Video Article

Transient Expression and Cellular Localization of Recombinant Proteins in Cultured Insect Cells

Jeffrey A. Fabrick[1], J. Joe Hull[1]

[1]U.S. Arid Land Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture

Correspondence to: Jeffrey A. Fabrick at jeff.fabrick@ars.usda.gov

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Abstract

Heterologous protein expression systems are used for the production of recombinant proteins, the interpretation of cellular trafficking/localization, and the determination of the biochemical function of proteins at the sub-organismal level. Although baculovirus expression systems are increasingly used for protein production in numerous biotechnological, pharmaceutical, and industrial applications, nonlytic systems that do not involve viral infection have clear benefits but are often overlooked and underutilized. Here, we describe a method for generating nonlytic expression vectors and transient recombinant protein expression. This protocol allows for the efficient cellular localization of recombinant proteins and can be used to rapidly discern protein trafficking within the cell. We show the expression of four recombinant proteins in a commercially available insect cell line, including two aquaporin proteins from the insect *Bemisia tabaci*, as well as subcellular marker proteins specific for the cell plasma membrane and for intracellular lysosomes. All recombinant proteins were produced as chimeras with fluorescent protein markers at their carboxyl termini, which allows for the direct detection of the recombinant proteins. The double transfection of cells with plasmids harboring constructs for the genes of interest and a known subcellular marker allows for live cell imaging and improved validation of cellular protein localization.

Video Link

The video component of this article can be found at https://www.jove.com/video/55756/

Introduction

The production of recombinant proteins using insect cell expression systems offers numerous benefits for the study of eukaryotic proteins. Namely, insect cells possess similar post-translational modifications, processing, and sorting mechanisms as those present in mammalian cells, which is advantageous for producing properly folded proteins[1,2,3]. Insect cell systems also typically require fewer resources and less time and effort for maintenance than mammalian cell lines[4,5]. The baculovirus expression system is one such insect cell-based system that is now widely used in many disciplines, including the production of recombinant proteins for protein characterization and therapeutics, the immunogenic presentation of foreign peptides and viral proteins for vaccine production, the synthesis of multi-protein complexes, the production of glycosylated proteins, *etc.*[1,2,4,6]. There are, however, situations in which baculovirus expression may not be applicable[3,7], and the use of nonlytic and transient insect expression systems may be more appropriate. Specifically, transient insect cell expression offers the possibility for the rapid synthesis of recombinant protein, requires less development and maintenance, does not involve viral-imposed cell lysis, and provides a means to better study cellular trafficking during protein synthesis[7,8,9,10].

This protocol describes the rapid generation of expression vectors using two-step overlap extension PCR (OE-PCR) [11] and the standard cloning of plasmid DNA in *Escherichia coli*. Plasmids are used to double-transfect commercially available cultured insect cells and to produce representative proteins. The protocol describes the production and use of two different fluorescently-labeled subcellular marker proteins and demonstrates colocalization with two aquaporin proteins from the insect *Bemisia tabaci*. The following protocol provides the basic methodology for OE-PCR, insect cell maintenance and transfection, and fluorescence microscopy for the cellular localization of target proteins.

Protocol

1. OE-PCR for the Construction of Expression Plasmids

Note: See **Table 1** for all primers used in OE-PCR. The use of a high-fidelity DNA polymerase is recommended for all amplifications. However, because these enzymes frequently do not leave a 3' A, it is necessary to perform a brief, non-amplifying incubation with a Taq DNA polymerase to "A-tail" the PCR products prior to cloning them into a TA insect cell expression vector. This protocol demonstrates a method to generate insect

expression plasmids harboring chimeric proteins, with the fluorescent proteins fused in-frame to the carboxyl terminus of the genes of interest (in this case, to two *Bemisia tabaci* aquaporin proteins) or to subcellular marker proteins (**Figure 1**).

