Video Article

Modifying Baculovirus Expression Vectors to Produce Secreted Plant Proteins in Insect Cells

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Abstract

It has been a challenge for scientists to express recombinant secretory eukaryotic proteins for structural and biochemical studies. The baculovirus-mediated insect cell expression system is one of the systems used to express recombinant eukaryotic secretory proteins with some post-translational modifications. The secretory proteins need to be routed through the secretory pathways for protein glycosylation, disulfide bonds formation, and other post-translational modifications. To improve the existing insect cell expression of secretory plant proteins, a baculovirus expression vector is modified by the addition of either a GP67 or a hemolin signal peptide sequence between the promoter and multiple-cloning sites. This newly designed modified vector system successfully produced a high yield of soluble recombinant secreted plant receptor proteins of *Arabidopsis thaliana*. Two of the expressed plant proteins, the extracellular domains of Arabidopsis TDR and PRK3 plasma membrane receptors, were crystallized for X-ray crystallographic studies. The modified vector system is an improved tool that can potentially be used for the expression of recombinant secretory proteins in the animal kingdom as well.

Video Link

The video component of this article can be found at https://www.jove.com/video/58283/

Introduction

It is imperative for a research laboratory to be capable of producing large quantities of homogeneous recombinant proteins for biochemical and biophysical characterizations, especially for X-ray crystallographic studies. There are many well-established heterologous expression systems such as *Escherichia coli*, yeast, insect cells, mammalian cells, plant cells, *etc.* Among them, the baculovirus-mediated insect cell expression system is one of the most commonly used techniques to produce large quantities of structurally folded large-sized recombinant eukaryotic proteins for protein crystallization[1].

The expression vectors of the baculovirus expression system are engineered to contain a strong polyhedrin or P10 promoter to produce a high yield of recombinant intracellular proteins[2,3]. To make a recombinant baculovirus, the gene of interest is cloned into an insect vector containing the polyhedrin (polh) locus of the *Autographa californica* multi-nucleopolyhedroviral genome. The resulting construct is then sequenced and its correct open reading frame (ORF) is verified. The correct construct is then introduced into the host insect cell through the process of transfection. The gene of interest is inserted into the viral genome by homologous recombination. This event results in the production of the recombinant viral genome, which then replicates to produce recombinant budded virus particles[1].

The insect cells that are most commonly used in the expression system are Sf9 and High Five (Hi5) cells. Sf9 cells are a clonal isolate of Sf21, derived from the pupal ovarian cells of *Spodoptera frugiperda*, and Hi5 cells are a clonal isolate derived from the parental *Trichoplusia ni* ovarian cell line TN-368[4,5]. Co-transfections, virus amplification, and plaque assays are conducted on Sf9 cells, while Hi5 cells are typically selected to produce higher quantities of recombinant proteins[6]. It is worth noting that the Hi5 cells are not suitable for the generation and amplification of virus progenies because of their tendency to produce mutant viruses. Traditionally, a temperature range of 25 - 30 °C is considered to be good for the cultivation of insect cells. However, it has been reported that 27 - 28 °C is the optimal temperature for the insect cell growth and infection[7,8].

The introduction of a strong signal sequence preceding the gene is needed for the high expression of the secreted proteins. The signal sequence would efficiently guide the translated recombinant protein into the endoplasmic reticulum for protein secretion and post-translational modifications necessary for proper folding and stabilization[3]. Signal peptide sequences, such as the baculovirus envelop protein GP64/67, honeybee melittin, and others, have been chosen to facilitate the expression of secretory recombinant proteins in the baculovirus-mediated expression systems[3]. The introduction of the signal peptide of GP67 has been shown to improve the expression yield of a secreted recombinant protein, in comparison to using the intrinsic signal peptide of the target gene[9]. Hemolin is a hemolymph protein of the giant silk moth *Hyalophora cecropia*, induced upon bacteria infection[10]. Due to the relatively high level of induced expression, the signal peptide sequence of the gene can be used to mediate the secretion expression of the recombinant proteins in the baculovirus-insect cell system.

