovirus*”.

Preferably, the inoculation is performed by injecting the pupa a specific amount of a solution comprising at least one baculovirus. The injection is preferably performed with a needle, which perforates the pupa and dispenses a specific amount of a solution comprising the baculovirus inside the body of the pupa. This step can also be automated; pupae may lay, e.g., in a matrix or array of alveolus, and an inoculation device or robot (described below) may automatically dispense the baculovirus inside the pupae. For instance, the pupa may lay in a matrix of alveolus (or array), the matrix including a top with a hole in the centre, so that the inoculation device or robot (comprising a needle, e.g., on a robotic arm) automatically positions the needle on the alveolus, and the needle access the pupa through the hole of the top. The needle penetrates the pupa's body, e.g., about 1-5 millimetres, preferably about 3 mm (this can also be automated) and dispenses the solution comprising the baculovirus inside the pupa. The device or robot may comprise a precision pump able to dispense an exact amount of baculovirus (e.g., about 0.5-10 microliter amounts of a solution comprising the baculovirus, preferably about 5 µl) into the pupae. The robot may comprise an arm comprising one or more needles able to inject the baculovirus into the pupae at precise

positions. Once this has been performed, the robot leaves the alveolus, and, thanks to the top of the matrix where the pupae lay, the pupa is easily left on the matrix alveolus, and it is not removed from it when the robot arm removes the needle from the pupa, since the hole on the top of the matrix is smaller than the pupa, so that only the needle can go through it, so the needle is removed from the pupa and from the alveolus matrix leaving the pupa in it. With this preferable disposition, the inoculation process is automatic, rapid, efficient and highly reproducible (every pupa receives the same amount of solution comprising the baculovirus, with the same procedure). The robot may have several inoculation needles (namely, needles able to inoculate or inject the baculovirus into the pupae at precise positions) which may be removable, and may inoculate baculovirus into the pupae at a speed of between about 3.000 and about 10.000 pupae per hour.

For example, an amount of more than about 50 plaque-forming units (PFUs) of baculovirus is inoculated into each pupa. For example, about 50, about 100, about 500, about 1,000, about 5,000, about 10,000, about 15,000, about 20,000, about 25,000, about 30,000, about 40,000, about 50,000, about 60,000, about 75,000 or more PFUs (even about 1,000,000) are inoculated into each pupa. For example, as already mentioned above, the baculovirus are comprised in a solution, so a certain amount of a solution comprising baculovirus is injected into the pupae. For example, the solution may be cell culture media comprising baculovirus. For example, the solution may be a buffered solution comprising baculovirus, such as a solution of PBS 1x pH 7.4, 1 mM PMSF (proteases inhibitor) and 5mM DTT comprising baculovirus. For example, the pupae can be inoculated with an amount of about 0.5 – 10 μ l of a solution comprising a baculovirus, such as about 0,5 μ l, such as about 1-10 μ l, such as about 1, about 2, about 3, about 5, about 7 or about 10 μ l. For example, each pupa receives 0.5 – 10 μ l of a solution comprising between 50 to 1,000,000 PFUs.

Thanks to the automation of the inoculation, the robot may inoculate about 3,000 pupae per hour or more, such as between about 3.000 and about 10.000 pupae per hour. The robot is suitable to deliver a fluid into pupae provided in a matrix or array.

Step (c) of the method of the present invention comprises incubating the inoculated pupa of step (b) for a period of time sufficient for the at least one recombinant protein to be expressed. This incubation step may be preferably performed for a period of time of at least about 72 h, such as about 72h, about 96h, about 100h, about 125h, about 150h, about 168h or more (post-inoculation). Preferably, the incubation time is about 96-168h post-inoculation or about 72-168h post-inoculation. The inoculation step may be preferably performed at a temperature of about at 22-28 °C, preferably in an incubator without any need for light or humidity control.

The inoculated pupae (preferably laying in the alveolus of the matrix) may be left on the alveolus of the matrix for the above period of time (incubation). In another embodiment, the pupae are removed from the matrix and placed anywhere else for the incubation time, such as bags or any recipient that allows transpiration (the incubation should be preferably performed in a container where there is an exchange of gases, not in a completely closed container).

The skilled person would calculate the required incubation time and incubation conditions for each pupae and each recombinant protein on the basis of previous experiments, depending on the protein to be expressed.

Step (d) of the method of the present invention comprises obtaining the pupae comprising the at least one recombinant protein which has been expressed during the incubation step (c). The pupae comprising the at least one recombinant protein may be collected from the matrix/array (or incubation place). These pupae comprising the at least one recombinant protein may be stored (frozen or lyophilized) for subsequent processing. For example, the pupae comprising the at least one recombinant protein may be packed in a package (preferably under vacuum). The package comprising the pupae may be stored, transported and/or further processed.

The present invention also provides a package comprising the pupae of the present invention, wherein preferably, the pupae comprise at least one recombinant protein. Preferably, the pupae are packaged under vacuum, and the package of the present invention thus comprises vacuum pupae, which are e.g., frozen or lyophilized, to avoid oxidation. This allows for an easier manipulation and longer storage periods.

The pupae obtained according to step (d) of the above method may also be frozen and stored for further processing. For instance, the pupae may be frozen immediately after collecting them at about -20°C, or at about -80°C, until they are further processed. For example, the pupae comprising the at least one recombinant protein may be frozen at a temperature of, for example, between about -20°C and -70°C, until the recombinant protein is extracted. The pupae comprising the recombinant protein may be stored frozen for long period of times, such as, for example, for more than 1 year.

(Optional) step (e) of the method of the present invention comprises, optionally, harvesting the at least one recombinant protein. The skilled person is aware of methods and protocols for harvesting the at least one recombinant protein comprised in the pupae obtained in step

(d). Of course, these methods and/or protocols may depend on the nature of the recombinant protein that has been expressed.

For example, the protein may be extracted by homogenising the pupae (e.g., in a homogenizer machine or homogenizer mixer, such as a blender homogenizer or a rotor-stator homogenizer, for at least several seconds/minutes), preferably in the presence of a neutral pH buffer, which buffer preferably comprises anti-oxidants (reducing agents), protease inhibitors and appropriate detergents. For example, the buffer comprises about 1-25 mM of a reducing agent and about 0.01%-2% (volume/volume) of a detergent product. Preferably, the buffer further comprises a mixture of protease inhibitors.

After homogenisation the extracts may be sonicated and/or centrifuged (e.g., 15,000-20,000 G) to eliminate the insect debris, and filtrated.

For example, the extraction means (methods and protocols for harvesting the at least one recombinant protein comprised in the pupae) comprise physically disrupting the pupae, centrifugations, tangential flow filtration steps, sonication, chromatographic methods and/or sterilizing filtrations.

In a preferred embodiment, the viscosity of the homogenate may be reduced through its incubation (filtration) with a diatomaceous earth (e.g., Celite). A protein precipitation step could be included.

The extract may be clarified through tangential flow filtration, using a filter that the skilled person is available to select depending on the nature of the recombinant protein. The buffer may be changed by a diafiltration process (e.g., in the same tangential filtration device).

(Optional) step (f) of the method of the present invention comprises, optionally, purifying the at least one recombinant protein. The skilled person is aware of protein purification techniques. For instance the purification may be achieved by chromatography purification, filtration or ultracentrifugation.

The purified recombinant protein may thus be obtained. A schema of one embodiment of this method is shown in Figure 17.

The recombinant protein obtainable by the method of the present invention has a high purity degree, and may be used in vaccines, diagnosis, cosmetics or in therapy.

Method for producing a recombinant baculovirus of the present invention

The present invention further provides a method for producing a recombinant baculovirus comprising the steps of:

- (a) Providing a pupa,
- (b) Transfecting the pupa of step (a) with a bacmid vector suitable for producing a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV);
- (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the recombinant baculovirus is produced;
- (d) Obtaining the pupae comprising the recombinant baculovirus;
- (e) Optionally, harvesting the recombinant baculovirus; and
- (f) Optionally, purifying the recombinant baculovirus.

Preferably, the pupa provided in step (a) is a silk-free pupa as defined above. The pupa preferably belongs to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*, more preferably to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia cynthia*, even more preferably to the species *Trichoplusia ni*.

In step (b), the pupa of step (a) is transfected with a bacmid vector suitable for producing a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV).

This step (b) may also be referred to as “*inoculation step*”, since it can be performed in a similar way as step (b) of the method for producing a recombinant protein described above (but instead of inoculating a baculovirus, a bacmid vector is inoculated), namely in an automatic or semiautomatic way, using the device of the present invention (also described below). The device may inoculate into the pupa (which is preferably placed in the alveolus of a matrix, comprising a tap with holes) a specific amount of a solution comprising the transfer vector and/or bacmid to be inoculated to the pupa, by means of injection with a needle, as described above.

The bacmid vector may be generated by procedures known to the skilled person, for example by generating sequentially a cloning vector, a donor vector, a transfer vector and, finally, a

bacmid vector. For example, the generation of a bacmid vector is described in the patent application published as WO 2014/086981.

In a preferred embodiment, this transfer vector and/or bacmid comprises or alternatively, consists of, a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein. These nucleic acid sequences have been already described above. For instance, the nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof is preferably selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

In addition, the promoter that drives the expression of said recombinant protein is preferably selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-14, preferably indicated in any of SEQ ID NOs: 11-13; and
- (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-14, preferably indicated in any of SEQ ID NOs: 11-13.

The recombinant homologous region (*hr*) is preferably the sequence indicated in SEQ ID NO: 21 (*hrI*). The nucleic acid sequence that comprises combinations of recombinant promoter, sequences encoding transcriptional regulators and enhancer regions are preferably selected from the group comprising SEQ ID NO: 15-20.

Preferably, the vector inoculated to the pupae is a bacmid.

Step (c) refers to the incubation of the inoculated pupa of step (b) for a period of time sufficient for the recombinant baculovirus is produced. For example, this period of time for pupa of *T. ni* may be from about 72h to about 7 days, depending on the virus dose and temperature. The skilled person is able to calculate the period of time sufficient for the recombinant baculovirus to be produced.

Then, the pupae comprising the recombinant baculovirus is obtained (step (d)). The pupae comprising the recombinant baculovirus may be stored for further processing. For instance, they may be vacuum packaged, to reduce the oxidation process and facilitate their manipulation and increase the safe storage time. For example, the pupae comprising the recombinant baculovirus may be frozen (e.g., at -20°C to -80°C) before they are further processed. For example, the pupae comprising the recombinant baculovirus may be lyophilized before they are further processed.

Optionally, the recombinant baculovirus may be harvested and purified (steps (e) and (f)).