1. **Use OE-PCR, which consists of two independent rounds of amplification, to generate: (1) sequences encoding the genes of interest (*B. tabaci* aquaporin 1: BtDrip1; *B. tabaci* aquaporin 2: BtDrip2_v1) fused in-frame with a green fluorescent protein variant called EGFP or (2) the subcellular markers (*Drosophila melanogaster* sex peptide receptor: DmSPR; *Homo sapiens* phospholipase A2: HsPLA2) with the mCherry coding sequence. Directly ligate the final OE-PCR products into the pIB/V5-His-TA plasmid.**
    1. For the first round of OE-PCR (indicated by A-D and A'-D' in **Figure 1**), use a gene-specific sense primer (shown in bold in **Table 1**) and a chimeric antisense primer (italicized in **Table 1**) corresponding to the final 15 bp (without the stop codon) of the gene of interest/ subcellular marker and to 15 bp of the 5'-end of the desired fluorescent protein (EGFP or mCherry) to generate a product with a 3' overhang.
    Note: See **Table 2** for PCR conditions.
    2. In a separate tube, use a gene-specific fluorescent protein antisense primer (shown with an underline in **Table 1**) and a chimeric sense primer that contains 15 bp from the 3'-end of the gene of interest/subcellular marker and 15 bp from the 5'-end of the desired fluorescent protein to generate a product with a 5' overhang.
    Note: See **Table 2** for the PCR conditions. The PCR template can either be a sequence-validated PCR product or, more preferably, plasmid DNA harboring the desired sequence. The study described here uses sequence-validated plasmids.
    3. Separate the PCR products with 1% agarose gel electrophoresis (using standard molecular-grade agarose).
    4. Excise and purify the products of the expected sizes (see **Table 2**) using a commercially available DNA gel extraction kit (see the table of materials for the specific kit used in this protocol).
    5. Use the gel-purified PCR products from step 1.1.4 to generate the final overlap extension products (indicated by A"-D" in **Figure 1**). Use a gene-specific sense primer for the gene of interest/subcellular marker and the corresponding gene-specific antisense primer for the fluorescent protein to generate the desired chimeric sequence.
    Note: See **Table 2** for the PCR conditions. It may be necessary to empirically determine the optimal amounts of first-round overlap extension products to add to the reaction mixture to yield the final, full-length chimeric PCR product.
    6. Separate the second-round OE-PCR products by 1% agarose gel electrophoresis. Excise and purify the products using a commercially available DNA gel extraction kit.
    7. Incubate gel-purified second-round OE-PCR products with 1 unit of Taq DNA polymerase master mix for 10 min at 72 °C to generate the 3' adenylated (*i.e.* A-tailed) products needed for TA cloning.
    8. Ligate the resulting adenylated products into the pIB expression vector and use standard molecular cloning techniques to transform chemically competent (heat shock) or electrocompetent (electroporation) *Escherichia coli* cells. Plate overnight on medium containing the appropriate selection marker (*i.e.,* ampicillin or carbenicillin).
    9. Perform colony PCR[12] using a vector-specific primer and an insert-specific primer to identify insert-positive colonies that have been transformed with expression plasmids harboring an insert in the 5'-3' orientation. Prepare overnight cultures of colonies generating PCR products of the expected sizes.
    10. Isolate plasmid DNA using a commercially available DNA purification kit according to the manufacturer's instructions (see the **Materials Table** for the specific kit used in this protocol). Validate the sequence integrity of each insert by direct DNA sequencing.

2. Insect Cell Culture Maintenance

Note: To maintain sterile conditions, conduct all cell manipulations requiring the opening of the tissue culture flask within a laminar flow hood. Turn on the laminar flow hood UV germicidal lamp at least 1 h prior to cell manipulations. Wear nitrile gloves and decontaminate the surface of the bench, pipettes, utensils, tubes, and flasks with 70% ethanol before their use. Familiarity with basic cell culture techniques is recommended[13].

1. Use Tni cells (an established cell culture line derived from *Trichoplusia ni* ovarian tissue) that are adapted to serum-free medium and maintain them as an adherent monolayer culture in serum-free insect medium at 28 °C in T25 tissue culture flasks.
2. Maintain Sf9 cells (an established cell culture line derived from *Spodoptera frugiperda* ovarian tissue) similarly but using TNM-FH insect culture medium supplemented with 10% Fetal Bovine Serum (FBS).
3. Seed the initial culture from frozen Sf9 or Tni cells by removing stock vials from -80 °C and allowing them to thaw in a 37 °C water bath. Decontaminate the vials with 70% ethanol after thawing and place them on ice.
4. Add 4 mL of insect cell medium to a new T25 flask and transfer 1 mL of the thawed insect cell suspension. Place the flask in a 28 °C, non-humidified incubator and allow the cells to attach for 30-45 min.
5. Replace the seeding medium with 5 mL of the appropriate medium and transfer the flasks to a 28 °C non-humidified incubator. Monitor cell confluence daily. Passage the cells when they reach 90% confluency.
Note: Complete coverage of T25 flask corresponds to ~5 x 10^6 cells.
6. **Insect cell passage.**
    1. To passage the cells, first remove the exhausted medium from the flask containing confluent cells using a sterile 5 mL serological pipette. Tilt the flask so that the medium flows to one corner, away from the cell monolayer. Carefully remove the medium using a pipette without disturbing the cells.
    2. Dislodge the Tni cells by gently rinsing the T25 flasks containing the confluent monolayer with 4 mL of serum-free insect medium using new, sterile, 5 mL serological pipette. Move the pipette tip across the flask and slowly irrigate to remove cells loosely attached to the flask bottom.
        1. Check for the adequate detachment of cells by removing all media, turning the flask over, and observing that the bottom of the flask is clear.
    3. For Sf9 cells, which adhere more tightly, add 4 mL of fresh TNM-FH medium and use a cell scraper to dislodge the attached cells. Use a 5 mL serological pipette to gently mix and reduce cell clumping.