The *A. thaliana* Tracheary Element Differentiation Inhibitory Factor Receptor (TDR) and Pollen Receptor Kinase 3 (PRK3) both belong to the plant Leucine-rich Repeat Receptor-like Kinase (LRR-RLK) family of proteins[11,12]. In order to study the structure and function of this family of plant receptor proteins, as well as to facilitate the structural and biochemical characterization of other plant secreted proteins, the baculovirus-insect cell expression system has been modified to improve protein quality and production yield. The extracellular domains of both TDR and PRK3 have successfully been expressed using two modified expression vectors in the baculovirus-insect cell expression system. Both the extracellular domains of TDR and PRK3 proteins have been crystallized. This article reports the expression and purification of large amounts of recombinant secreted plant proteins with two modified baculovirus expression vectors by incorporating either a GP67 or a hemolin signal sequence between the promoter and multiple cloning sites.

Protocol

NOTE: An insect cell/baculovirus system with modified expression vectors for secretory plant protein expression and crystallization is used.

1. Modification of a Baculovirus Expression Vector with the GP67 Signal Peptide for Plant Protein Secretion Expression

1. Synthesize a DNA fragment containing a 5' BglII cutting site[13], the GP67 secretion signal sequence, and a multi-cloning site with NotI, BamHI, EcoRI, StuI, SalI, SpeI, XbaI, PstI, and XhoI (**Figure 1A**).
   NOTE: Since both BglII and BamHI digested DNA resulted in the same adhesive ends, the linearized vector can ligate to the digested DNA fragment while both the BglII and BamHI sites in the annealed ligation sequence will be mutated to a sequence that is not cleavable by either BglII or BamHI[14].
2. Digest 4 µg of the DNA with BglII and XhoI, and 4 µg of an expression vector DNA with BamHI and XhoI[14]. Mix 10 units of each restriction enzyme with the DNA in a 1x concentration reaction buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 100 µg/mL BSA, pH 7.9 at 25 °C) and incubate the mixture at 37 °C for 4 h.
   1. Purify the excised DNA fragment and the linearized vector DNA by a DNA gel extraction kit[15].
3. Mix 100 ng of the linearized vector DNA with 400 ng of the digested DNA fragment in a 10-µL ligation reaction mixture containing 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM DTT, pH 7.5 at 25 °C) and 5 units of T4 DNA ligase. Incubate the reaction mixture at 16 °C for 16 h.
4. Transform 5 µL of the ligation mixture into 100 µL of DH5α competent cells following a standard transformation protocol and select the resulting colonies on an ampicillin-LB agar plate[16]. Pick up 5 - 10 colonies and grow each colony in 2 mL of LB medium containing 100 µg/mL ampicillin at 220 rpm and 37 °C in a shaking incubator for 16 h.
5. Extract each plasmid DNA with a DNA miniprep kit[17] and confirm the clones by DNA sequencing with a primer AGTATTTTACTGTTTTCGTAACAG.
6. PCR-amplify the *A. thaliana* TDR gene fragment encoding residues 30 - 642 from a synthetic TDR gene construct (forward primer: CATG GCGGCCGC CTCAAGTTTTCACCTCAACTCTTGTC, reverse primer: GC GGATCC CTA GTG GTG ATG GTG GTG GTG ACCGTCTATATCTGCATTTCCGGC). Amplify the DNA for 30 cycles in a DNA polymerase reaction buffer containing a 0.5 mM dNTP mix, 0.2 µM primers, 0.1 µg of template DNA, 0.4 mM $MgCl_2$, and 1 reaction unit of the DNA polymerase.
   1. For the PCR cycle steps, set the initial denaturation at 95 °C for 30 s, and then 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min.
7. Digest both the PCR fragment and the modified vector with NotI and BamHI restriction endonuclease enzymes. Ligate the gene fragment with the linearized vector. Mix 100 ng of the linearized vector DNA with 400 ng of the digested DNA fragment in a 10-µL ligation reaction mixture containing T4 DNA ligase reaction buffer and 5 units of T4 DNA ligase. Incubate the reaction mixture at 16 °C for 16 h.
8. Transform 5 µL of the ligation mixture into 100 µL of DH5α competent cells following a standard transformation protocol and select the resulting colonies on an ampicillin-LB agar plate[16]. Confirm the correct clones by DNA sequencing.

