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Full Length Research Paper

Comparative analysis of the activity of two promoters in insect cells

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Insect cell expression system is widely used for the production of many thousands of recombinant proteins. Non-lytic vector-based expression provides a reliable result for investigating gene functions and molecular pathways, because baculovirus-mediated expression will lyse cells eventually. There are currently two major commercial vector providers. The pIB/V5-His vector from invitrogen uses the OpIE2 promoter and the pIEX-4 vector from novagen uses the IE1 promoter and hr5 enhancer. To compare the activity of these two promoters, we replaced the OpIE2 promoter in the pIB/V5-His vector with the hr5-IE1 promoter from pIEX-4. By measuring the activity of β-galactosidase under control of these two promoters, we found that the hr5-IE1 promoter showed about 20% higher activity than the OpIE2 promoter for both transient and stable expression. To facilitate protein expression in the secretion pathway, we cloned an AKH signal peptide downstream of the hr5-IE1 promoter. By comparing the cytosolic expression and localization of GFP, AKH-GFP and AKH-GFP-GPI, we found that the AKH signal peptide was sufficient to guide the passenger protein into the secretion pathway. Furthermore, to allow researchers to use different antibiotics for stable expression, we replaced the blasticidin resistance ORF with the zeocin resistance ORF and found that the two vectors yielded similar expression levels. Taken together, the hr5-IE1 is a stable and more effective combination to direct expression in insect cell. Vectors constructed in this study will provide a new choice for biological researchers using insect cell model systems.

Key words: Insect cells, vector, secretion, expression.

INTRODUCTION

Insect cell systems provide an efficient means for expressing proteins that require specific post-translational modifications to retain solubility and activity. Insect cells are a higher eukaryotic system than yeast and are able to carry out more complex post-translational modifications. There are many advantages to using insect cells for heterologous gene expression. As with other eukaryotic expression systems, insect cell expression of heterologous genes permits folding, post-translational modification and oligomerization in ways that are often identical to those that occur in mammalian cells. The insect cytoplasmic environment allows proper folding and disulfide bond formation, unlike the reducing environment of the *Escherichia coli* cytoplasm. Post-translational processing identical to that of mammalian cells has been reported for many proteins. These include proper proteolysis, N- and O-glycosylation (Chen et al., 1991; Paul et al., 1990), acylation (Murphy et al., 1988), amidation (Suzuki et al., 1990), carboxymethylation (Cha et al., 1997), phosphorylation (Piwnica-Worms et al., 1990) and prenylation (Buss et al., 1991). Proteins may be secreted from cells or targeted to different subcellular locations (Alnemri et al., 1991). Single polypeptides, dimeric proteins and trimeric proteins have been expressed in insect cells (Ingley et al., 1991).

The most commonly used expression systems in insect cells include baculovirus-mediated expression and

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vector-based expression. The baculovirus-mediated expression system is a lytic expression system and is mainly used for a high level of expression of recombinant proteins, whereas vector-based expression is a non-lytic system which provides a reliable means for investigating gene functions and molecular pathways (Olczak and Olczak, 2006). In vector-based expression system, cells are directly (transiently or stably) transfected using an expression vector. In the case of secreted proteins, directly transfected insect cells allow the production of a homogenous product because the protein production and processing machinery of the cell are not compromised by a viral infection (Olczak and Olczak, 2006). Studies demonstrated that recombinant proteins may be produced with high yield using the nonlytic insect cell system compared with the baculovirus-infected insect cell system (Olczak and Olczak, 2006). There are currently two major commercial vector providers. The vector from invitrogen (invitrogen, CA) uses the OpIE2 promoter, whereas the vector from novagen (novagen, WI) uses the IE1 promoter with hr5 enhancer. The OpIE2 promoter was derived from the gene of *Orgyia pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early 2, whereas the IE1 promoter was derived from the *Autographa california* multicapsid nuclear polyhedrosis virus (AcNPV) immediate early gene and has been used to express several eukaryotic proteins, including glycoproteins in insect cells (Jarvis et al., 1996). The hr5 is an AcNPV enhancer element that stimulates transcription from the IE1 promoter through a DNA-protein interaction. Both companies claim that their vector can highly express a gene under a stronger promoter. We occasionally found that the novagen vector expressed more protein than the invitrogen vector for transient expression in insect cells. In this study, we systematically analyzed the activity of these two promoters, and found that the IE1 promoter with the hr5 enhancer had slightly stronger activity than the OpIE2 promoter. A series of expression vectors was constructed with the IE1 promoter and hr5 enhancer. These vectors will serve as an additional tool for biological researchers using an insect cell model system.

MATERIALS AND METHODS

DNA manipulation

To construct the pIEX-B vector, a DNA fragment containing the hr5 enhancer and the IE1 promoter was amplified by PCR using the pIEX-4 vector (Novagen, WI, www.ebiotrade.com/buyf/ productsf/ Novagen/71235-000.pdf) as template. The forward primer was 5'-GGATCCTCATGAC GCGTAAAACACAATCAAGTATGA-3' and the reverse primer was 5'-CTCGAGAAGCTTTGG TCACTTGGTT-GTTCACGATCT-3'. PCR products were cloned into the pIB/V5-His (Invitrogen, CA, http://pef.aibn.uq.edu.au/support/material/ download/pibv5his_map.pdf) vector by replacing the OpIE2 promoter using BspH I and Hind III.

To construct the pIEX-Z vector, an ORF conferring zeocin resistance was amplified by PCR using the pBudCE4.1 vector (Invitrogen, CA, http://tools.invitrogen.com/content/sfs/manuals/ pbudce4_1man.pdf) as template. The forward primer was 5'-GGTACCACCATGGCCAAGTT GACCAGTGCCGTTCCG-3' and the reverse primer was 5'-GGATAACCCGGGTCAGTCCTG CTCCTCGGCCACGAA-3'. PCR products were digested by Nco I and Sma I and then were cloned into pIEX-B using Nco I and Sma I.

To construct the pIES-B and pIES-Z vectors, DNA encoding AKH signal peptide was synthesized. The forward primer was 5'-GTGACTATGTACAAGCTCACAGTCTTCCTGATGTTCATCGCTTT CGTCATCATC-3' and the reversed primer was 5'-GGATCCAAGCT TGGACTGGGCCTCAGCGATGATGACGAAAGCGATGAACATCA G-3'. Two oligos were annealed together and digested by BsrG I and HindIII. DNA fragments were cloned into pIEX-B and pIEX-Z under the IE1 promoter using BsrG I and Hind III.

To construct pIB/V5-His-mGFP and pIEX-B-mGFP, the ORF conferring blasticidin resistance in pIB/V5-His and pIEX-B was replaced with sequence encoding mGFP from pmGFP-N1 using Nco I and Bgl II.

To construct a β-galactosidase expression vector, the gene encoding β-galactosidase from pBudCE4.1/LacZ/CAT (Invitrogen, CA, http://tools.invitrogen.com/content/sfs/manuals/ pbudce4_1man. pdf) was subcloned into pIB/V5-his, pIEX-B, pIEX-Z, pIES-B, pIES-Z, pIB/V5-His-mGFP and pIEX-B-mGFP via Hind III and Xba I (Supplementary figure 1. pIB/V5-His).

To construct the pIE1-B-mGFP-gal vector, the Ac-IE1 promoter was amplified by PCR using pIEX-B as template. The forward primer was 5'-GGATCGTCATGATCGATGTCTTTGTGATGCGC GCGA-3' and the reverse primer was 5'-GGTACCAAGCTTTGG TTGTTCACGA TCTTGTCG-3'. PCR products were digested by BspH I and Hind III and then were cloned into pIEX-B-mGFP-gal.

To construct cytosolic mGFP (pIEX-B/mGFP) and secreted mGFP (pIES-B/mGFP) expression vectors, the DNA encoding mGFP was subcloned from pmGFP-N1 into pIEX-B and pIES-B via Hind III and Xba I.