Device of the present invention

The present invention also provides a device (also referred to in the present invention as robot) comprising a precision pump, a mobile mechanic arm and a (removable) needle suitable for injecting a fluid (preferably a solution comprising baculovirus or bacmid vectors) into a pupa (preferably to the pupa of the present invention, which is preferably a silk-free pupa and which preferably belongs to the order *Lepidoptera*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the genus *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis* or *Helicoverpa*, more preferably to the species *Trichoplusia ni*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Heliothis virescens*, *Helicoverpa armigera*, *Helicoverpa Zea*, *Manduca sexta*, *Ascalapha odorata* or *Samia cynthia*).

As described in detail above, the pupa may preferably be provided in a matrix (or array), namely the pupa lays in the alveolus of a matrix, so that the device can easily locate the pupae

in an automatic manner. Preferably, the matrix comprises a taps, with wholes that are smaller than the pupa (namely the pupae cannot pass through the holes).

The device may further comprise a computer program for defining the position of the needle (and/or the position of the mechanic arm) and/or for calculating the distance from the tip (end) of needle to the pupa and/or the distance of penetration of the needle into the pupa and/or the volume of liquid (preferably a solution comprising baculovirus or bacmid vectors) to be inoculated into the pupa. In addition, the device of the present invention may further comprise a computer program for calculating the inoculation time and/or the time between different inoculations into the pupae. The device may further comprise a camera for defining the position of the needle.

In a preferred embodiment, the fluid injected by the device of the present invention comprises a recombinant baculovirus. In another embodiment, the fluid injected by the device of the present invention comprises a bacmid vector suitable for producing a recombinant baculovirus derived from AcMNPV, as described above in this description.

The device of the present invention is suitable for performing step (b) of the method for producing a recombinant protein of the present invention and step (b) of the method of providing a recombinant baculovirus of the present invention.

For example, the device of the present invention is suitable to inject (inoculate) into the pupa an amount of fluid which is in the range of from about 0,5 to about 10,0 μL , such as about 0,5 μl , such as about 1-10 μl , such as about 1, about 2, about 3, about 5, about 7 or about 10 μL .

For example, the needle comprised in the device of the present invention is able to penetrate into the pupa a distance of from about 1 to about 4,5 mm, preferably about 3 mm.

For example, the device of the present invention comprises several removable inoculation needles, and is able to inoculate fluid (preferably a solution comprising baculovirus or bacmid vectors) at speeds of between about 3.000 and 10.000 pupae per hour.

For example, the fluid injected by the device of the present invention comprises baculovirus, preferably in an amount of from 50 to 10^6 PFUs/dose injected into each pupa. For example, the device of the present invention injects (inoculates) to each pupa an amount of more than about 50, or more than about 100, or more than about 500 plaque-forming units (PFUs) of baculovirus. For example, about 50, about 100, about 300, about 500, about 1,000, about

5,000, about 10,000, about 15,000, about 20,000, about 25,000, about 30,000, about 40,000, about 50,000, about 100,000, about 500,000 or more PFUs are inoculated into each pupa, such as about 1,000,000 PFUs.

The device of the present invention preferably comprises high precision pumps which enable the device to inoculate the desired volume of fluid (preferably a solution comprising baculovirus or bacmid vectors) into the pupae with a high precision.

The device of the present invention is suitable to deliver a fluid into pupae provided in a matrix or array.

Preferably, the device of the present invention further comprises a computer program for calculating the inoculation time and/or the time between inoculations into the pupae, which should be enough time to dispense the liquid containing the baculovirus to each pupa.

Preferably, the device of the present invention further comprises a camera for defining the position of the needle.

The present invention further provides a device for silk removal (silk-removal device). A schematic representation of an example of silk-removal device of the present invention is represented in Figure 3 (semi-automatic device for silk removal from the *T. ni* cocoons).

The silk-removal device of the present invention (also referred to as “silk-removal machine”) comprises at least one container which contains a silk-dissolving solution, as explained above. For example, the container comprises hypochlorous acid. The first container may preferably also comprise a system to project the liquid through the rearing modules containing the cocoons (it helps to dissolve more efficiently the silk surrounding the pupa). The dissolving solution is preferably applied to the cocoon with pressurized air turbulences, to reduce the time needed for dissolving the silk cocoon.

Preferably, the silk-removal device of the present invention further comprises a second container, which is a wash-container and comprises and/or disperses a solution suitable for removing traces of silk and silk-dissolving solution from the pupae, such as water. The solution (preferably water) is preferably sprayed over the chrysalises (pupae). Preferably, on top of this container, there is a system that dispenses air to dry the pupae. Accordingly, after the washing of the pupae, they are preferably dried with air. At the end of the process, the pupae are free of silk and ready to be infected or to stored (refrigerated) until use.

Summary of sequences

SEQ ID NO:	Name:
1	Complete <i>Ac-ie-01</i> cDNA
2	Protein coding sequence (CDS) of IE-1
3	CDS of IE-0
4	CDS of the IE-1 N-terminal domain
5	CDS of the IE-0 N-terminal domain
6	IE-1 protein
7	IE-0 protein
8	IE-1 N-terminal domain protein
9	IE-0 N-terminal domain protein
10	<i>polh</i> (promoter)
11	<i>p10</i> (promoter)
12	<i>pB2₉p10</i> (promoter)
13	<i>p6.9p10</i> (promoter)
14	<i>pB2₉</i> (promoter)
15	<i>polhAc-ie-01/hr1p10</i>
16	<i>polhAc-ie-01/hr1pB2₉p10</i>
17	<i>polhAc-ie-01/hr1p6.9p10</i>
18	<i>pB2₉Ac-ie-01/hr1p10</i>
19	<i>pB2₉Ac-ie-01/hr1pB2₉p10</i>
20	<i>pB2₉Ac-ie-01/hr1p6.9p10</i>
21	Homologous region enhancer <i>hr1</i>
22	<i>polhAc-ie-01</i>
23	<i>polhGFP</i>
24	<i>hr1pB2₉p10</i>
25	ORF2 from porcine circovirus type 2
26	Capsid protein (Cap) from porcine circovirus type 2
27	<i>polhAc-ie-01/hr1p6.9p10Cap</i> (including the polyadenylation signal from the <i>p10</i> gene after the <i>Cap</i> gene)
28	<i>polhCap</i>
29	<i>polhAc-ie-01/hr1p6.9p10Cap</i>
30	Hemagglutinin protein without transmembrane domain from an H1 influenza virus, PR8 strain (MelHAHis)

SEQ ID NO:	Name:
31	<i>polhAc-ie-01/hrIp6.9p10HA1 (including the polyadenylation signal from the p10 gene after the H1 gene) (polhAc-ie-01/hrIp6.9p10MelHA)</i>
32	<i>VP60 from RHDV AST789 (genogroup G1)</i>
33	<i>VP60 from RHDV N11 (genogroup RHDVb)</i>
34	<i>polhAc-ie-01/hrIp6.9p10VP60G1 (including the polyadenylation signal from the p10 gene after the H1 gene)</i>
35	<i>polhAc-ie-01/hrIp6.9p10VP60RHDVb (including the polyadenylation signal from the p10 gene after the H1 gene)</i>
36	<i>VP60 from RHDV AST789 (genogroup G1) (amino acid sequence)</i>
37	<i>VP60 from RHDV N11 (genogroup RHDVb) (amino acid sequence)</i>
38	<i>polhAc-ie-01/hrIp6.9p10GFP</i>

EXAMPLES

Example 1. Production of *T. ni* pupae

Insect larvae were reared in re-usable or single-use rearing boxes containing several hundreds of larvae that were allowed to evolve for 15-18 days from egg to pupa (**Figure 1**). Then, whole rearing boxes or collected pupae were immersed or sprayed with a solution of sodium hypochlorite at concentrations of 0.1% to 5% W/V to dissolve the silk fibers of the cocoons. Silk was dissolved in a few minutes and pupae were then washed with water to remove traces the hypochlorite. Pupae were subsequently dried and stored at 4°C until baculovirus inoculation. This process is more simple with respect to the same operation with *Bombyx mori* cocoons because of the lower density of silk treats of *T. ni* Lepidoptera, as can be seen in **Figure 2**. *Bombyx mori* cocoons require manual intervention to liberate the pupae, while *T. ni* silk is easy to dissolve and remove by automatic or semiautomatic procedures, facilitating scale-up the obtaining of pupae ready to be injected with a recombinant baculovirus for recombinant protein production.

Example 2. Dual recombinant baculoviruses containing an enhancer sequence (*hrI*) operatively linked to a chimeric promoter (*p6.9* and *p10*) and the over-expressed IE-1 and IE-0 transactivation factors are highly efficient in producing recombinant proteins in insect pupa

The recombinant protein expression driven under the control of a chimeric promoter enhanced by the baculovirus homologous repeated sequence *hrI* and trans-activated by the over-expressed IE-1 and IE0 factors was compared in insect pupa (*T. ni*) to that obtained by

using a conventional baculovirus. The encoding gene for the recombinant Green fluorescent protein (GFP) was cloned in a conventional AcMNPV baculovirus under the control of polyhedrin promoter by conventional means (**Figure 4A**). Another AcMNPV baculovirus modified by the cassette containing the above mentioned regulatory elements (TB expression cassette) was also generated containing the GFP encoding gene (**Figure 4B**). Pupae were infected with 50,000 PFUs of each baculovirus by an inoculation robot (**Figure 5A**), comprising a precision pump able to dispense microliter amounts of the virus inoculum and a robotic arm able to inject the virus in precise positions. Pupae were allocated in a matrix of alveolus with a top with a hole in the center of every alveolus (**Figure 5B**). The inoculation needle (removable) accessed to the pupae through the hole and penetrated the pupae several millimeters to inject the baculovirus (**Figure 5C**). The recombinant virus contained in 0.5 to 10 microliters (μ l) was dispensed into the pupae and during retraction of the needle the pupae were retained in the alveolus due to the top. This inoculation robot showed an inoculation speed of at least 3,000 pupae per hour, at least 6 times more speed than manual inoculation by a skilled person.

After an incubation period after inoculation of 96-168h, the pupae were collected and protein was extracted in a homogenizer machine in presence of a neutral pH buffer containing anti-oxidants, protease inhibitors and non-ionic detergents (e.g., PBS1X pH 7,4 + 5mM Dithiothreitol (DTT) + 1mM phenylmethylsulfonyl fluoride (PMSF) + 0,1% Brij 35 + 0,5% Sarkosyl. Extracts were centrifuged at 15,000-20,000 g and filtered. Extracts were analyzed by SDS-PAGE electrophoresis and gels were stained with Coomassie blue (**Figure 6A**). The production yields (expressed as milligrams per biomass unit) of the GFP protein produced by each baculovirus in the infected pupae were calculated by densitometry using a bovine serum albumin (BSA) curve. This analysis rendered an increase of around 5.6 times of GFP productivity in pupae obtained by the baculovirus genetically modified with the TB cassette with respect to that obtained with the conventional baculovirus (namely a baculovirus comprising, e.g., the polyhedrin promoter without any other regulatory element in the expression cassette) (**Figure 6B**).