2. Modification of a Baculovirus Expression Vector with the Insect Hemolin Signal Peptide for Plant Protein Secretion Expression

1. Synthesize a DNA fragment containing a 5' BglII cutting site[13], the insect hemolin secretion signal sequence, and a multi-cloning site with NotI, BamHI, EcoRI, StuI, SalI, SpeI, XbaI, PstI, and XhoI (**Figure 1B**).
2. Digest 4 µg of the DNA with BglII and XhoI, and 4 µg of an expression vector DNA with BamHI and XhoI[14]. Mix 10 units of each restriction enzyme with the DNA in a 1x concentration reaction buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 100 µg/mL BSA, pH 7.9 at 25 °C) and incubate the mixture at 37 °C for 4 h.
   1. Purify the excised DNA fragment and the linearized vector DNA by a DNA gel extraction kit[15].
3. Mix 100 ng of the linearized vector DNA with 400 ng of the digested DNA fragment in a 10-µL ligation reaction mixture containing 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM DTT, pH 7.5 at 25 °C) and 5 units of T4 DNA ligase. Incubate the reaction mixture at 16 °C for 16 h.
4. Transform 5 µL of the ligation mixture into 100 µL of DH5α competent cells and select the colonies on an ampicillin-LB agar plate. Pick up 5 - 10 colonies and grow each colony in a 2-mL LB medium containing 100 µg/mL of ampicillin at 220 rpm and 37 °C in a shaking incubator for 16 h.
5. Extract plasmid DNA with a DNA miniprep kit[17] and confirm the clones by DNA sequencing with a primer AGTATTTTACTGTTTTCGTAACAG.
6. PCR-amplify *A. thaliana* Pollen Receptor Kinase 3 (PRK3) gene fragment encoding residues 20 - 237 from a synthetic PRK3 gene construct (forward primer: CATG GCGGCCGC CTACAAAACGTTAGTGAATCGGAACC, reverse primer: CGC GGATCC CTA GTG GTG ATG GTG

GTG GTG TGAAGGTTTCTCATCGCATTCTATATTC). Amplify the DNA for 30 cycles in a DNA polymerase reaction buffer containing a 0.5 mM dNTP mix, 0.2 µM primers, 0.1 µg of template DNA, 0.4 mM $MgCl_2$, and 1 reaction unit of the DNA polymerase.

1. For the PCR cycle steps, set the initial denaturation at 95 °C for 30 s, and then 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s within each cycle, and the final extension at 72 °C for 5 min.

7. Digest both the PCR fragment and the modified vector with NotI and BamHI restriction endonuclease enzymes. Ligate the gene fragment with the linearized vector. Mix 100 ng of the linearized vector DNA with 400 ng of the digested DNA fragment in a 10-µL ligation reaction mixture containing T4 DNA ligase reaction buffer and 5 units of T4 DNA ligase. Incubate the reaction mixture at 16 °C for 16 h.
8. Transform 5 µL of the ligation mixture into 100 µL of DH5α competent cells following a standard transformation protocol and select the resulting colonies on an ampicillin-LB agar plate[16]. Confirm the correct clones by DNA sequencing.