To construct the GPI-anchored mGFP expression vector (pIES-B/mGFP-GPI), the DNA encoding the mouse prion protein GPI-anchor signal sequences was amplified by PCR from mouse genomic DNA. The forward primer was 5'-GGATCCTTGTACAAG GACGGGAGA AGATCCAGCAGCACC-3' and the reverse primer was 5'-TGTACCTCTAGACTCGAGT GCGGCCGCTCATCCCAC GATCAGGAAGAT-3'. PCR products were digested by BsrG I and Xba I and cloned into pIES-B/mGFP expressing vector at the C-terminus of mGFP.

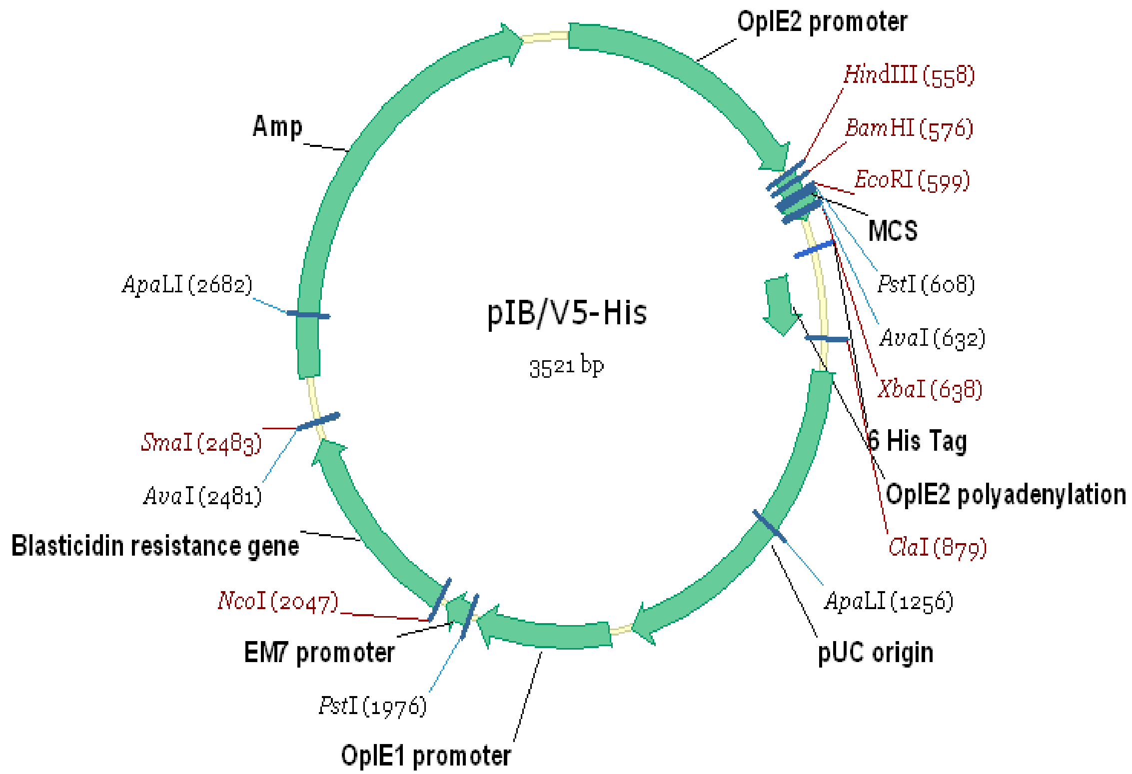
All inserts were verified by DNA sequencing using BigDye 3.1. And all maps of main vectors used in this study can be found in supplemental file.

Insect cell culture and transfection

All insect cells used in this study were purchased from Invitrogen and cultured in Grace's insect cell medium (Invitrogen, CA) with supplements of 10% heat-inactivated fetal calf serum, Yeastolate, lactalbumin hydrolysate and 50 µg/ml of gentamicin. Log-phase Sf9 cells or Sf21 cells with > 95% viability were plated at 8×10^5 cells/well in Sf9-900 II SFM medium (Invitrogen, CA) in six-well plates. Cellfectin II Reagent (Invitrogen, CA) was used for transfection following the manufacturer's instructions.