Example 3. Expression of different proteins by TB-modified baculoviruses in *T. ni* pupae

To analyze the benefit of using the TB (TopBac) expression cassette (SEQ ID NO.: 17) for the expression of further proteins, two genes were cloned in the TB cassette and the corresponding recombinant baculoviruses were obtained, as well as conventional baculoviruses expressing the genes under the control of polyhedrin promoter (*polh*) (**Figure 7**). The two genes used for obtaining the recombinant baculoviruses were those encoding for

the proteins Cap from Porcine circovirus type 2 derived from the PCV2a GER3 strain (SEQ IS NOs: 25 and 27) and the hemagglutinin from influenza virus (H1) derived from the virus strain A/PR/8/34 (SEQ ID NOs: 30 and 31). The resulting TB(-) (namely a conventional baculovirus that includes the polyhedrin promoter to express the protein in the expression cassette but which was not modified by the TopBac (TB) expression cassette) and TB(+) baculoviruses were compared for their productivity in *T. ni* pupae using the same infection and protein extraction protocols as the ones used and described in Example 2.

The comparison of production yields (expressed as milligrams per biomass unit) in infected pupae mediated by conventional (TB(-)) or TB-modified (TB(+)) baculoviruses showed that for both proteins the expression cassette TB increased the production yield. In the case of the porcine circovirus Cap protein, an increase of 2.79 times in protein production when the pupa was infected with a TB-modified baculovirus as compared with pupa infected with conventional baculovirus (Figure 8). For the HA protein from influenza virus this increase was of 2.04 times (Figure 9). These results confirmed that the TB cassette significantly increases the production of recombinant proteins in *T. ni* pupae.

Example 4. Production of recombinant proteins in Baculovirus infected *T. ni* pupae is more efficient than larvae

The comparative expression of five recombinant proteins in TB(+) baculoviruses in *T. ni* pupae and larvae was carried out. The expressed proteins were the following: GFP (SEQ ID NO.: 23), Cap (SEQ ID NO.: 25), HA (SEQ ID NO.: 30) and the VP60 (capsid protein) from two rabbit hemorrhagic calicivirus strains (RHDV genogroup 1 and RHDVb; SEQ ID NOs: 32 and 33) (Figure 10). Pupae and larvae were infected with 50,000 PFUs of the corresponding TB(+) baculoviruses. Infected insects (larva and pupa) were collected at 96h post-infection. Soluble protein extracts were analyzed by SDS-PAGE electrophoresis stained with Coomassie blue (Figures 11A, 12A, 13A and 14A). Recombinant proteins were quantified by densitometry using a BSA curve and production yields were expressed as milligrams per biomass unit (Figures 11B, 12B, 13B and 14B). In all cases, pupae expressed higher quantities of recombinant protein than larva, with increasing ratios from 1.06 to 3.64.

Example 5. Production of recombinant virus-like particles (VLPs) in *T. ni* pupae

To demonstrate the production of VLPs in infected pupae, protein extracts from infected pupae with the TB(+) baculoviruses expressing the VP60 protein from the two rabbit calicivirus strains analyzed in Example 4 were processed for VLPs purification. The VLPs were extracted from infected pupae at 96 h post-infection by centrifugation in the presence of detergents (2% sarkosyl (Sigma) and 5 mM EDTA (Sigma) in a PBS (0.2 M sodium

phosphate, 0.1 M NaCl, pH 6.0) and protease inhibitors (Complete®, Roche) and incubated overnight at 4°C. Then, they were treated with DNase I (Roche Diagnostics) for 1 h at 37°C. After an additional centrifugation (2,000 x g, 5 min), supernatants were subjected to ultracentrifugation (131,453 x g; 2.5 h). Sediments were extracted twice in Vertrel (Sigma) and submitted to second ultracentrifugation (131,453 x g; 2.5 h). Finally, sediments were resuspended in PBS 1X and stored at 4°C until analysis.

Sediments were analyzed by transmission electron microscopy performed by conventional means. Briefly, purified VLPs (approximately 5 µl) were applied to glow-discharged carbon-coated grids for 2 min. Samples were negatively stained with 2% (w/v) aqueous uranyl acetate. Micrographs were recorded with an EM 2000 Ex microscope (JEOL, Japan). As shown in **Figure 14**, VLPs of the expected size and shape corresponding to RHDV were observed, demonstrating that a corrected folding and self-assembling is carried out in the baculovirus-infected pupae tissues (**Figure 15**).

Example 6. Virus inoculum production in infected *T. ni* pupae

Spodoptera frugiperda (Sf21 and Sf9) cell lines were cultured at 27°C in TNMFH medium (PAN Biotech GmbH, Germany) with 10% heat-inactivated fetal bovine serum (PAN Biotech GmbH) and gentamicin (50 µg/ml) (PAN Biotech GmbH). Cell density and viability were assessed by Trypan blue staining. Cell viability was calculated on the basis of the percentage of living cells with respect to the total number of cells at various times post-infection.

The Sf9 cells, which were cultured in suspension, were infected in spinner flasks (80 ml of culture media) at a cell density of 2×10^6 cells/ml. Cell viability at the time of infection was >99% in suspension. Sf9 cells were infected *in vitro* with recombinant baculoviruses at a multiplicity of infection (MOI) of 0.01 to 0.1. After 72-96 h post-infection, the virus inoculum was recovered from supernatants after centrifugation. The virus titer was calculated by a plaque forming unit (pfu) assay, obtaining virus titers between 10^7 to 10^8 viruses per ml.

The viruses were used to infect *T. ni* pupae with doses ranging from 5×10^2 to 10^4 pfu. After 3-7 days, the pupae were homogenized to collect the infective virus in cell culture media or in a specific PBS buffer containing DTT and protease inhibitor PMSF. Pupae homogenates were centrifuged to eliminate the pupae debris at 5,000 x g during 30 min. Then, the supernatant containing the virus was filtered sequentially by a filter of 0.45 and 0.22 microns and the resulting virus preparation was preserved by mixing with glycerol and then it could be frozen or lyophilized. The virus stocks were titrated in *T. ni* fifth instar larvae using the Karber method concretely, LID50 (larvae infectious dose 50). The statistical meaning of one

infectious dose calculated in this way is that a population of larvae infected with an ID50 will show a 50% of individual infected. Larvae were observed for at least 96 h in order to detect clinical signs and to follow their evolution to pupa. Typical virus titers were between 10^6 to 10^8 pfu/ml of virus preparation. Additionally, baculovirus inoculum can be obtained without a previous baculovirus vector generation in cell cultures. Pupae can be transfected with a bacmid obtained in bacteria by a transposition procedure. **Figure 16** represents the general procedure to obtain a virus stock in pupa by a cell-free system.

Example 7. Cell culture and viruses

The *Spodoptera frugiperda* Sf21 or Sf9 cell lines were cultured in 6-well tissue culture plates (1×10^6 cells/well) in TNM-FH insect medium (Pan Biotech™, Germany) containing 10 % heat-inactivated fetal bovine serum (Pan Biotech™, Germany) at 27°C. AcMNPV recombinant baculoviruses were obtained by the “Bac-to-Bac®” Baculovirus Expression System (invitrogen™, USA). The different TB(+) transfer vectors containing the recombinant DNA regulatory elements were generated using the pFastBac™-DUAL plasmid (invitrogen™). These transfer vectors were used to transfect Sf21 cells with Cellfectin® (invitrogen™, USA). The resulting recombinant baculoviruses from the infection of Sf21 cells were then passaged twice in cells and titered by the plaque assay method. The obtained gene constructs of the TB (+) baculovirus expression cassettes are schematically shown in **Figures 4, 7 and 10**, showing the combinations of genetic regulatory elements involved in the genes expression (*polhAc-ie-01/hr1p6.9p10*, SEQ ID NO.: 17, plus the sequence of the gene coding for the desired protein, e.g., SEQ ID NOs: 26, 30, 32 and 33). The different expression cassettes were used to generate the recombinant baculoviruses used in the examples shown in **Figures 6, 8, 9, 11, 12, 13, and 14**.

Example 8. Generation of the cloning vector

The cloning vector is a small piece of DNA containing the TB(+) baculovirus expression cassette into which a foreign DNA fragment can be inserted by treating the vehicle and the foreign DNA with a restriction enzyme that creates the same overhang, then ligating the fragments together. The essential characteristics of the cloning vector are that it must include a synthetic multiple cloning site (MCS) to facilitate the insertion of foreign genes directed in a chosen orientation, a selectable marker, such as an antibiotic resistance to allow the selection of positively transformed cells and a functional origin of replication (ORI) for propagation in bacteria

Example 9. Generation of the donor vector containing the baculovirus expression cassette of the present invention

A donor vector consists of a cloning vector, for example a pUC57 plasmid, containing the baculovirus expression cassette, into which a foreign gene has been cloned using the appropriate restriction enzymes. The TB(+) baculovirus expression cassette used was synthesized by ligating the following DNA sequences: (i) the baculovirus transcriptional regulator encoding sequence *Ac-ie-01* (e.g., SEQ ID NOs: 1-5) downstream of a promoter sequence, such as the *polh* promoter (e.g., SEQ ID NO.: 10), and upstream of the HSV TK polyadenylation signal and (ii) in another locus an enhancer sequence, for example, the homologous region *hr1*, upstream of (iii) a promoter sequence, for example, *p6.9p10* (e.g., SEQ ID NO.: 13), followed by a multiple cloning site (MCS) for cloning the gene of interest and the *p10* polyadenylation signal downstream of the MCS (Figures 4, 7 and 10). The baculovirus expression cassette is flanked by specific restriction sites (for example *Bgl*III and *Bst*Z171 at the 5'-terminal end and *Bgl* II and *Sgf* I at the the 3'-terminal end) to facilitate subcloning into a transfer vector of a commercial baculovirus generation system (based on transposition, for example the “Bac-to-Bac[®]” system (invitrogen[™]), or based on homologous recombination, for example “flashBAC[™]” (Oxford Expression Technologies[™]), “Baculogold[™]” (BD Biosciences[™]), “BacPAK6[™]” (Clontech[™]), “Bac-N-Blue DNA[™]” (invitrogen[™]).

The encoding foreign genes were cloned into the MCS of the cloning vector using the *Nco* I and *Spe* I restriction sites, generating the donor plasmid vectors.

Example 10. Generation of the transfer vector containing the baculovirus expression cassette of the present invention

The transfer vector was generated by digesting a donor vector with *Bst*Z171 of the 5'-flanking site and with *Xba* I and cloning it into the transfer vector pFastBac[™]1 that was also digested with the same enzymes. In this case, as a result of the subcloning, the SV40 polyadenylation signal of the baculovirus expression cassette is exchanged by the *p10* polyadenylation signal from the transfer vector. Apart from this, all the elements of the expression cassette are included in the pFastBac transfer vector, substituting the *polh* promoter and MCS of the original commercial transfer vector.