3. Production and Amplification of Baculovirus Constructs Harboring the Recombinant Protein Expression Cassette

1. Use 100 ng of the construct plasmid to transform 40 µL of DH10Bac competent cells following the protocol of the baculovirus expression system[1]. Incubate the transformation plates at 37 °C for 48 h.
2. Pick up two white colonies from each transformation plate, inoculate each colony into 2 mL of LB medium containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline. Follow the protocol of the baculovirus expression system[1] to extract and verify the bacmid DNA. Aliquot each DNA in two tubes with 20 µL each, and store the tubes at -20 °C.
   NOTE: The following steps require an aseptic operation.
3. Grow a monolayer of Sf9 cells in the culture media with 10% fetal bovine serum (FBS) and 100 µg/mL (or 100 I.U./mL) penicillin-streptomycin antibiotics at 27 °C to 80% confluence in a 6-well plate. Estimate the percentage of confluence based on the percentage of covered area on the tissue culture plate.
4. Prepare the following solutions in two sterile 1.5-mL centrifuge tubes.
   1. Tube 1: For each transfection, dilute 20 µL of bacmid DNA into 100 µL of unsupplemented Sf9 cell culture medium without antibiotics. Mix the dilution gently by flicking the side of the tube.
   2. Tube 2: For each transfection, dilute 8 µL of lipid transfection reagent into 100 µL of unsupplemented Sf9 cell culture medium without antibiotics. Mix the dilution thoroughly by pipetting up and down for 6x.

5. Combine the two solutions, mix them gently by pipetting slowly up and down for 3x, and incubate them for 20 - 40 min at room temperature.
6. Wash each well of the Sf9 monolayer cells 2x with 3 mL of unsupplemented Sf9 cell culture medium without antibiotics. For each transfection, add 0.8 mL of unsupplemented Sf9 cell culture medium without antibiotics to each tube containing the lipid-DNA mixture. Mix the contents of the tubes gently by pipetting slowly up and down for 3x. Aspirate the wash media from the plates and overlay the samples with the transfection mixture.
7. After the addition of the transfection mixture, incubate the transfected plate for 5 h at 27 °C. Replace the transfection media with the complete Sf9 cell culture medium containing 10% FBS and 100 µg/mL (or 100 I.U./mL) penicillin-streptomycin antibiotics. Incubate the transfected plate at 27 °C for 4 d.
8. Harvest and amplify the recombinant baculovirus.
   1. Collect the supernatant of the infected plate in a sterile 15-mL conical tube after 4 d of incubation. Spin the supernatant at 1010 x *g* for 5 min to remove the cell debris. Discard the pellet and decant the supernatant to a clean tube and store it at 4 °C for up to 1 year.
      NOTE: This is the P0 virus. It can be aliquoted and stored at -80 °C for a longer period of time.
   2. To amplify each recombinant virus, grow a monolayer of Sf9 cells on a 150-mm plate to 80% confluence. Aspirate the media from the plate, add 2 mL of the P0 virus to the cells adherent to the plate and incubate the plate for 1 h in a 27 °C incubator. Rock the plate every 15 min to mix the medium with the cells.
      1. Add 25 mL of complete Grace's media containing 10% FBS and 100 µg/mL (or 100 I.U./mL) penicillin-streptomycin antibiotics. Incubate the plate for 3 d in a 27 °C incubator to obtain the P1 passage virus.

   3. Collect the supernatant of the infected plate in a sterile 50-mL conical tube and spin at 1010 x *g* for 5 min to remove the cell debris. Decant the supernatant to a clean tube and store it at 4 °C for up to 1 year.
      NOTE: The P1 virus can be aliquoted and stored at -80 °C for a longer period of time.
   4. To amplify the virus from P1 to P2 passage, grow a monolayer of Sf9 cells on a 150-mm plate to 90% confluence, aspirate the media of the plate, add 2 mL of the P1 virus to the plate, and incubate it for 1 h in a 27 °C incubator.
   5. Repeat steps 3.8.2 and 3.8.3 to obtain the P2 and P3 passages of the viruses. Do not store the P3 virus for more than 2 months.