GFP and β-galactosidase assay

GFP assays were carried out using GFP quantification kits (Biocat, Beidelberg, Germany) according to the manufacturer's manual. β-Galactosidase assays were performed using the ß-Galactosidase enzyme assay system (Promega, WI) following the manufacturer's instructions.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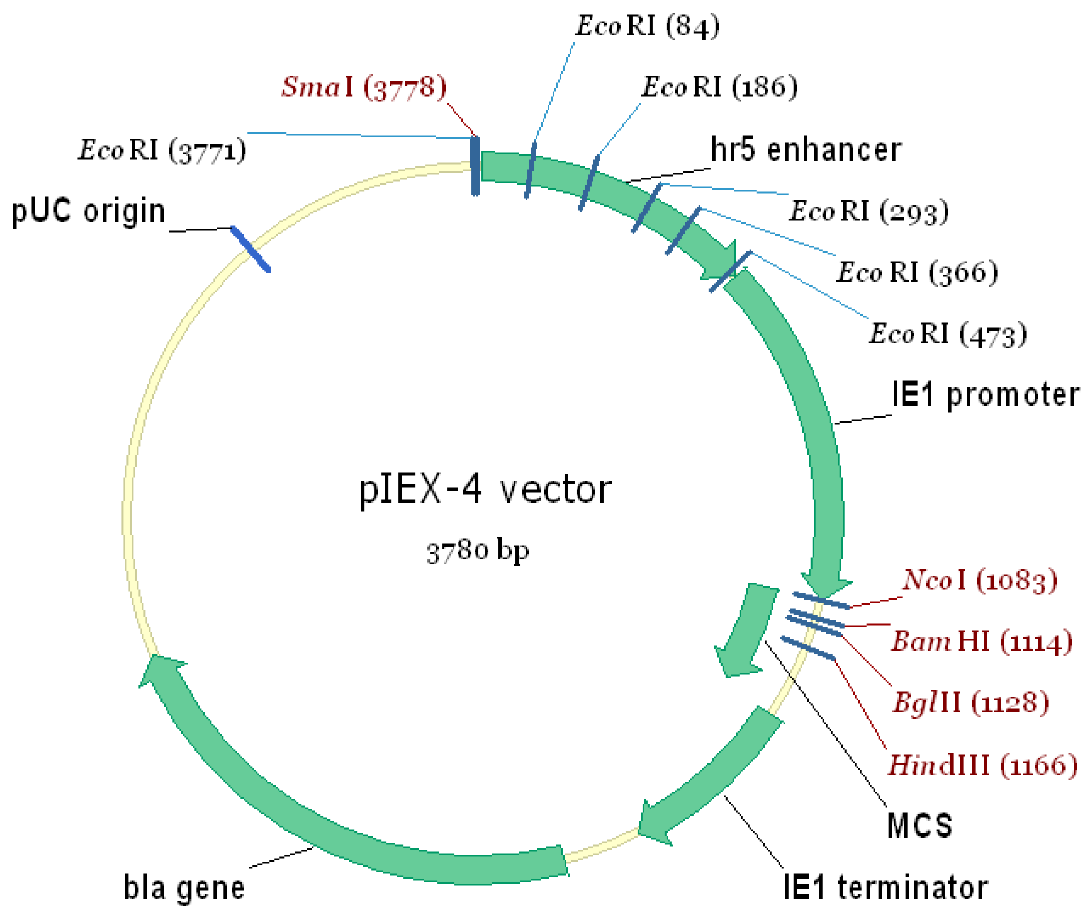