Example 11. Generation of the baculovirus expression vector containing the baculovirus expression cassette of the present invention using the “Bac-to-Bac[®]” system

The modified transfer vector pFastBac[™]1 and the TB(+) baculovirus expression cassette were used to generate the recombinant baculovirus by using the “Bac-to-Bac[®]” Baculovirus

Expression System. More specifically, the modified transfer vector was used to transform the *E. coli* host strain DH10Bac™ that contains a baculovirus shuttle vector (bacmid) and a helper plasmid, and allows the generation of a recombinant bacmid following transposition of the expression cassette. The DNA of the recombinant bacmid containing the TB(+) baculovirus expression cassette of the present invention and the different foreign encoding genes were then used to transfect insect cells, for example, Sf21 cells, using Cellfectin®. Also the bacmid was used to transfect insect pupae. *Trichoplusia ni* (Cabbage looper) pupae at an age of 1 to 5 days were used for this experiment. 72 hours post-transfection, cells or pupae were harvested or processed and the first recombinant baculovirus generation was obtained. This recombinant baculovirus could then be further amplified and/or titered following conventional protocols. Similar procedures can be used to generate recombinant baculoviruses with other transfer vectors provided by commercial BEVSs.

Example 12. Infection of insect pupae

Trichoplusia ni (Cabbage looper) pupae at an age of 1 to 5 days were used for all experiments. The standard weight of each pupa was approximately 200-300 mg and pupae were injected manually or by a specifically designed robot with 1 to 10 µl of recombinant baculoviruses diluted in cell culture media or PBS 1X as to reach the number of plaque forming units (PFU) per dose selected. Pupae were collected at 72-168 h post-infection. The pupae collected were frozen immediately to be stored at -20°C or -80°C until they were processed for recombinant protein quantification. Total soluble, non-denatured proteins (TSNDPs) from frozen *T. ni* pupae infected by the baculoviruses were obtained by homogenization in presence of a extraction buffer using a blender or a homogenizer mixer for several min.

Example 13. Downstream processing of insect pupae

Frozen pupae are disrupted by a homogenizer to obtain a crude extract containing a reducing agent in a concentration of 1-25mM, a detergent in a concentration of 0,01%-2% and a mixture of protease inhibitors. The viscosity of the crude extract is reduced by its incubation with a diatomaceous earth for a specific period of time and then centrifuged to eliminate the insect debris and filtered to eliminate the diatomaceous earth. Then, the extract is clarified through tangential flow filtration using an appropriate filter depending of the recombinant protein nature. Finally, a diafiltration process is performed in the same tangential filtration device to change the buffer before further chromatography purification. **Figure 17** represents the general procedure to obtain a soluble recombinant protein extract from baculovirus-infected pupae.

Items of the present invention (I):

1. A pupa comprising a recombinant baculovirus and/or a bacmid derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV).
2. The pupa according to item 1, wherein the pupa belongs to the order *Lepidoptera*.
3. The pupa according to item 1, wherein the pupa belongs to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*, *Spodoptera littoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.
4. The pupa according to any one of items 1 to 3, wherein the baculovirus comprises a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein.
5. A pupa comprising a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein.
6. The pupa according to item 5, wherein the pupa belongs to the order *Lepidoptera*.
7. The pupa according to item 6, wherein the pupa belongs to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*, *Spodoptera littoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.
8. The pupa according to any one of items 1 to 7, wherein the pupa belongs to the species *Trichoplusia ni*.

9. The pupa according to any one of items 4 to 8, wherein the nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof is selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NOs: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NOs: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NOs: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NOs: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

10. The pupa according to any one of items 4 to 9, wherein the promoter that drives the expression of said recombinant protein is, selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NOs: 10-14, preferably indicated in any of SEQ ID NOs: 11-13; and
- (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NOs: 10-14, preferably indicated in any of SEQ ID NOs: 11-13.

11. The pupa according to any one of items 4 to 10, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 21 (*hr1*).

12. The pupa according to any one of items 4 to 11, wherein the nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions are selected from the group comprising SEQ ID NOs: 15-20.

13. The pupa according to any one of items 4 to 12, wherein the pupa further comprises a nucleic acid sequence encoding a recombinant protein.
14. The pupa according to item 13, wherein the recombinant protein is selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and the green fluorescent protein (GFP).
15. The pupa according to item 14, wherein the recombinant protein is a virus-like particle protein selected from the group consisting of:
 - (a) Porcine circovirus capsid protein, preferably from porcine circovirus type 2 (e.g., SEQ ID NO.: 26),
 - (b) Foot and mouth disease virus VP1, VP3 or VP0 protein,
 - (c) Canine parvovirus VP1 and VP2 proteins,
 - (d) Porcine parvovirus VP1 and VP2 proteins,
 - (e) Human norovirus (genogroup I or II) capsid protein,
 - (f) Calicivirus capsid protein,
 - (g) Human papillomavirus L1 protein, preferably from human papillomavirus 16,
 - (h) Hepatitis E protein E2,
 - (i) Infectious bursal disease virus VP1, VP2 and VP3 proteins,
 - (j) Astrovirus ORF2-encoded proteins,
 - (k) Influenza virus HA (e.g., SEQ ID NO.: 30), NA and M1 proteins,
 - (l) Hepatitis B core and surface antigens,
 - (m) Human parvovirus VP1 and VP2 proteins, and
 - (n) Rabbit calicivirus VP60 protein, preferably from rabbit haemorrhagic disease virus (e.g., SEQ ID NOs: 32 and 33).
16. Use of the pupa as defined in any one of items 1 to 15 for the expression of recombinant proteins.
17. The use according to item 16, wherein the recombinant protein is selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and the green fluorescent protein (GFP).

18. The use according to item 17, wherein the recombinant protein is a virus-like particle protein selected from the group consisting of:

- (a) Porcine circovirus capsid protein, preferably from porcine circovirus type 2 (e.g., SEQ ID NO.: 26),
- (b) Foot and mouth disease virus VP1, VP3 or VP0 protein,
- (c) Canine parvovirus VP1 and VP2 proteins,
- (d) Porcine parvovirus VP1 and VP2 proteins,
- (e) Human norovirus (genogroup I or II) capsid protein,
- (f) Calicivirus capsid protein,
- (g) Human papillomavirus L1 protein, preferably from human papillomavirus 16,
- (h) Hepatitis E protein E2,
- (i) Infectious bursal disease virus VP1, VP2 and VP3 proteins,
- (j) Astrovirus ORF2-encoded proteins,
- (k) Influenza virus HA (e.g., SEQ ID NO.: 30), NA and M1 proteins,
- (l) Hepatitis B core and surface antigens,
- (m) Human parvovirus VP1 and VP2 proteins, and
- (n) Rabbit calicivirus VP60 protein, preferably from rabbit haemorrhagic disease virus (e.g., SEQ ID NOs: 32 and 33).

19. A method for producing at least one recombinant protein comprising the steps of:

- (a) Providing a pupa;
- (b) Inoculating the pupa of step (a) with a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV);
- (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the at least one recombinant protein to be expressed;
- (d) Obtaining the pupae comprising the at least one recombinant protein;
- (e) Optionally, harvesting the at least one recombinant protein; and
- (f) Optionally, purifying the at least one recombinant protein.

20. The method according to item 19, wherein the pupa belongs to the order *Lepidoptera*.

21. The method according to item 20, wherein the pupa belongs to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*,

Spodoptera littoralis, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* and, *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.

22. The method according to item 21, wherein the pupa belongs to the species *Trichoplusia ni*.

23. The method according to any one of items 19 to 22, wherein the recombinant baculovirus comprises a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

24. The method according to item 23, wherein the nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof is selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

25. The method according to any one of items 23 to 24, wherein the promoter that drives the expression of said recombinant protein is, selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-14, preferably indicated in any of SEQ ID NOs: 11-13; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-14, preferably indicated in any of SEQ ID NOs: 11-13.

26. The method according to any one of items 23 to 25, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 21 (*hr1*).

27. The method according to any one of items 23 to 26, wherein the nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions are selected from the group comprising SEQ ID NO: 15-20.

28. The method according to any one of items 19 to 27, wherein the pupa further comprises a nucleic acid sequence encoding a recombinant protein.

29. The method according to item 28, wherein the recombinant protein is selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and the green fluorescent protein (GFP).

30. The method according to item 29, wherein the recombinant protein is a virus-like particle protein selected from the group consisting of:

- (a) Porcine circovirus capsid protein, preferably from porcine circovirus type 2 (e.g., SEQ ID NO.: 26),
- (b) Foot and mouth disease virus VP1, VP3 or VP0 protein,
- (c) Canine parvovirus VP1 and VP2 proteins,
- (d) Porcine parvovirus VP1 and VP2 proteins,
- (e) Human norovirus (genogroup I or II) capsid protein,
- (f) Calicivirus capsid protein,
- (g) Human papillomavirus L1 protein, preferably from human papillomavirus 16,
- (h) Hepatitis E protein E2,
- (i) Infectious bursal disease virus VP1, VP2 and VP3 proteins,
- (j) Astrovirus ORF2-encoded proteins,
- (k) Influenza virus HA (e.g., SEQ ID NO.: 30), NA and M1 proteins,

- (l) Hepatitis B core and surface antigens,
 - (m) Human parvovirus VP1 and VP2 proteins, and
 - (n) Rabbit calicivirus VP60 protein, preferably from rabbit haemorrhagic disease virus (e.g., SEQ ID NOs: 32 and 33).
31. The method according to any one of items 19 to 30, wherein the pupa is a silk-free pupa.
32. The method according to item 31, wherein the silk-free pupa is obtainable by dissolving the silk of cocoons comprising the pupae of *T. ni* with a solution of a salt of hypochlorous acid, preferably sodium hypochlorite.
33. The method according to any one of items 19 to 32, wherein the inoculation of the pupa with a recombinant baculovirus is performed by injecting the baculovirus into the pupa.
34. The method according to any one of items 19 to 33, wherein the inoculated pupa of step (b) is the pupa as defined in any one of items 1 to 15.
35. The method according to any one of items 19 to 34, wherein the pupa is inoculated with baculovirus in an amount of from 50 to 10⁶ PFUs/pupa.
36. A method for producing a silk-free pupa belonging to the order *Lepidoptera* comprising the steps of:
- (a) Providing a pupa contained in a cocoon;
 - (b) Treating the cocoon containing a pupa with a solution of a salt of hypochlorous acid, preferably sodium hypochlorite; and
 - (c) Obtaining a silk-free and disinfected pupa.
37. The method according to item 36, wherein the pupa does not belong to the species *Bombyx mori*.
38. The method according to any one of items 36 to 37, wherein the pupa belongs to the species *Trichoplusia ni*.
39. The method according to any one of items 36-38, wherein the pupae are provided in a matrix (or array).