4. Use an automated cell counter to estimate the number of viable insect cells per volume of medium. Transfer 0.1 mL of cell/medium mixture to a 1.5-mL microfuge tube. In a separate 0.5 mL microfuge tube, add 10 µL of cell/medium mixture to 10 µL of trypan blue.
5. Remove a cell counter chamber slide from its packaging and add 10 µL of the cell/medium/trypan blue mixture to each side of the counting slide. Insert the slide into the cell counter and determine the cell density and viability.
6. Using the cell density, calculate and add the proper volume of insect cell medium (up to 5 mL) to new, sterile T25 flasks.
7. Transfer approximately 1-1.5 x 10^6 cells to T25 flasks with fresh media; label the flasks with the cell line, date, medium used, number of cells added, and passage number (P_n + 1 generation, where P_n is the passage number for the previous generation of cells); and place the flasks in a 28 °C incubator for up to 72 h.
   Note: Insect cells may be continuously propagated, although cells may be less receptive to transfection and/or heterologous protein expression after 30 passages. Treat all discarded cells/media with 10% bleach solution and autoclave disposable plastic ware before disposal.

3. Insect Cell Transfection

1. Seed a T25 flask with up to 1 x 10^6 Tni or Sf9 cells in an appropriate insect cell medium (serum-free insect medium for Tni and TNM-FH for Sf9) and grow to confluency for 72 h at 28 °C.
2. Remove and discard the old medium and dislodge the cells with 4 mL of fresh serum-free insect medium (see steps 2.6.2 and 2.6.3, above).
3. Estimate the cell density using an automated cell counter (see step 2.6.4, above).
4. Add approximately 7 x 10^5 cells to individual 35 mm glass-bottom dishes and allow the cells to attach for 20-25 min at 28 °C.
5. For each transfection, add 2 µg of plasmid DNA (either from one plasmid for single transfections or 2 µg from each of the two plasmids for double transfections) to 0.1 mL of serum-free insect medium (without FBS for both Sf9 and Tni transfections) in a sterile 1.5 mL microfuge tube.
6. In a separate tube, mix 8 µL of transfection reagent with 0.1 mL of serum-free insect medium and then transfer that solution to the tube containing the plasmid DNA of interest. Lightly vortex and incubate at RT for 20 - 30 min.
7. Dilute the plasmid-transfection mixture from steps 3.5 and 3.6 with 0.8 mL of serum-free insect medium so that the total volume equals 1 mL.
8. Carefully remove the media from the glass dishes containing attached cells. Overlay the attached cells with the diluted plasmid-transfection medium.
9. Incubate the cells at 28 °C for 5 h.
   Note: The conditions for transfection require empirical optimization for maximal transfection efficiency *(e.g.,* overnight transfection rather than 5 h, the amount of plasmid DNA used, the chemistry of the transfection reagent used, *etc.*).
10. Remove and discard the transfection medium and gently wash the cells with 1 mL of serum-free insect medium, being careful not to dislodge cells.
11. Add 2 mL of fresh insect cell medium (serum-free insect medium for Tni and TNM-FH for Sf9) and incubate at 28 °C for 48-72 h.
    Note: Again, conditions require empirical optimization for maximal heterologous protein expression.