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IE1 promoter
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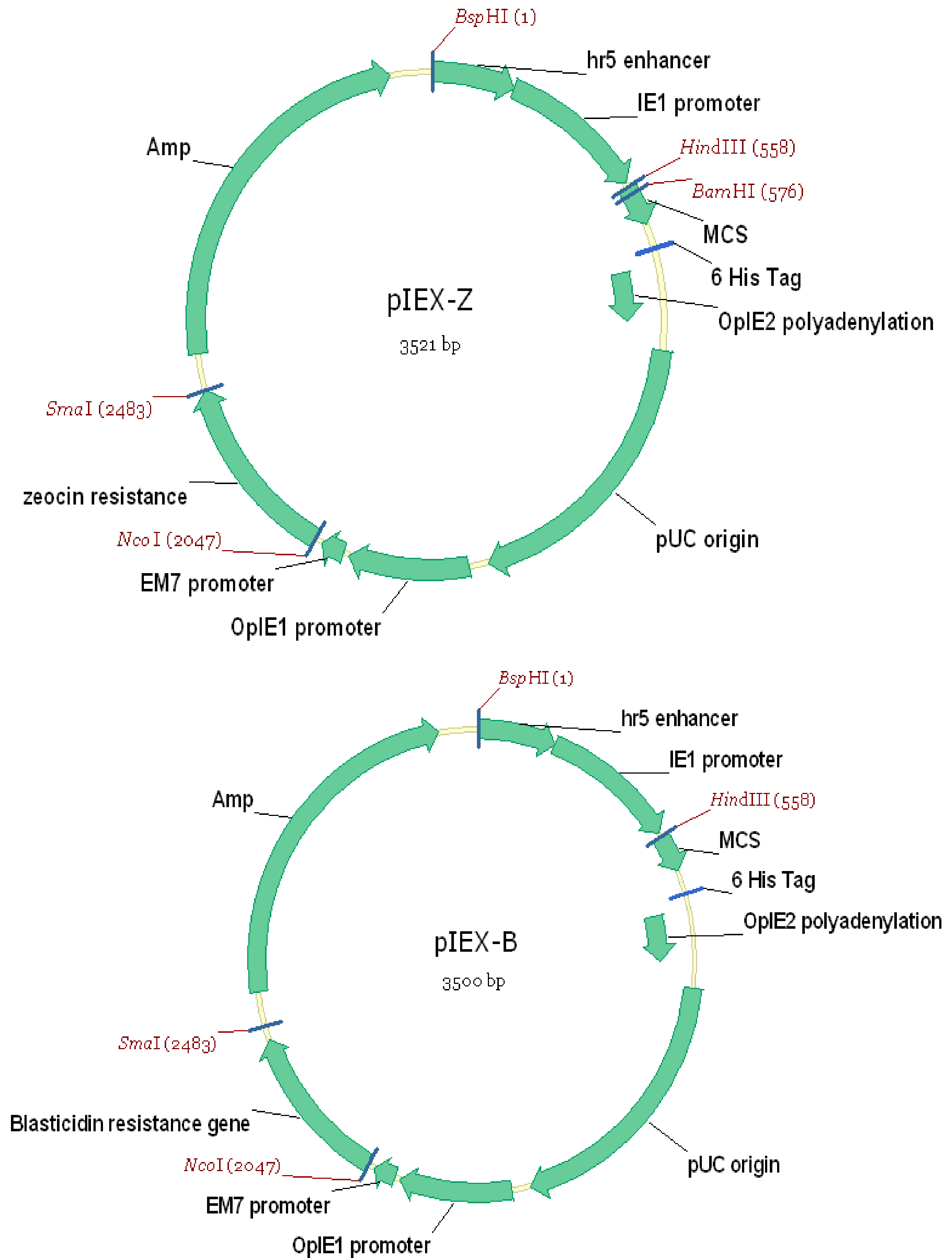
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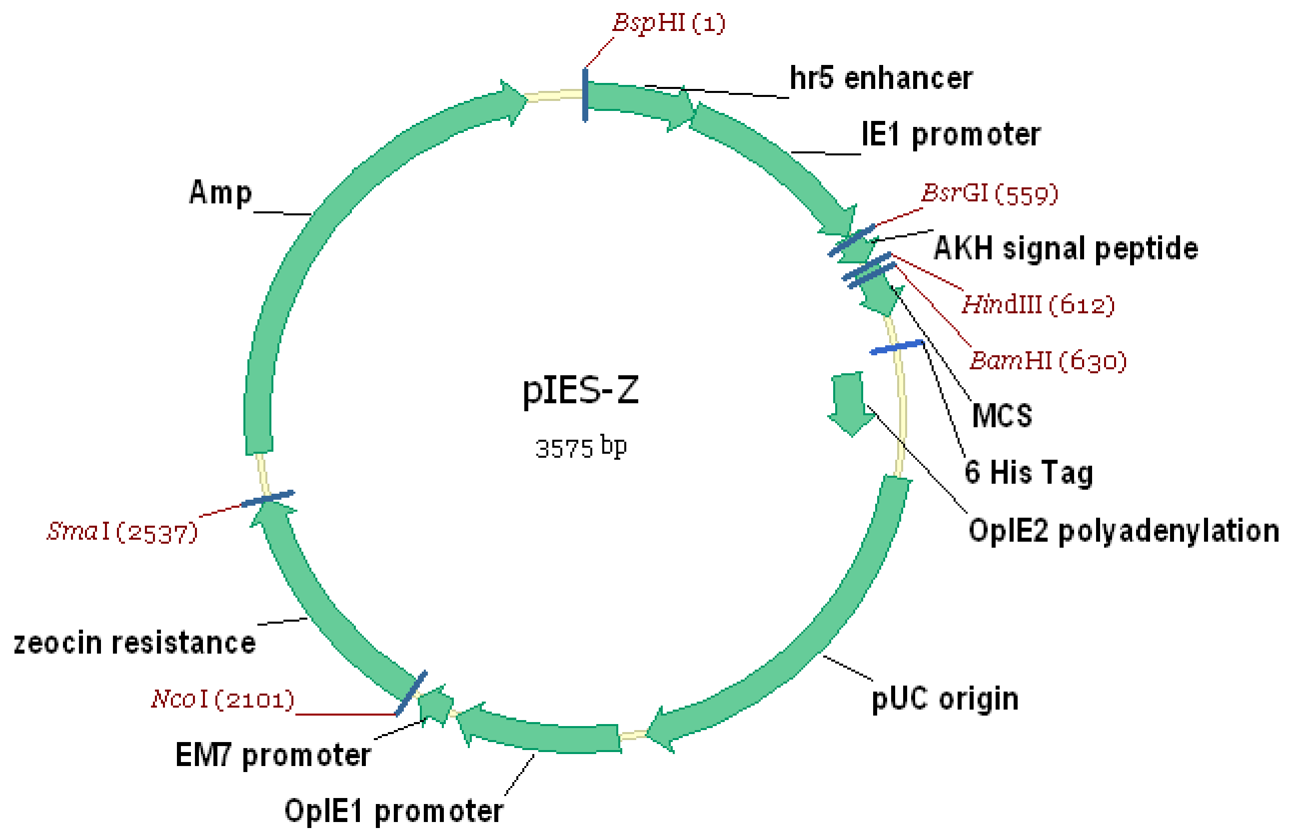
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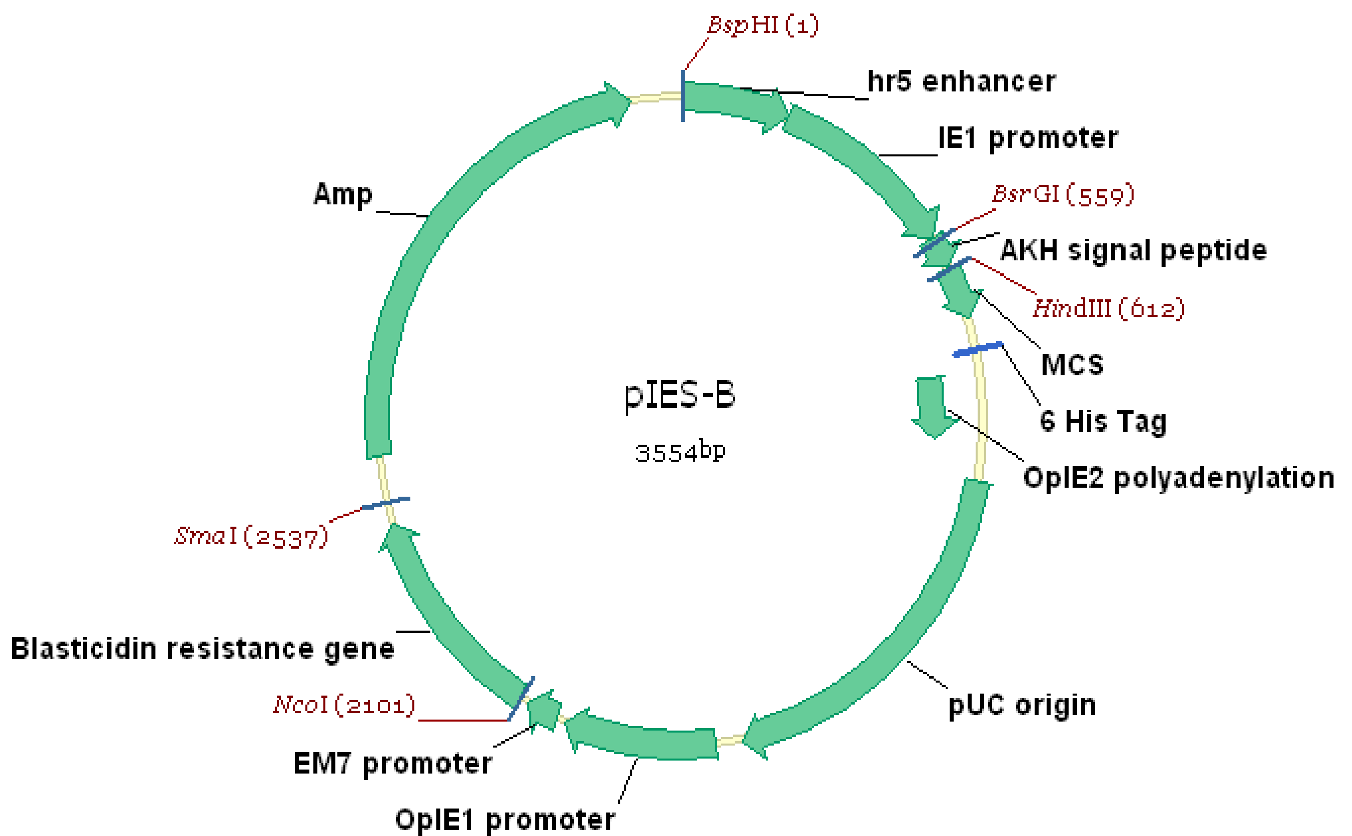
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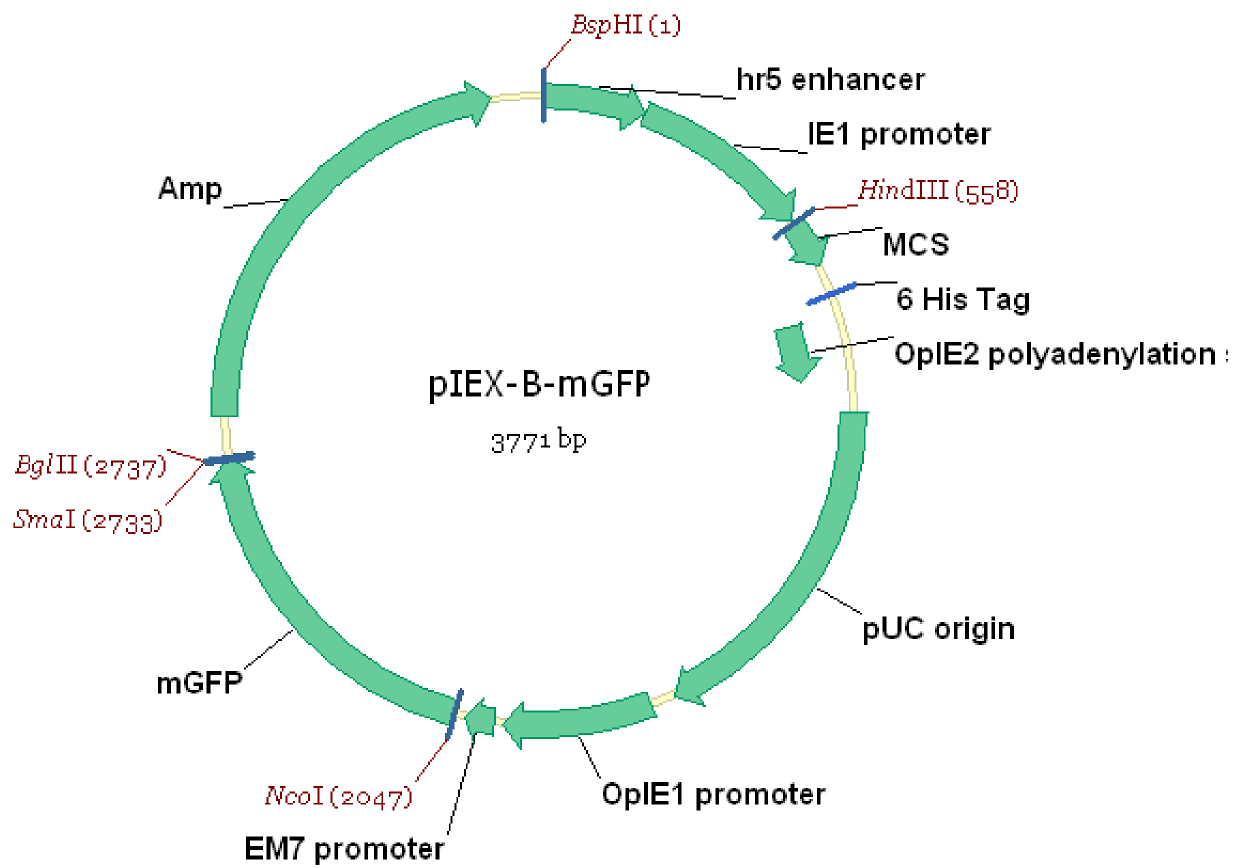