40. A method for producing a recombinant baculovirus comprising the steps of:
- (a) Providing a pupa;
 - (b) Transfecting the pupa of step (a) with a bacmid suitable for producing a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV);
 - (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the recombinant baculovirus is produced;
 - (d) Obtaining the pupae comprising the recombinant baculovirus;
 - (e) Optionally, harvesting the recombinant baculovirus; and
 - (f) Optionally, purifying the recombinant baculovirus.
41. The method according to item 39, wherein the pupa is the pupa as defined in any one of items 36-39.
42. The method according to any one of items 40-41, wherein the pupa belongs to the order *Lepidoptera*.
43. The method according to item 42, wherein the pupa belongs to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*, *Spodoptera littoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.
44. The method according to item 43, wherein the pupa belongs to the species *Trichoplusia ni*.
45. The method according to any one of items 40 to 44, wherein the bacmid suitable for producing a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV) comprises or alternatively, consists of, a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

46. The method according to item 45, wherein the wherein the nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof is selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

47. The method according to any one of items 45 to 46, wherein the promoter that drives the expression of said recombinant protein is, selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-14, preferably indicated in any of SEQ ID NOs: 11-13; and
- (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-14, preferably indicated in any of SEQ ID NOs: 11-13.

48. The method according to any one of items 45 to 47, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 21 (*hr1*).

49. The method according to any one of items 45 to 48, wherein the nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions are selected from the group comprising SEQ ID NO: 15-20.

50. The method according to any one of items 40 to 49, wherein the pupa is a silk-free pupa.
51. The method according to item 50, wherein the silk-free pupa is obtainable by dissolving the cocoons comprising the pupae of *T. ni* with a solution of a salt of hypochlorous acid, preferably sodium hypochlorite.
52. The method according to any one of items 40 to 51, wherein the transfected pupa of step (b) is the pupa as defined in any one of items 5 to 15.
53. The method according to any one of items 40 to 52, wherein the pupae are provided in a matrix.
54. A device comprising a precision pump, a mobile mechanic arm and a (removable) needle suitable for injecting a fluid into a pupa belonging to the order *Lepidoptera*, preferably to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, more preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*, *Spodoptera littoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.
55. The device according to item 54, wherein the pupa is provided in a matrix or array.
56. The device according to any one of items 54 to 55, wherein the device further comprises a computer program for defining the position of the needle and/or for calculating the distance from the needle to the pupa and/or the distance of penetration of the needle into the pupa and/or the volume of liquid to be inoculated into the pupa and/or determining the coordinates of the inoculation needle and/or calculating the inoculation volume and/or time and/or the time between inoculation of the pupae.
57. The device according to item 56, further comprising a computer program for calculating the inoculation time and/or the time between inoculation of the pupae.
58. The device according to any one of items 54 to 57, wherein the device further comprises a camera for defining the position of the needle.

59. The device according to item 54, wherein the pupa is the pupa as defined in any one of items 1 to 15.

60. The device according to any one of items 54 to 59, wherein the injected fluid comprises a recombinant baculovirus or bacmid.

61. The device according to any one of items 54 to 60, wherein the device is suitable for performing step (b) as defined in any one of items 19 and/or 40.

62. The device according to any one of items 54 to 61, wherein the injected fluid into the pupa is in the range of from about 0,5 to about 10,0 μ L, preferably about 5 μ L.

63. The device according to any one of items 54 to 62, wherein the needle penetrates into the pupa a distance of from about 1 to about 5 mm, preferably about 3 mm.

64. The device according to any one of items 54 to 63, wherein the fluid injected into the pupa comprises baculovirus, preferably in an amount of from 50 to 10^6 PFUs/dose injected into each pupa.

Items of the present invention (II):

1. A pupa comprising a recombinant baculovirus and/or a bacmid derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV), wherein preferably the pupa belongs to the order *Lepidoptera*, preferably to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, even more preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*, *Spodoptera littoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.

2. The pupa according to item 1, wherein the baculovirus comprises a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

3. A pupa comprising a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein, wherein preferably the pupa belongs to the order *Lepidoptera*, preferably to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, even more preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*, *Spodoptera litoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.

4. The pupa according to any one of items 2 to 3, wherein the nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof is selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NOs: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NOs: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NOs: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NOs: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus;

wherein the promoter that drives the expression of said recombinant protein is, selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NOs: 10-14, preferably indicated in any of SEQ ID NOs: 11-13; and
- (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably

at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NOs: 10-14, preferably indicated in any of SEQ ID NOs: 11-13; and

wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 21 (*hr1*).

5. The pupa according to any one of items 2 to 4, wherein the nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions are selected from the group comprising SEQ ID NOs: 15-20.

6. The pupa according to any one of items 2 to 5, wherein the pupa further comprises a nucleic acid sequence encoding a recombinant protein, preferably selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and the green fluorescent protein (GFP).

7. Use of the pupa as defined in any one of items 1 to 6 for the expression of recombinant proteins, preferably selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and the green fluorescent protein (GFP).

8. A method for producing at least one recombinant protein comprising the steps of:

- (a) Providing a pupa, preferably a silk-free pupa;
- (b) Inoculating the pupa of step (a) with a recombinant baculovirus derived from *Autographa californica multicaudatus nucleopolyhedrovirus* (AcMNPV);
- (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the at least one recombinant protein to be expressed;
- (d) Obtaining the pupae comprising the at least one recombinant protein;
- (e) Optionally, harvesting the at least one recombinant protein; and
- (f) Optionally, purifying the at least one recombinant protein,

wherein preferably the pupa belongs to the order *Lepidoptera*, preferably to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, even more preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera*

frugiperda, *Spodoptera littoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* and, *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV.

9. The method according to item 8, wherein the recombinant baculovirus comprises a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein, wherein preferably the nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and the recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein are as defined in claim 4.

10. The method according to item 11, wherein the nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions are selected from the group comprising SEQ ID NO: 15-20.

11. The method according to any one of items 8 to 10, wherein the pupa further comprises a nucleic acid sequence encoding a recombinant protein, preferably selected from the group consisting of subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone, diagnostic protein reagents and the green fluorescent protein (GFP).

12. A method for producing a silk-free pupa belonging to the order *Lepidoptera*, preferably to the species *T. ni*, comprising the steps of:

- (a) Providing a pupa contained in a silk cocoon;
- (b) Treating the silk cocoon containing a pupa with a solution of a salt of hypochlorous acid, preferably sodium hypochlorite; and
- (c) Obtaining a silk-free and disinfected pupa.

13. A method for producing a recombinant baculovirus comprising the steps of:

- (a) Providing a pupa, preferably a silk-free pupa preferably belonging to the order *Lepidoptera*, preferably to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, even more preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*,

Spodoptera littoralis, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV;

- (b) Transfecting the pupa of step (a) with a bacmid suitable for producing a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV);
- (c) Incubating the inoculated pupa of step (b) for a period of time sufficient for the recombinant baculovirus is produced;
- (d) Obtaining the pupae comprising the recombinant baculovirus;
- (e) Optionally, harvesting the recombinant baculovirus; and
- (f) Optionally, purifying the recombinant baculovirus.

14. The method according to item 13, wherein the bacmid suitable for producing a recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV) comprises or alternatively, consists of, a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein, wherein preferably the nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and the recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein are as defined in claim 4.

15. The method according to item 14, wherein the nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions are selected from the group comprising SEQ ID NO: 15-20.

16. A device comprising a precision pump, a mobile mechanic arm and a (removable) needle suitable for injecting a fluid into a pupa belonging to the order *Lepidoptera*, preferably to the genera *Trichoplusia*, *Rachiplusia*, *Spodoptera*, *Heliothis*, *Manduca*, *Helicoverpa*, *Ascalapha* or *Samia*, even more preferably to the species *Trichoplusia ni*, *Manduca sexta*, *Spodoptera frugiperda*, *Spodoptera littoralis*, *Ascalapha odorata*, *Helicoverpa zea*, *Heliothis virescens*, *Rachiplusia nu* or *Samia cynthia*, or any other pupa susceptible to be infected by a recombinant baculovirus and/or a bacmid derived from AcMNPV, wherein preferably the pupa is provided in a matrix or array.

17. The device according to item 16, wherein the device further comprises a computer program for defining the position of the needle and/or for calculating the distance from the needle to the pupa and/or the distance of penetration of the needle into the pupa and/or the volume of liquid (preferably comprising a recombinant baculovirus or bacmid) to be inoculated into the pupa, and wherein the device preferably further comprises a computer program for, e.g., determining the coordinates of the inoculation needle and/or calculating the inoculation volume and/or time and/or the time between inoculation of the pupae.

CLAIMS

1. A package comprising lyophilized or frozen silk-free pupae, wherein each pupa belongs to the genus *Trichoplusia* and comprises a recombinant baculovirus and/or bacmid derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV).

2. The package according to claim 1, wherein the recombinant baculovirus and/or bacmid comprises at least one nucleic acid sequence encoding at least one recombinant protein.

3 The package according to claim 2, wherein the baculovirus and/or bacmid further comprises at least one nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein,

wherein the at least one nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof is selected from the group consisting of:

- (a) a nucleic acid sequence indicated in any one of SEQ ID NOs: 1-5;
 - (b) a nucleic acid sequence having a sequence identity of at least 70% with the nucleotide sequence indicated in any one of SEQ ID NOs: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
 - (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any one of SEQ ID NOs: 6-9; and
 - (d) a nucleic acid sequence encoding an amino acid sequence having a sequence identity of at least 70% with the amino acid sequence indicated in any one of SEQ ID NOs: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.
4. The package according to claim 3, wherein the promoter that drives the expression of said recombinant protein is selected from the group of nucleic acids consisting of:
- (a) a nucleic acid containing the nucleotide sequence indicated in any one of SEQ ID NOs: 10-14; and
 - (b) a nucleic acid able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70% with the nucleotide sequence indicated in any one of SEQ ID NOs: 10-14; and/or

wherein the recombinant homologous region has a sequence indicated in SEQ ID NO: 21 (*homologous region I*).

5. The package according to claim 4, wherein the promoter, nucleic acid sequence encoding the transcriptional regulator and homologous region (*hr*) form part of a single nucleic acid sequence indicated in any one of SEQ ID NOs: 15-20.

6. A method for producing at least one recombinant protein comprising the steps of:

- (a) Providing a silk-free pupa;
- (b) Inoculating the silk-free pupa of step (a) with a recombinant baculovirus derived from *Autographa californica multicausid nucleopolyhedrovirus* (AcMNPV) comprising at least one nucleic acid sequence encoding the at least one recombinant protein;
- (c) Incubating the inoculated silk-free pupa of step (b) for a period of time sufficient for the at least one recombinant protein to be expressed; and
- (d) Obtaining the silk-free pupa comprising the at least one recombinant protein,

wherein the pupa belongs to the genus *Trichoplusia*.