4. Confocal Fluorescence Microscopy

1. At 48-72 h post-transfection, wash the cells once with 1 mL of IPL-41 insect medium and then cover with 2 mL of IPL-41 for imaging.
   Note: This wash step reduces the background auto-fluorescence observed with insect cell medium used in normal cell maintenance.
2. Add 4 drops of Hoechst live-cell staining reagent (see the table of materials for the specific nuclear stain used in this protocol) to the medium and incubate at 28 °C for 20-25 min.
   Note: Additional nuclear stains may be substituted, although the dye should have a fluorescence profile unique from EGFP and mCherry.
3. Place a 35 mm dish into the self-enclosed laser scanning confocal microscope (see the table of materials for the specific instrument used in this protocol).
4. Adjust the microscope for Hoechst, EGFP, and mCherry observation conditions: Hoechst excitation/emission - 359/461 nm; EGFP excitation/emission - 489/510 nm; mCherry excitation/emission - 580/610 nm.
5. Perform an initial scan using a 10x objective to confirm fluorescent expression and then switch to scanning mode using a 60X phase contrast water-immersion objective.
6. Adjust the laser power (5-7%), detector sensitivity (47-49%), scanning speed, Z-axis depth, and digital zoom to optimize the image contrast and resolution. Image the cells at 1.5X digital zoom to give a total of 90X amplification.
   Note: Microscope parameters require empirical adjustment for optimal image collection and may be specific to the instrument in use.
7. Export the raw data as TIFF image files and modify (crop and overlay) for figure generation.

Representative Results

OE-PCR

OE-PCR allows for the synthesis of chimeric DNA products that, once inserted into an expression vector, allow for the production of recombinant chimeric proteins corresponding to any test gene of interest and fluorescent marker protein. **Figure 1** represents a general scheme for the production of pIB expression vectors containing *B. tabaci* aquaporin coding sequences (BtDrip1 and BtDrip2_v1) in-frame with the fluorescent protein marker EGFP. In this scenario, two BtDrip chimeric proteins containing EGFP at their carboxyl termini were produced. This protocol also demonstrated methods for developing two insect subcellular marker proteins, DmSPR and PLA2G15 (**Figure 1**), produced as chimeras with mCherry at their carboxyl termini. EGFP and mCherry were chosen because their emission spectra are sufficiently divergent, allowing for the independent detection and co-localization of signals from the test proteins (*e.g.,* BtDrip-EGFP) and the DmSPR- and PLA2G15-mCherry-labeled marker proteins. All PCR reactions produced bands of the expected sizes and were successfully cloned into pIB. All plasmids were confirmed by DNA sequencing to contain the appropriate inserts in the correct orientation and in-frame with fluorescent protein markers.

Transient recombinant protein expression in insect cell culture

Following the construction of expression vectors corresponding to pIB/BtDrip1-EGFP, pIB/BtDrip2_v1-EGFP, pIB/DmSPR-mCherry, and pIB/PLA2G15-mCherry, cells were transfected and the expression of recombinant proteins in live cells was visualized using confocal fluorescence microscopy (**Figure 2**). Successful transfection and expression of recombinant BtDrip1-EGFP is evident by the presence of green fluorescence on the surface of Tni cells (**Figure 2B, 2F**). Green fluorescence is observed within Tni cells transfected with BtDrip2_v1-EGFP (**Figure 2J, 2N**), indicating the intracellular expression of BtDrip2_v1. Likewise, Tni cells transfected with pIB/DmSPR-mCherry (**Figure 2C, 2K**) or pIB/PLA2G15-mCherry (**Figure 2G, 2O**) show red fluorescence, indicating the expression of the respective chimeras.

Co-localization of recombinant BtDrip proteins and subcellular markers

The double transfection of Tni cells allows for the co-expression of two fluorescent-labeled proteins. Overlaid images of transfected Tni cells that are orange/yellow indicate that cells harbor plasmids producing both EGFP and mCherry and suggest that the proteins are co-localized within the same subcellular structures (**Figure 2D, 2H, 2L & 2P)**. This confirms previous results that BtDrip1 and DmSPR are expressed on the cell surface[14,15,16]. An overlay of Tni cells double-transfected with pIB/BtDrip1-EGFP and pIB/DmSPR-mCherry shows overlap of the green and red fluorescence signals (**Figure 2D**), suggesting co-localization of BtDrip1-EGFP and DmSPR-mCherry on the cell surface. Previously, Maroniche *et al*.[17] demonstrated that PLA2G15 is a marker of intercellular lysosomes when expressed in Sf9 cells as a chimera with mCherry. Although it was predicted that the recombinant BtDrip2_v1-EGFP protein would translocate to the cell surface, it was previously shown that the chimeric protein was primarily localized intracellularly within transfected Tni cells[15]. In support of this, there was little evidence for the co-localization of green and red fluorescent signals when BtDrip2_v1-EGFP was co-expressed with pIB/DmSPR-mCherry (**Figure 2L**). In contrast, the co-expression of pIB/BtDrip2_v1-EGFP and pIB/PLA2G15-mCherry resulted in a significant overlap in the cytoplasmic green and red fluorescent signals (**Figure 2P**), strongly suggesting that BtDrip2_v1 is likely trafficked to intracellular lysosomes.