4. Protein Expression, Purification with NiNTA, and Crystallization

1. Culture 1 L of Hi5 insect cells in the culture media with 100 µg/mL (or 100 I.U./mL) penicillin-streptomycin antibiotics to a density of 2 x 10^6 at 100 rpm in a 27 °C incubator shaker. Spin down the cells at 570 x *g* for 5 min, decant the supernatant, resuspend the cells in 20 mL of the freshly produced P3 virus, and keep it at room temperature for 1 h. Gently shake the spin bottle to resuspend the cells 1x every 15 min.
2. Transfer the cells to 1 L of culture media with 100 µg/mL (or 100 I.U./mL) penicillin-streptomycin antibiotics, culture the cells at 100 rpm in a 27 °C incubator shaker for 72 h. Spin down the cells at 1,010 x *g* for 5 min and collect the supernatant.
3. Use pH indicator strips to adjust the pH of the supernatant to 8.0 by adding either 0.1 M HCl or 0.1 M NaOH and add binding buffer at a final concentration, containing 20 mM Tris buffer at pH 8.0, 100 mM NaCl, and 5 mM imidazole. Add a certain amount of NiNTA resin (10 - 40 mL) based on the estimated yield of the expressed His-tagged recombinant protein.
   1. Mix the solution on a magnetic stir with low speed at 4 °C for 1 h. Wash the resin and elute the bound protein following the standard NiNTA purification protocol[18].

4. Subject the purified protein to ion exchange and gel filtration chromatography, as well as other protein purification methods, to yield a homogeneous sample.
   NOTE: For the TDR ectodomain, 20 mM Tris, pH 8, 100 mM NaCl were used as gel filtration buffer. For the PRK3 ectodomain, 20 mM bis-Tris, pH 6, 100 mM NaCl were used as the preferred gel filtration buffer.

Representative Results

As shown in **Figure 1**, two modified pFastBac1 baculovirus expression vectors were used to express the secreted proteins with either the GP67 or the hemolin signal sequence to replace the intrinsic signal sequence of the target gene. The viral GP67 and the insect hemolin genes have been demonstrated to have high secretion expression levels in the cells. Fusion proteins with either of these two signal sequences are expected to have greatly improved secretion expression levels. Identical multiple cloning sites (MCS) were engineered in these two modified vectors. A NotI site was deliberately placed on the most 5'-side of the MCS, because NotI has an eight-nucleotide sequence rather than the most common six-nucleotide sequence present in many other restriction sites. As such, a NotI site is less present in the target genes than most other restriction sites, making restriction digestion and the cloning of most target genes more feasible in comparison to using other restriction sites. Cloning the target gene between the NotI and a downstream restriction site will introduce only three amino acid residues, GGR, preceding the target gene.

The pBac1-GP67 and pBac1-Hem have been successfully utilized to express the extracellular domains of the *A. thaliana* receptors TDR and PRK3, respectively (**Figure 2**). After the recombinant proteins were purified by NiNTA using an engineering C-terminal 6-histidine tag, the average protein yield was consistent around 20 mg/L of Hi5 cells. The purity of each protein is close to or higher than 50%, examined with SDS-PAGE and Coomassie staining.

The recombinant proteins of the extracellular domains of TDR and PRK3 were further purified by size exclusion chromatography (**Figure 2**). The purified protein was concentrated to 5 mg/mL and then subjected to a crystallization screening. The conditions, which yielded preliminary crystals of each protein, were optimized until protein crystals with a size bigger than 20 µm were observed (**Figure 3**). The crystal structures of both proteins have been reported[11,12].