7. The method according to claim 6, wherein the recombinant baculovirus further comprises at least one nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection and a recombinant homologous region (*hr*) operably linked to any promoter that is suitable for driving the expression of a recombinant protein,

wherein the nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof is selected from the group consisting of:

- (a) a nucleic acid sequence indicated in any one of SEQ ID NOs: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70% with the nucleotide sequence indicated in any one of SEQ ID NOs: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any one of SEQ ID NOs: 6-9; and

- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence identity of at least 70% with the amino acid sequence indicated in any one of SEQ ID NOs: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.
8. The method according to claim 7, wherein the promoter, nucleic acid sequence encoding the transcriptional regulator and homologous region (*hr*) form part of a single nucleic acid sequence indicated in any one of SEQ ID NO: 15-20.
9. The method according to any one of claims 6-8, wherein the silk-free pupa is obtained through a method for producing a silk-free pupa, comprising the steps of:
- (a1) Providing a pupa contained in a silk cocoon;
 - (a2) Treating the silk cocoon containing a pupa with a solution of a salt of hypochlorous acid; and
 - (a3) Obtaining the silk-free and externally disinfected pupa.
10. The method according to any one of claims 6-9, wherein step (b) is performed by a device comprising a precision pump, a mobile mechanic arm and a needle suitable for inoculating the silk-free pupa belonging to the genus *Trichoplusia* with the recombinant baculovirus derived from *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV).
11. The method according to claim 10, wherein the device further comprises a computer program for:
- (i) defining the position of the needle;
 - (ii) calculating the distance from the needle to the pupa;
 - (iii) calculating the distance of penetration of the needle into the pupa;
 - (iv) calculating the volume of liquid to be inoculated into the pupa; or
 - (v) two or more of (i) to (iv).

Figure 1

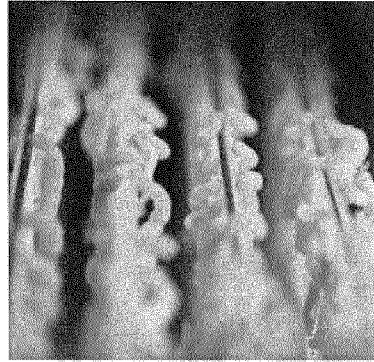
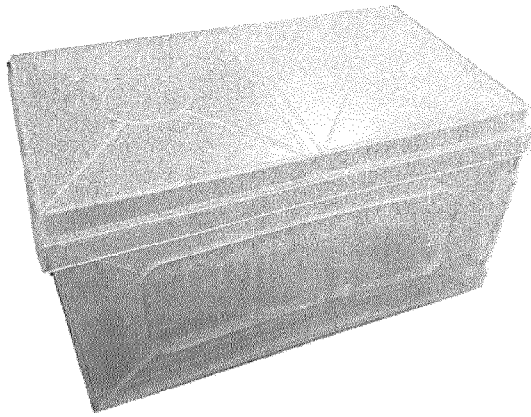


Figure 2

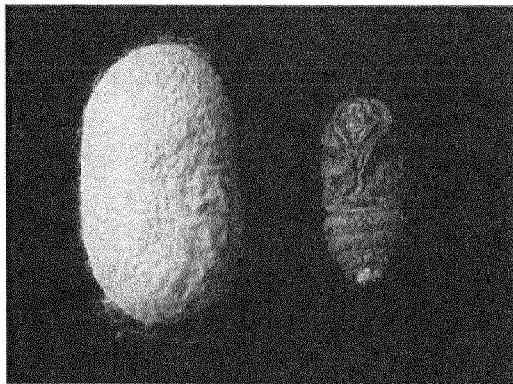
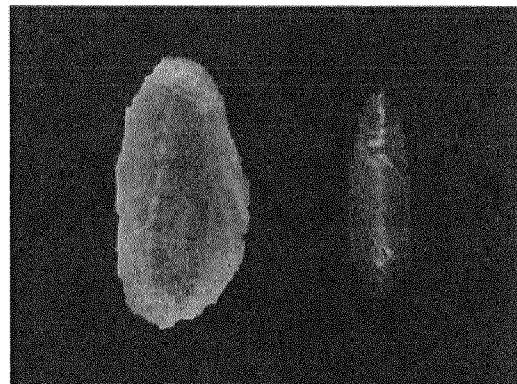
Bombyx mori*Trichoplusia ni*