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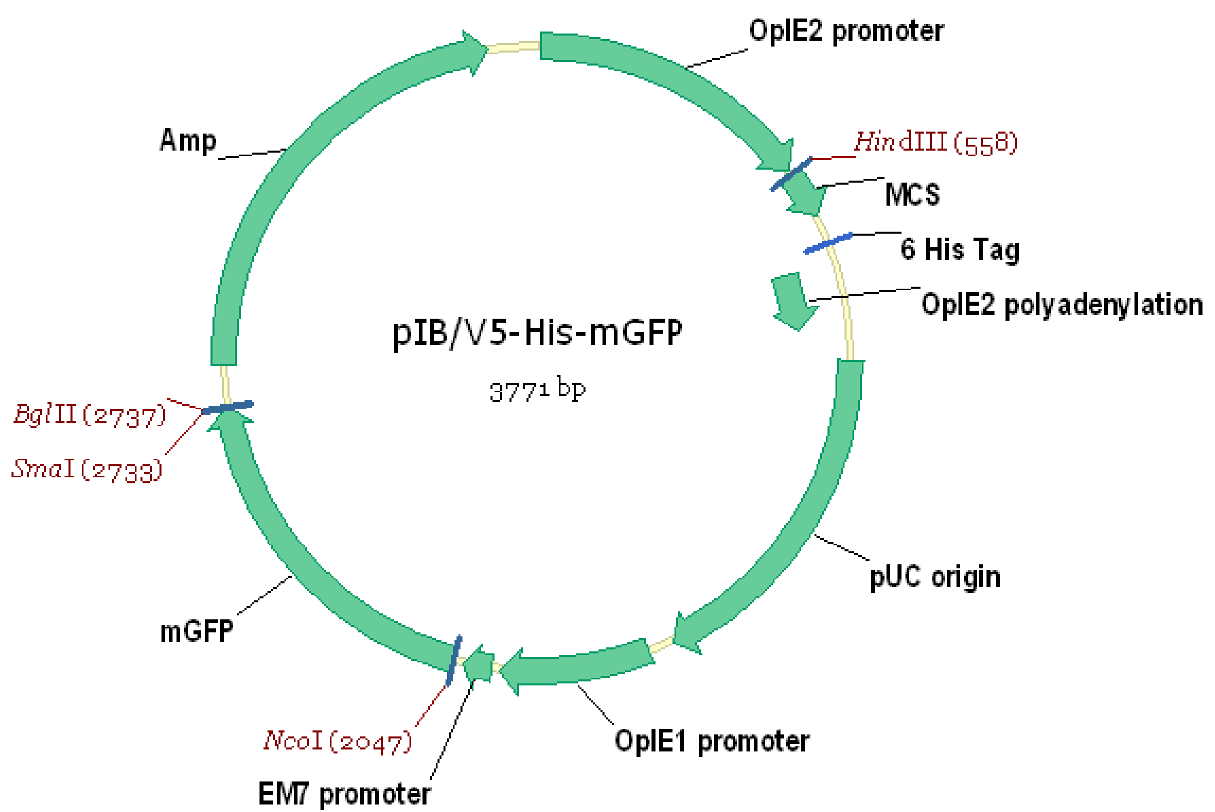
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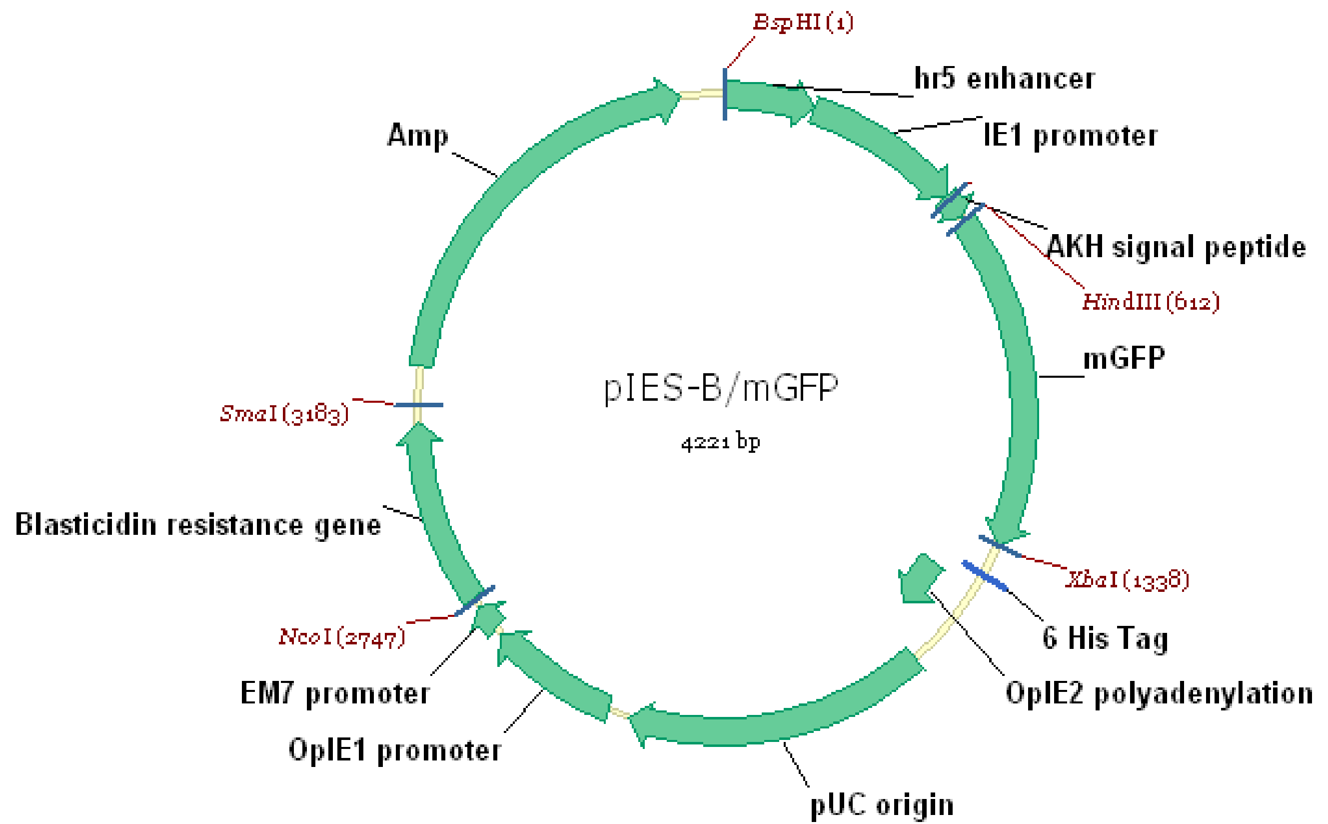
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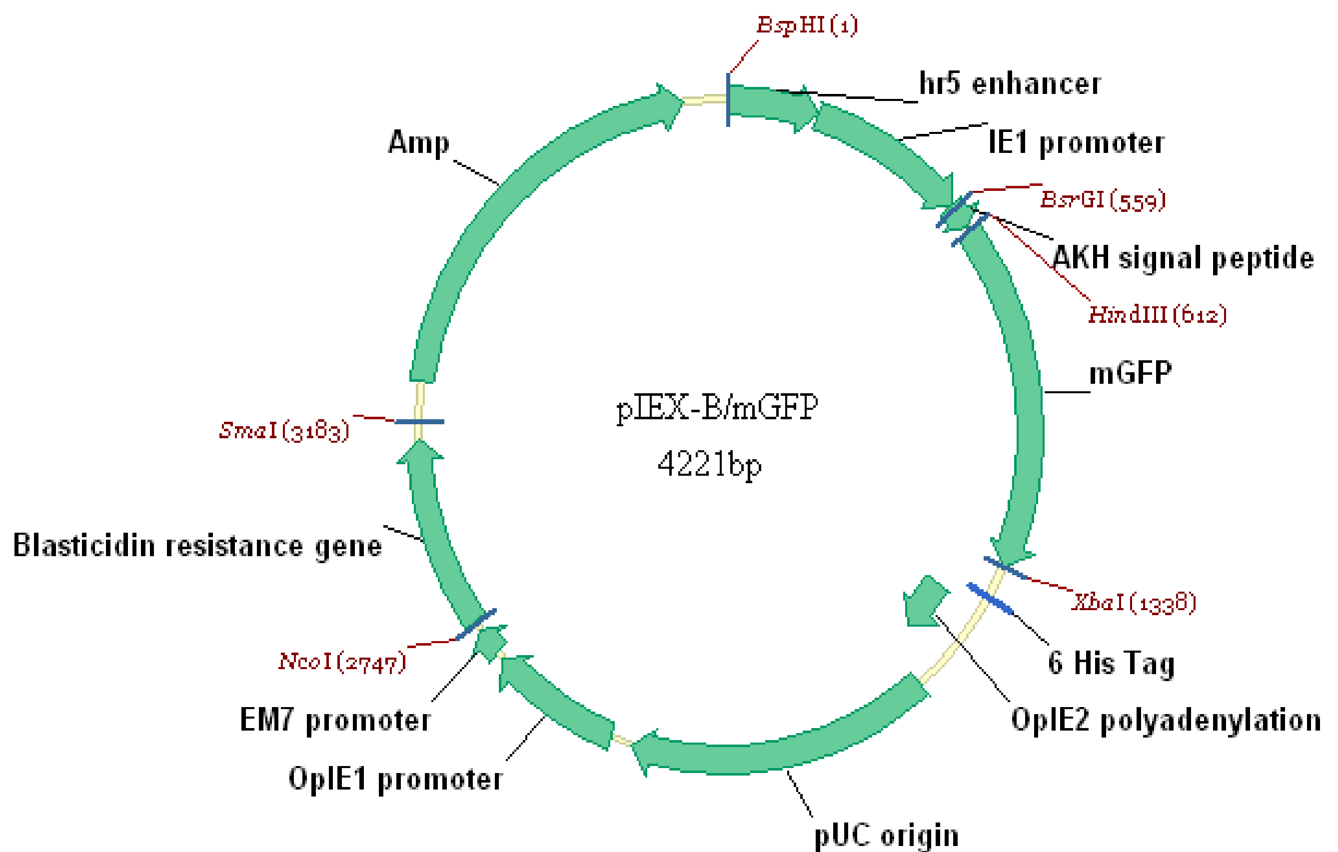
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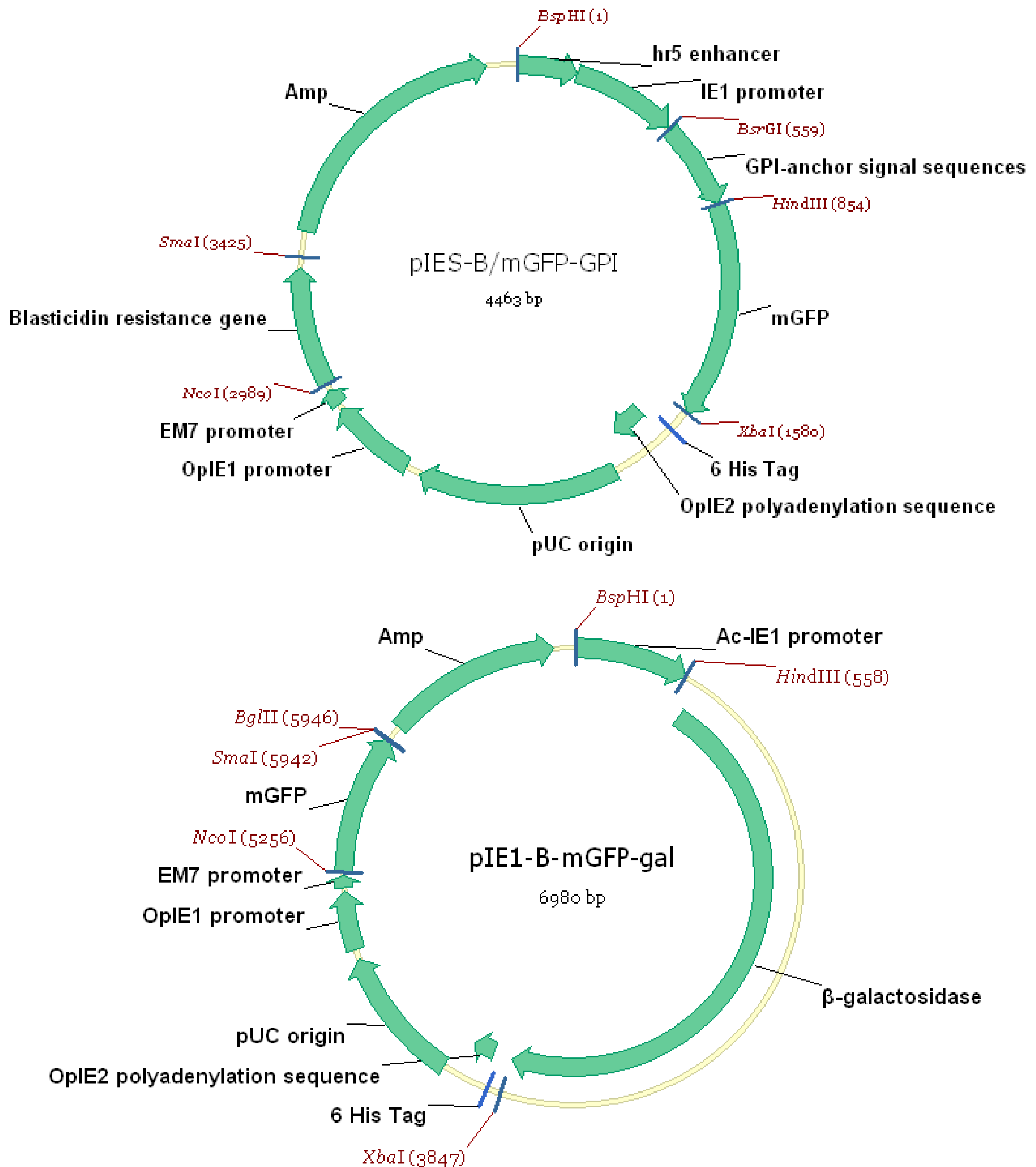