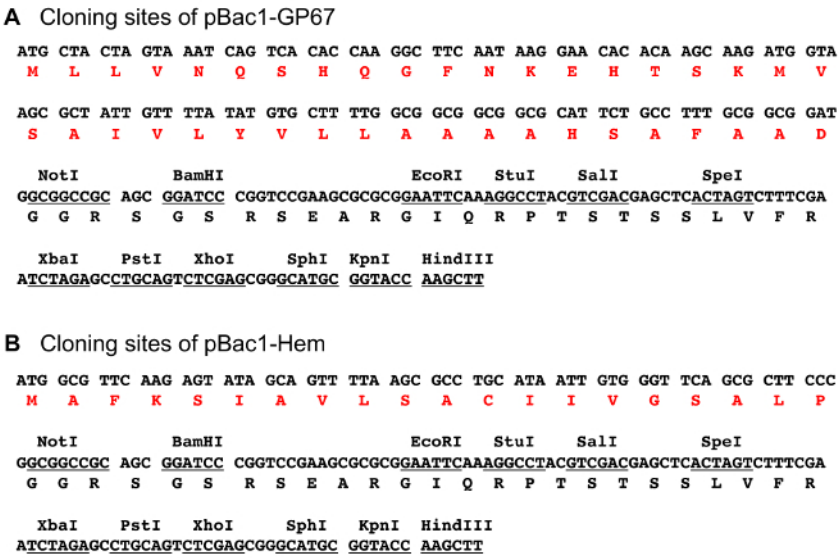

Figure 1: Maps of the modified pFastBac1 vectors. The DNA sequences shown in these panels were inserted downstream of the polyhedrin promoter of the pFastBac1 vector, to possess the signal peptide sequence and the multiple cloning sites (MCS) for target genes. Both vectors contain the same MCS. (**A**) This panel shows a sequence of the cloning sites downstream of the polyhedrin promoter in the pBac1-GP67 vector. The translated GP67 signal peptide amino acid sequence is colored in red. Each unique restriction site in the MCS is underlined, with the name of the site labeled above. (**B**) This panel shows a sequence of the cloning sites downstream of the polyhedrin promoter in the pBac1-Hem vector. The translated hemolin signal peptide amino acid sequence is colored in red. Each unique restriction site in the MCS is underlined, with the name of the site labeled above. Please click here to view a larger version of this figure.

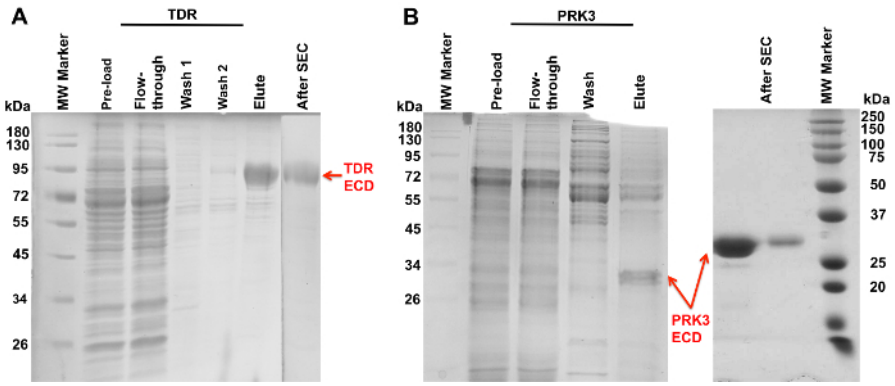

Figure 2: Protein expression with the modified pFastBac1 vectors in the baculovirus-insect cell system. (**A**) The ectodomain of TDR was expressed in Hi5 insect cells, purified by nickel-affinity and size exclusion chromatography (S), and resolved on SDS-PAGE gel. The nickel resin was washed once with a buffer containing 5 mM imidazole (wash 1), and a second time with 20 mM imidazole (wash 2). (**B**) This is an SDS-PAGE analysis showing the expression and nickel affinity purification (NiNTA), as well as the size exclusion chromatography of the PRK3 ectodomain. Molecular weight (MW) markers with the corresponding sizes are labeled. The red arrows in each panel denote the expressions and the expected sizes of the recombinant TDR and PRK3 ectodomain proteins. The multi-band natures of the expressed proteins are likely due to heterogeneous glycosylation. Please click here to view a larger version of this figure.