Figure 3

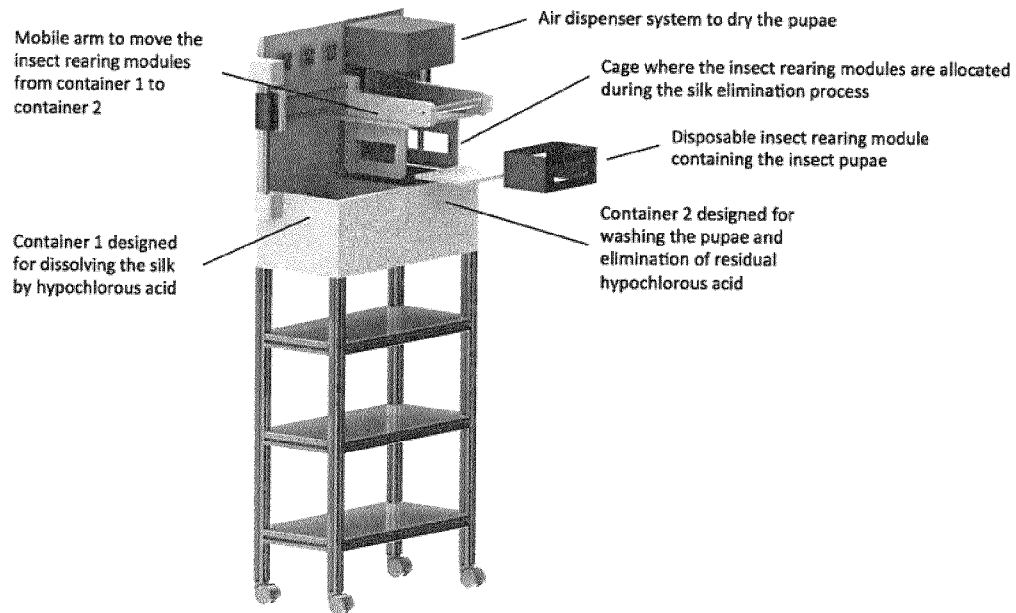


Figure 4

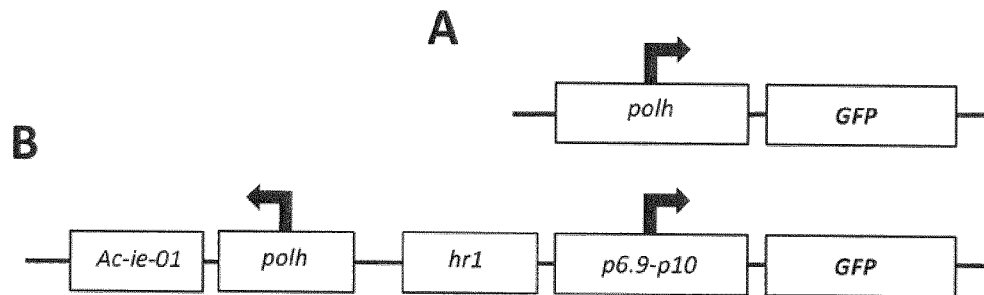


Figure 5

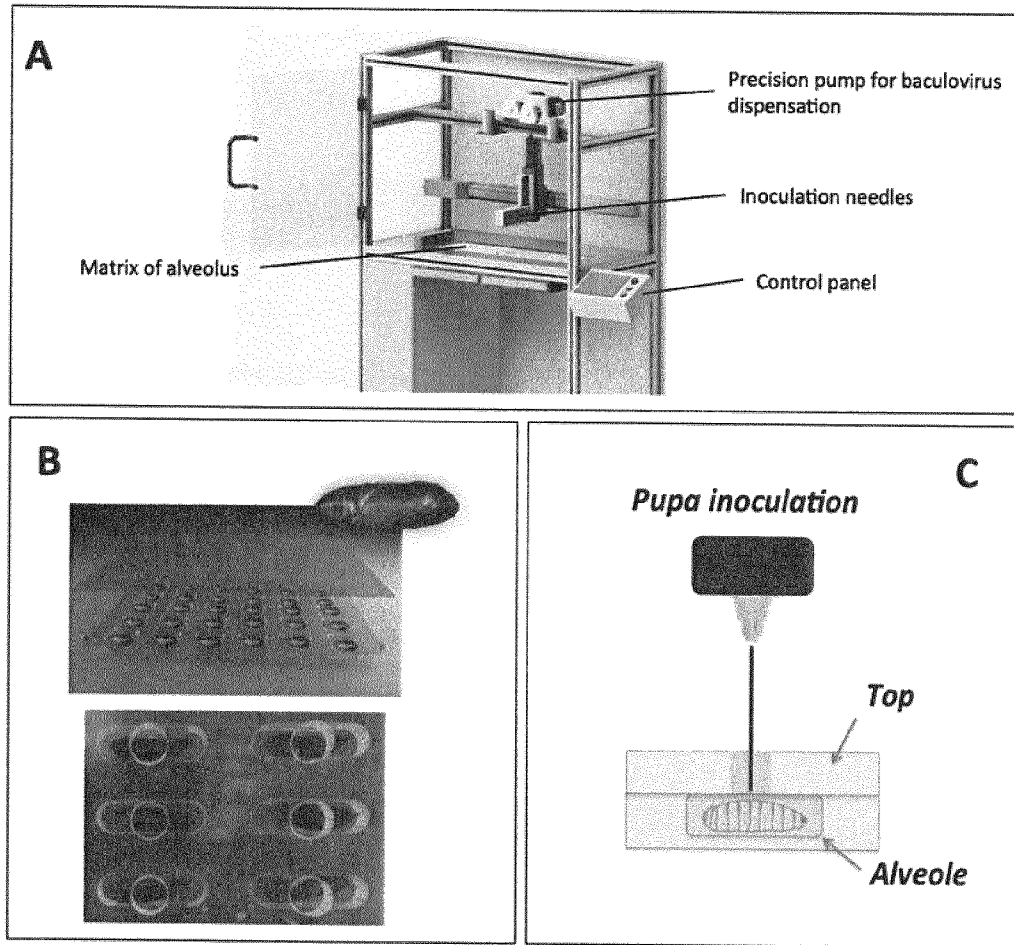


Figure 6

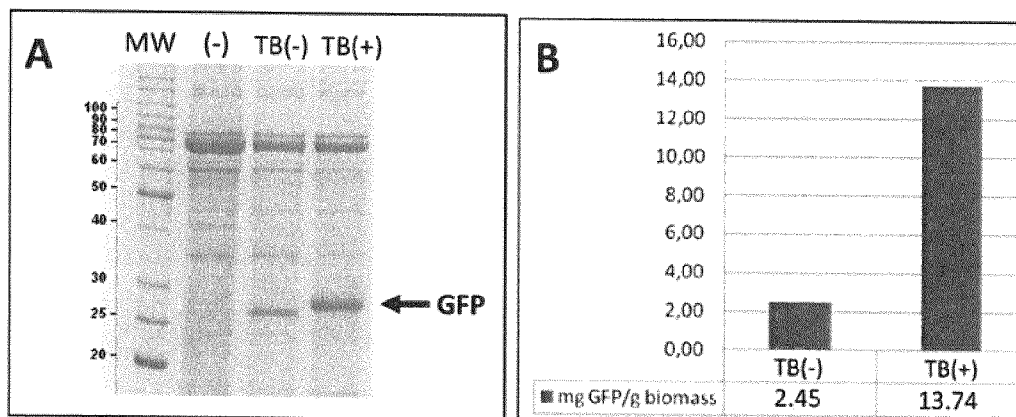


Figure 7

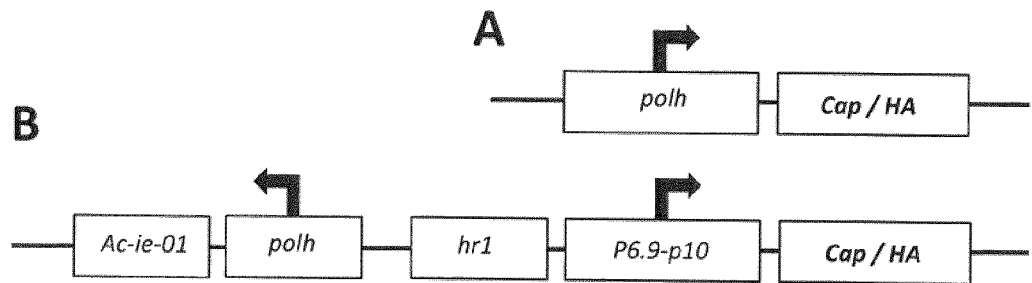


Figure 8

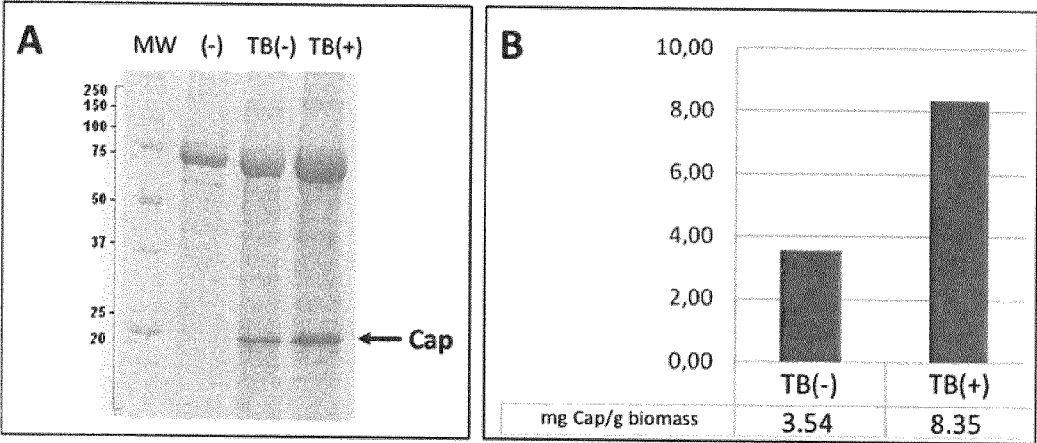


Figure 9

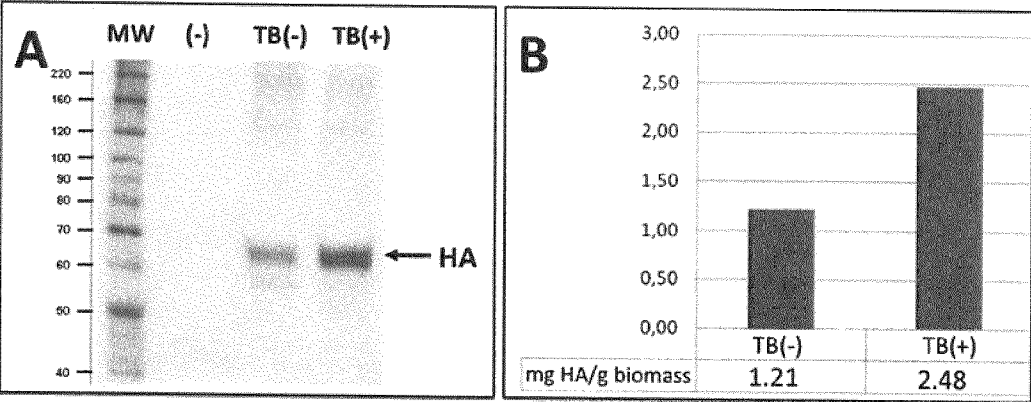


Figure 10

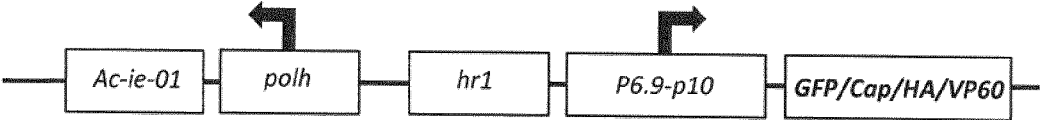


Figure 11

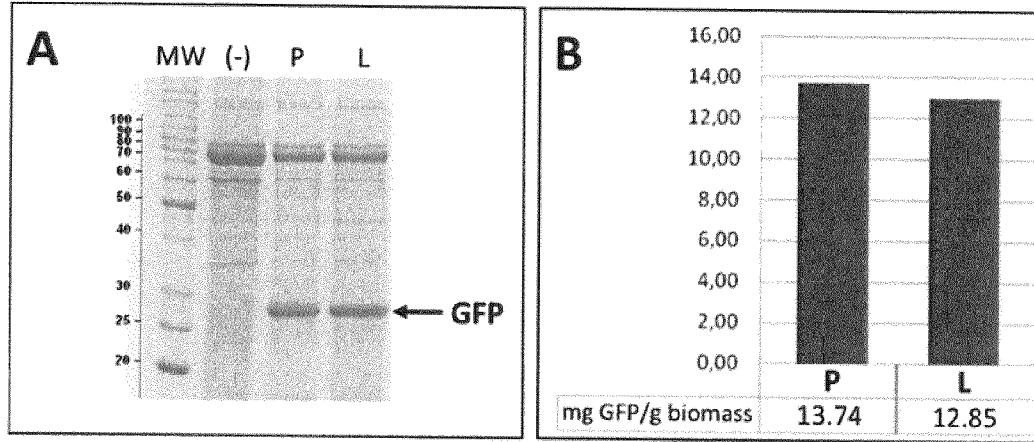