Supplementary Figure 1. Vector maps. Note: all of these vector maps were constructed by vector nti 10.3.0 (Invitrogen Corportation).

Western blot

Sf9 cells expressing GFP, β-galactosidase or vector control were collected by centrifugation. Media were also collected by cold methanol precipitation. Cell pellets and methanol-precipitated materials were resuspended in lysis buffer D (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% DOC and 0.5% NP-40). Samples were lysed on ice for 30 min. Post-nuclear fractions were prepared by centrifu-

gation at 1000×*g* at 4°C. Samples were mixed with 4× sample loading buffer and proteins were denatured by heating for 6 min at 100°C. Proteins were separated by SDS-12% PAGE and transferred onto a PVDF membrane. The transferred membrane was blotted with either polyclonal anti-GFP antibody (Abcam, MA) or monoclonal anti-β-galactosidase antibody (Promega, WI). Signals were visualized using an ECL kit (GE Healthcare, UK).

Statistical analysis

All data were analyzed by using GraphPad Prism 5.0 software. $P < 0.05$ was considered as a significant difference.

RESULTS AND DISCUSSION

hr5-IE1 promoter had higher activity than OpIE2

The pIB/V5-His vector from Invitrogen has an OpIE2 promoter and a blasticidin selectable marker. The pIEX-4 vector from Novagen has an IE1 promoter and a hr5 enhancer (hr5-IE1), but does not have an antibiotic-expressing cassette to ensure stable expression. We occasionally found that the Novagen vector expressed more protein than the Invitrogen vector when transiently expressing prion protein in insect cells. To systematically compare the activity of these two promoters in same backbone, we replaced the OpIE2 promoter with the hr5-IE1 promoter sequences in the pIB/V5-His vector and named this new vector pIEX-B. A gene encoding β-galactosidase was cloned into the pIB/V5-his and pIEX-B vectors via multiple cloning sites, and generated pIB/V5-His-gal and pIEX-B-gal plasmids. To eliminate differences caused by transfection efficiencies, monomeric GFP (mGFP) was used as an internal control. The gene encoded blasticidin resistance in pIB/V5-His, pIEX-B, pIB/V5-His-gal and pIEX-B-gal was replaced with the gene encode mGFP and these new plasmids were designated as pIB-mGFP, pIEX-mGFP, pIB/V5-His-gal-mGFP and pIEX-B-gal-mGFP, respectively. Sf9 cells were transiently transfected with the plasmids indicated in Figure 1a. Both GFP and the activity of β-galactosidase were measured at 48 h post-transfection. The relative activity of β-galactosidase was obtained by normalizing to mGFP. The result showed that the hr5-IE1 promoter had about 20% higher activity than the OpIE2 promoter (Figure 1a). By Western blot analysis using an anti-GFP or anti-gal antibody, which allows better quantitative comparison, hr5-IE1 promoter was found to be > 20% more activity than the OpIE2 promoter (Figure 1b). Furthermore, stably expressing cell lines were established by blasticidin selection. The activity of β-galactosidase from three stable cell lines (Figure 1c) was measured and showed that the hr5-IE1 promoter also had 20% more activity than the OpIE2 promoter (Figure 2c). In addition, a second insect cell line, Sf21, was also tested in the study. Similar results were obtained (data not shown).