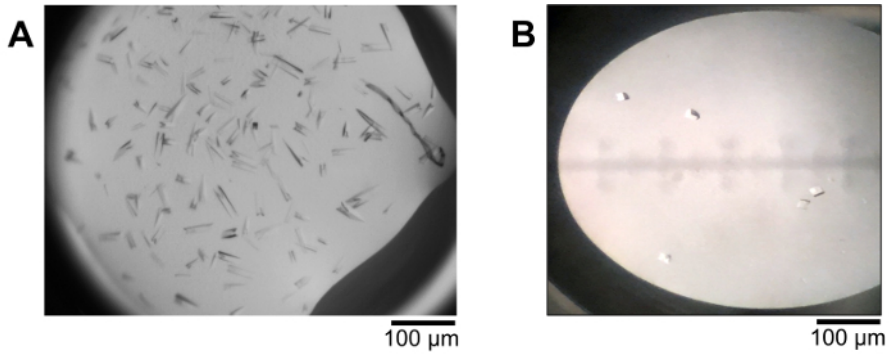

Figure 3: Protein crystals of the extracellular domains of *Arabidopsis thaliana* TDR and PRK3. (**A**) This panel shows the protein crystals of the extracellular domains of *A. thaliana* TDR. (B) This panel shows the protein crystals of the extracellular domains of *A. thaliana* PRK3. A scale bar is shown below each picture. Please click here to view a larger version of this figure.

Discussion

Given the diversity in size and stability of the thousands of proteins present in the biological systems, it is often empirical for a research laboratory to decide which heterologous expression system has to be chosen for the expression of a specific protein. The *E. coli* expression system is often the first choice for protein expression due to the short life cycle of the bacteria, low cost of the culture media, and relative ease to scale up[19]. For the expression of large eukaryotic proteins with sizes more than 60 kDa, however, using the *E. coli* system often results in insoluble proteins in the inclusion body or aggregation[20]. For the expression of those difficult proteins, and for other secreted proteins, the baculovirus-insect cell expression system may be more advantageous than the *E coli*. Especially when expressing secreted eukaryotic proteins, this modified baculovirus-insect cell expression system could be a preferred choice.

Many secreted eukaryotic proteins, including plant secreted proteins, need complex glycosylation, disulfide formation, and other post-translational modifications during protein folding and secretion[21]. *E. coli* systems do not have the cellular machinery to process the complex modifications required for many of the eukaryotic proteins[22]. Yeast has a relatively more advanced glycosylation system than *E. coli*[23]. However, for the expression of the secreted proteins in higher eukaryotic species and plants, the baculovirus-insect cell system presents a significant advantage. Since glycosylation affects protein folding and stability[24], many of the secreted recombinant proteins expressed in the *E. coli* and yeast systems have a low yield and tend to aggregate, presumably due to incorrect protein folding. The baculovirus-insect system has been modified to improve protein glycosylation, which makes it an ideal system for the expression of secreted eukaryotic proteins[25]. In this study, both the GP67 and the hemolin signal peptides have been successfully used to guide the secretion expression of two plant receptor proteins for protein crystallization. With these studies in mind though, the choice of either signal peptide is a critical step, and it needs more systematic comparative studies with a set of target genes from different organisms.

The idea of using both GP67 and hemolin signal peptides to enhance protein secretion expression was driven by the high expression yields of both genes. However, if the protein secretion and posttranslational modification machinery of the expression host are overloaded with the expressed recombinant proteins, the secreted recombinant proteins may not have adequate modifications, especially glycosylation. Both modified vectors have been used to successfully express numerous plant secretory proteins[11,12,26], many of which tend to aggregate, which is probably due to the incorrect or inadequate glycosylation. Therefore, for the expression of those difficult proteins, both strong and weak signal peptide sequences have to be tested and the quality of the expressed recombinant proteins needs to be compared. Keep in mind that a weak

signal peptide sequence will lower the overall yield of the protein; however, it may give the secretion machinery of the expression host enough capacity to process the protein secretion and modifications.

In addition to the expression of heterologous secreted proteins in insect cells, the mammalian cell and plant cell expression systems have been used successfully in the overexpression of recombinant mammalian and plant proteins, respectively[27,28,29]. In comparison to the heterologous systems, the near endogenous expression condition of either the mammalian or the plant secreted proteins will have the almost native modification machineries in the host cells to yield well-folded proteins. The caveat of using the near-native expression system is that the expressed recombinant proteins may interfere with the physiological function of the cells, which may potentially have adverse effects on the final expression yield of the proteins.

Disclosures

The authors have nothing to disclose.

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