Figure 12

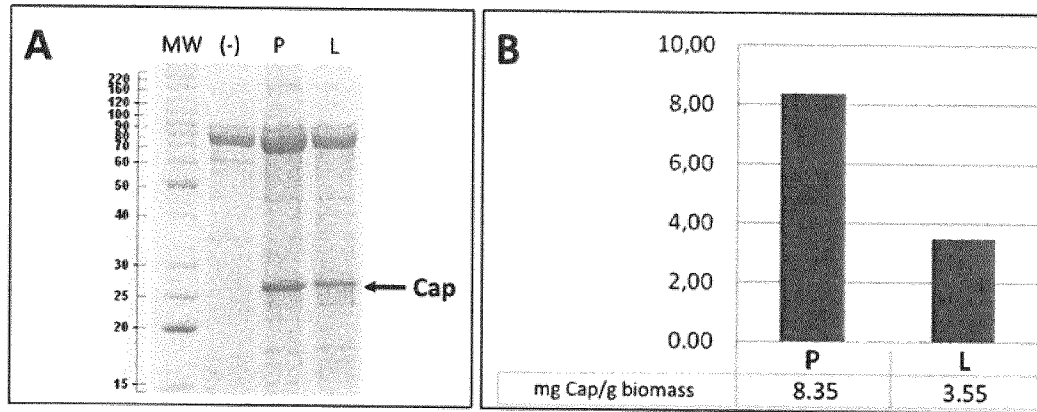


Figure 13

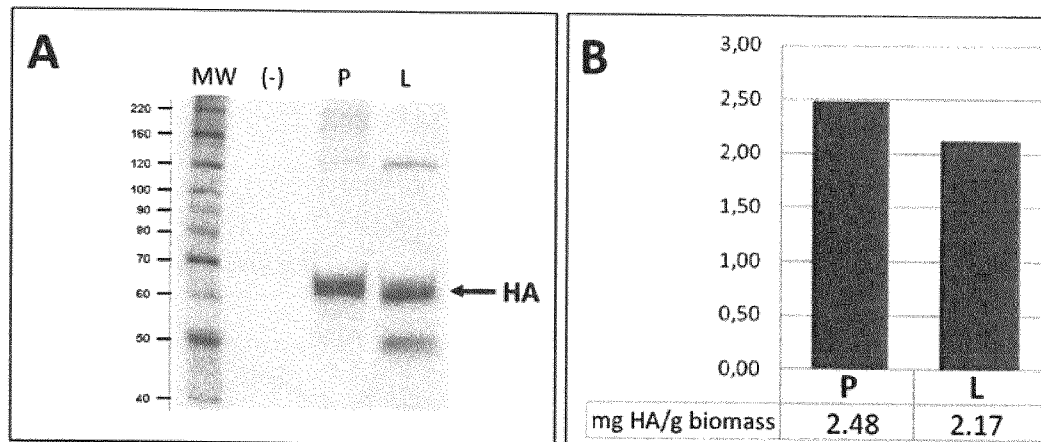


Figure 14

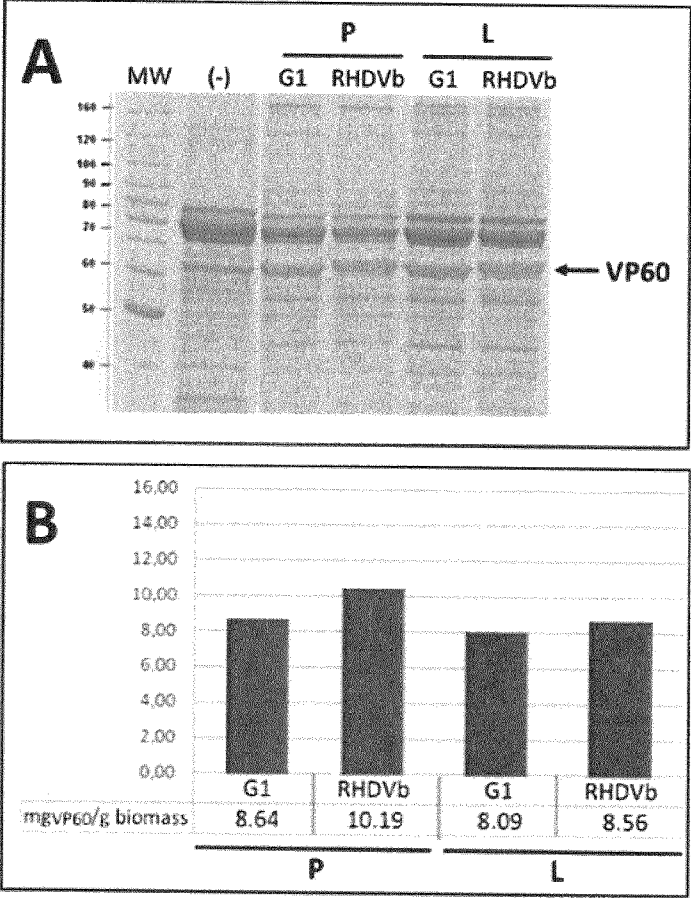


Figure 15

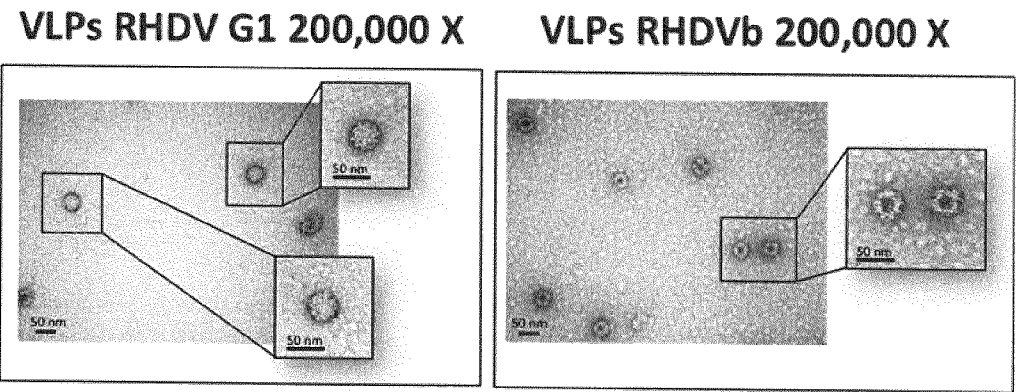


Figure 16

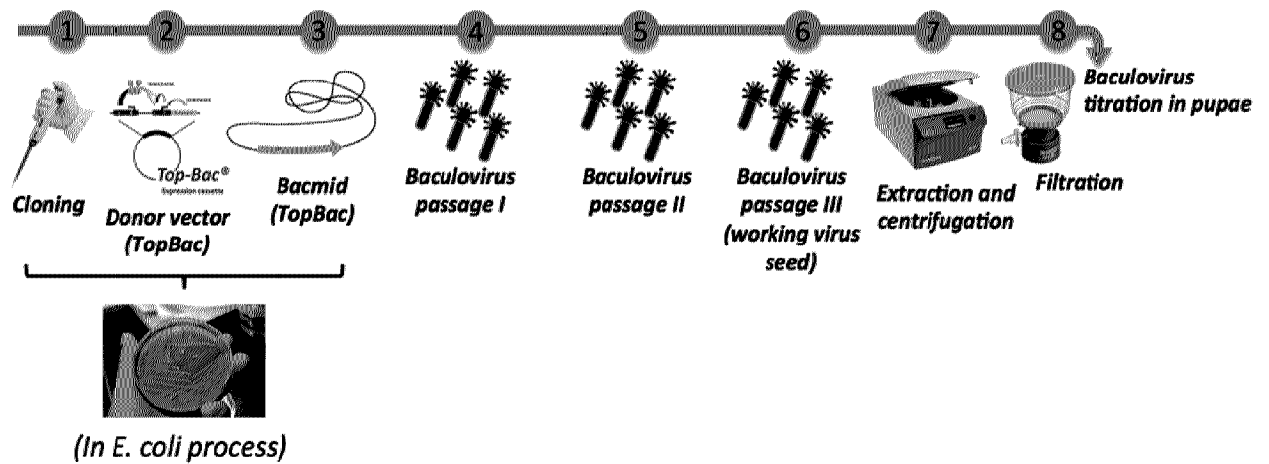


Figure 17

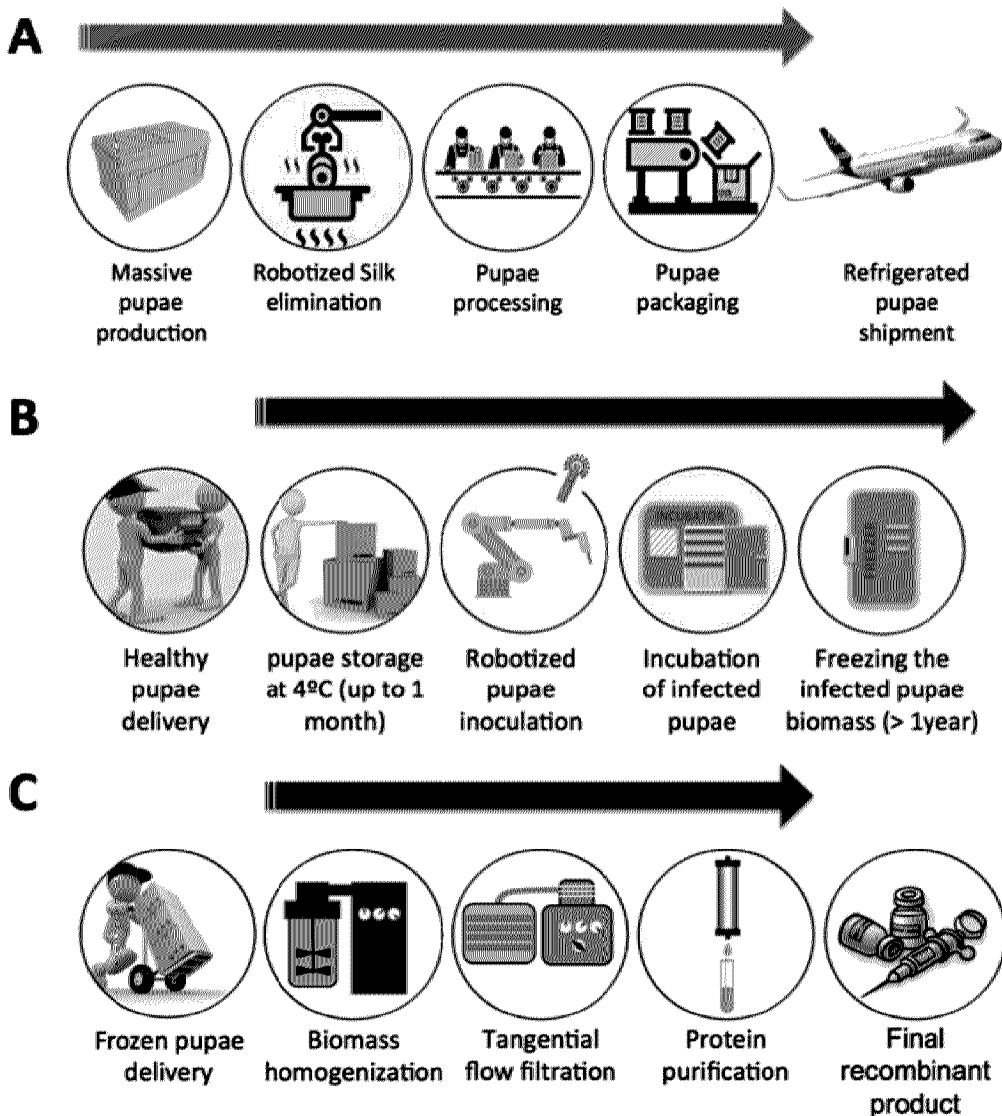


Figure 18

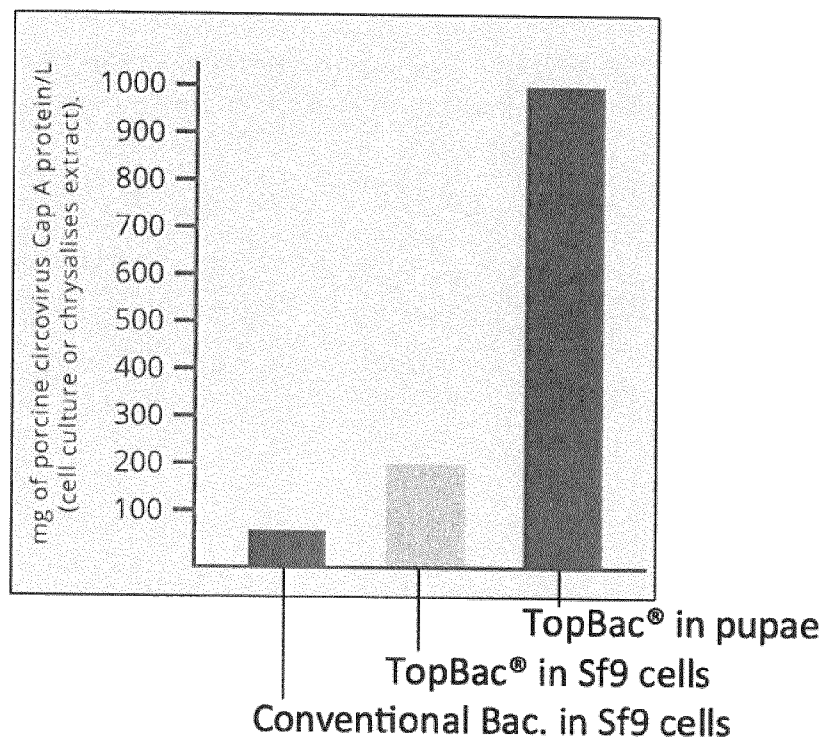


Figure 19.

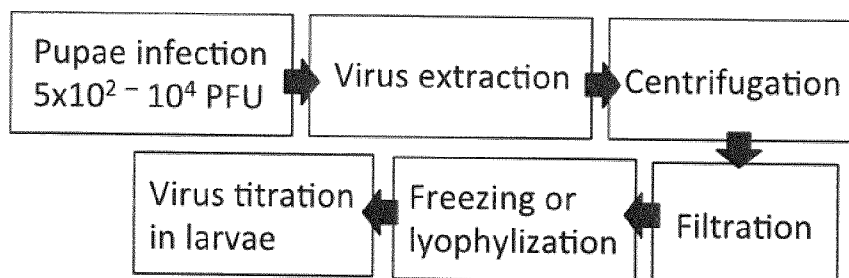


Figure 20.