The OpIE2 promoter is derived from the *O. pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early 2 genes, while the IE1 promoter is derived from the *A. california* multicapsid nuclear polyhedrosis virus (AcNPV) immediate early gene. Prior to this study, there was no direct comparison of activity between the OpIE2 and IE1 promoters, but a previous study showed that the OpIE2 promoter had five- to ten-fold higher activity than the OpIE1 (*O. pseudotsugata* multicapsid nucleopolyhedrosis virus immediate-early 1) (promoter Pfeifer et al., 1997). Because the OpIE1 protein has 21% identity at the N-terminal region and 55% identity at C-terminal region to the IE1 protein (Theilmann and Stewart, 1991), the IE1 promoter itself should be much weaker than the OpIE2 promoter. To directly compare the activity of the Ac-IE1 and OpIE2 promoters, we created a pIE1-B-mGFP-gal construct by removing the hr5 enhancer from pIEX-B-mGFP-gal. We found that the activity of the Ac-IE1 promoter was seven-fold lower than OpIE2 promoter (data not shown). The hr5 is an enhancer element of AcNPV and stimulates transcription from the IE1 promoter through DNA-protein interaction (Guarino and Dong 1994). In the paper, the activity of IE1 promoter itself might be lower than OpIE2, but the combination of hr5 and IE1 produced a stronger gene expression activity than OpIE2 in both the transiently and stably experiments. These results suggest that hr5 enhancer enhances the activity of IE1 and that the hr5-IE1 combination uses the endogenous insect cell transcription machinery to direct expression.

AKH signal peptide effectively led passenger protein into the secretion pathway

To date, all mammalian secretion signals tested have functioned properly in insect cells. For some applications, researchers need to express non-secreted protein into the secretion pathway or need to target proteins to organelles in secretion pathway. To meet this need, we constructed two vectors (pIES-B and pIES-Z) with an AKH signal peptide under the hr5-IE1 promoter. AKH is an insect adipokinetic hormone that is synthesized in the corpora cardiaca, a neurohemal organ that is considered the functional equivalent of the vertebrate pituitary gland (O'Shea and Rayne, 1992). The AKH signal peptide has been widely used for expressing proteins in the secretion pathway in insect cells (Olczak and Olczak, 2006; Olczak et al., 2006). To test AKH signal peptide function, we cloned mGFP into pIEX-B and pIES-B downstream of the AKH signal peptide. In contrast to cytosolic mGFP (Figure 2a, b), mGFP with the AKH signal peptide was secreted into the medium (Figure 2a to d, high green background). Western blotting confirmed that the majority of the mGFPs were in the medium fraction (Figure 2b; upper panel and middle panel). To further test the function of the AKH signal peptide, we fused mouse prion protein GPI-anchor signal sequences to the C-terminus of mGFP. The results clearly demonstrated that mGFPs

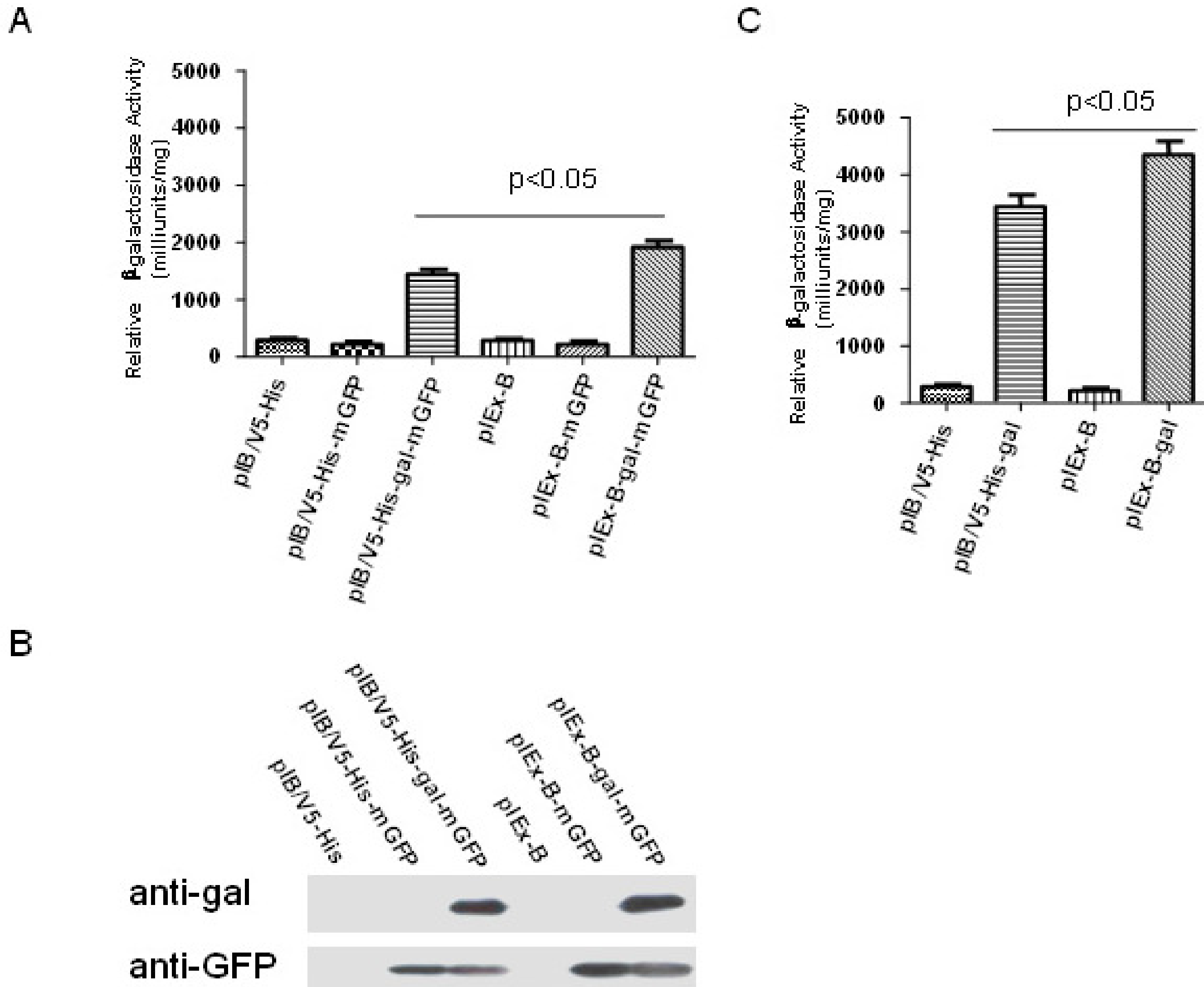

Figure 1. The hr5-IE1 promoter had greater activity than OpIE2. (A) The control vector and plasmids encoding either mGFP alone under the control of OpIE1 promoter or mGFP together with β-galactosidase (gal) were transiently transfected into Sf9 cells. mGFP and β-galactosidase activity were measured. The activity of β-galactosidase was normalized to the mGFP expression level to eliminate differences due to transfection efficiencies. The relative activity of β-galactosidase was shown in milliunits per milligram. The relative activity of β-galactosidase between pIB/V5-His-gal-mGFP and pIEX-B-gal-mGFP was significantly different ($p < 0.05$). Each error bar represents three independent experiments; (B) cell extracts from each transient transfection as indicated in the figure were blotted with either anti-β-galactosidase antibody (upper panel) or anti-GFP antibody (lower panel); (C) the control vector and plasmids encoding β-galactosidase were stably transfected into Sf9 cells. The activity of β-galactosidase from three stable cell lines was measured. There was a significant difference in activity between pIB/V5-His-gal and pIEX-B-gal ($p < 0.05$). Each error bar represents three independent experiments.

were located at the surface of the cells (Figure 2a to f). Western blotting showed similar expression profiles to cytosolic mGFP (Figure 2b; lower panel). Taken together, these results strongly suggest that the AKH signal peptide sufficiently led the passenger mGFP into the secretion pathway. Moreover, GPI anchor signal sequences from mouse prion protein functioned properly in insect cells. In addition, the same methods were applied to the pIES-Z vector and similar results were obtained (data not shown).

Vector with blasticidin resistance and vector with zeocin resistance had similar activity

Zeocin, a member of the phleomycin family of antibiotics isolated from *Streptomyces verticillus*, exhibits toxicity toward a broad range of prokaryotic and eukaryotic organisms and is thus, ideal for the development of vectors using a single antibiotic selection system. For some applications, it might be advantageous to use zeocin selection for stable expression. To meet this need, we

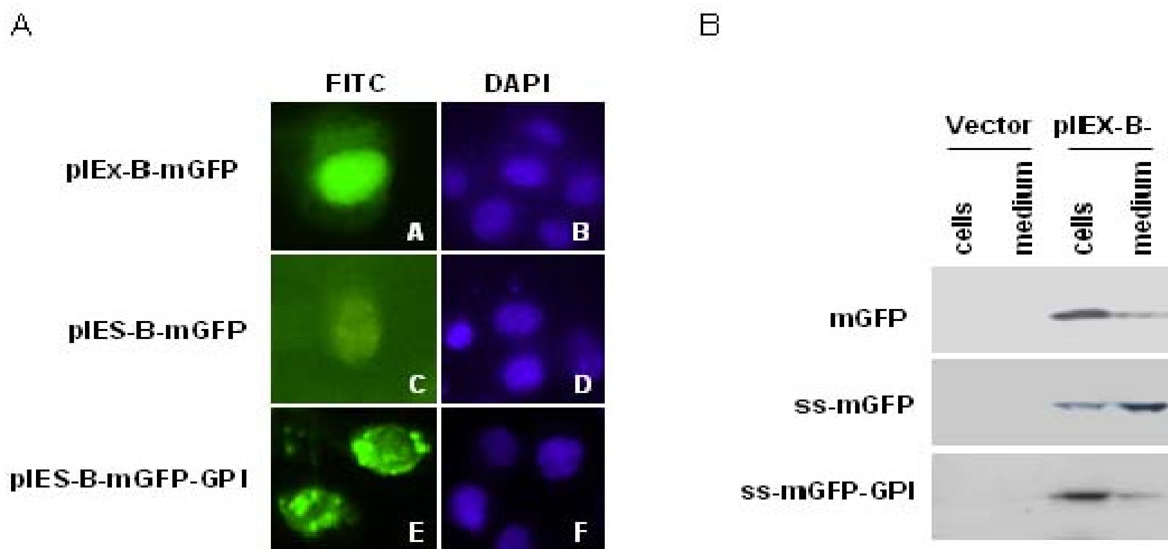

Figure 2. AKH signal peptide sufficiently led passenger mGFP into the secretion pathway. (A) Sf9 cells were transiently transfected with either plasmid pIEX-B-mGFP (a and b), pIES-B-mGFP (c and d), or pIES-B-mGFP-GPI (e and f). Cells were fixed and stained with DAPI at 48 h post-transfection. Images were taken under a fluorescent microscope; (B) expressed proteins in both the medium and the cells were confirmed by western blot using anti-GFP antibody.

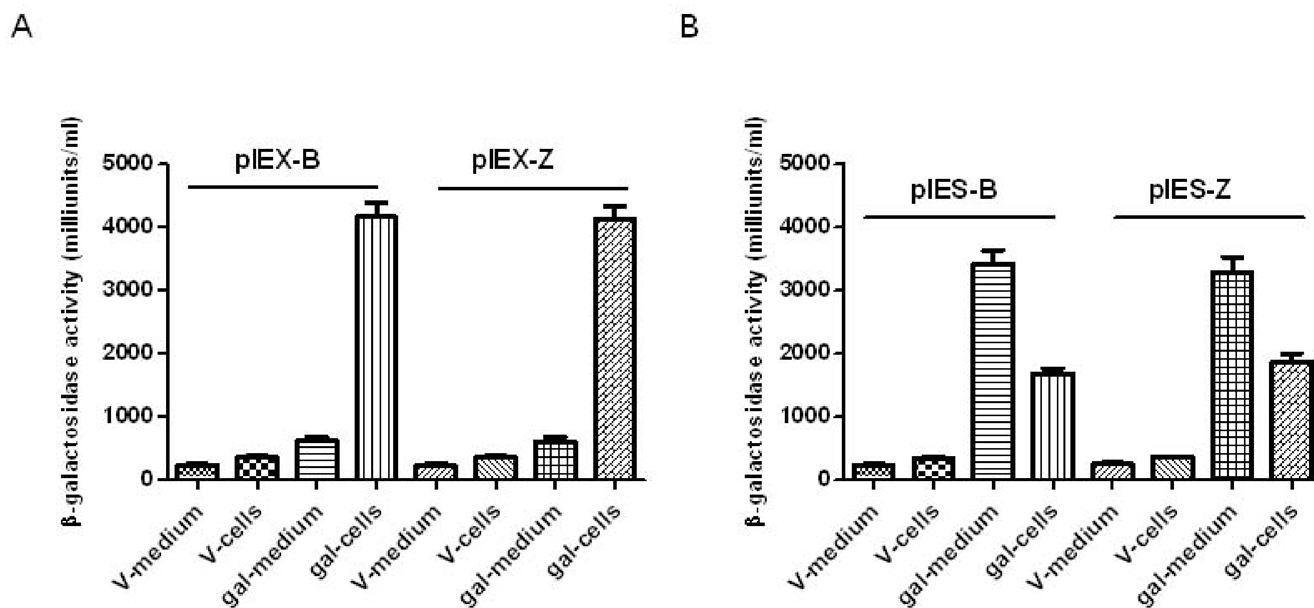

Figure 3. Blasticidin and zeocin resistance expression cassettes did not alter hr5-IE1 promoter activity. The control vector and plasmids encoding β-galactosidase were stably transfected into Sf9 cells. The activity of β-galactosidase from three stable cell lines was measured. V-medium and V-cells denote control vector expressed in medium and in cells, respectively and gal-medium and gal-cells denote β-galactosidase-encoding plasmids expressed in medium and in cells, respectively. (A) There was no significant difference in activity between pIEX-B-gal and pIEX-Z-gal ($p > 0.05$) in medium or in cells. Each error bar represents three independent experiments; (B) there was no significant difference in activity between pIES-B-gal and pIES-Z-gal ($p > 0.05$) in medium or in cells. Each error bar represents three independent experiments.

structed pIEX-Z and pIES-Z vectors by replacing gene encoded the blasticidin resistance in pIEX-B and pIES-B with gene encoded zeocin resistance. β-Galacto-sidase assays showed that the two new vectors, pIEX-Z and pIES-Z, had activity similar to that of their respective parental vectors, pIEX-B and PIES-B (Figure 3a, b).

These results suggest that the antibiotic-resistance genes did not affect the activity of the hr5-IE1 promoter and the hr5-IE1 is a more effective combination to direct expression in insect cell.

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