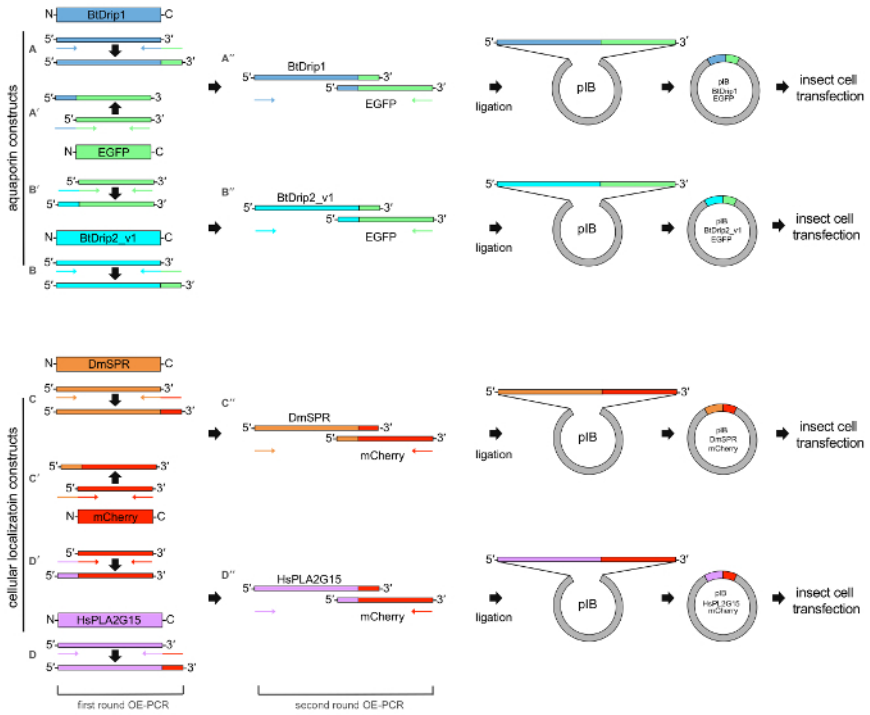

Figure 1: Schematic for Building Expression Constructs using Overlap Extension PCR (OE-PCR). OE-PCR is used to build pIB expression vectors containing the *Bemisia tabaci* aquaporin-1/EGFP (BtDrip1-EGFP), *B. tabaci* aquaporin-2_variant 1/EGFP (BtDrip2_v1-EGFP), *Drosophila melanogaster* sex peptide receptor/mCherry (DmSPR-mCherry), and *Homo sapiens* phospholipase-A2/mCherry (HsPLA2G15-mCherry) products. The first round of OE-PCR generates fragments corresponding to the full-length cDNAs of interest, BtDrip1 (**A**), BtDrip2_v1 (**B**), DmSPR (**C**), and HsPLA2G15 (**D**), containing a small 3' overlapping region corresponding to the 5'-end of either the EGFP or mCherry fluorescent marker proteins. Simultaneously, full-length cDNAs from EGFP and mCherry are PCR amplified and contain a small 5' overlapping region corresponding to the 3'-ends of BtDrip1 (**A'**), BtDrip2_v1 (**B'**), DmSPR (**C'**), or HsPLA2G15 (**D'**). Note that all primers used to amplify the cDNAs of interest (*i.e.* BtDrip1, BtDrip2_v1, DmSPR, and HsPLA2G15) in the first round of OE-PCR lack the stop codon normally found at the 3'-end of each coding sequence. All first-round OE-PCR products are amplified, separated by agarose gel electrophoresis, and gel-purified. The second round of OE-PCR uses pooled pairs of the gel-purified PCR products from the first round of OE-PCR as templates for the amplification of the full-length chimeric products. For each reaction, the sense primer corresponds to the 5'-end of the cDNA of interest coding sequences and the antisense primer is complementary to the 3'-end of EGFP or mCherry, with its stop codon intact (**A''**, **B''**, **C''** and **D''**). The four second-round OE-PCR products are gel purified, adenylated with Taq polymerase, and ligated into the pIB/V5-His- expression vector. Plasmids are propagated in *E. coli*, and purified plasmid DNA is used to transfect insect cells. The *B. tabaci* aquaporins BtDrip1 and BtDrip2_v1 are shown in dark blue and cyan, respectively, and the subcellular markers DmSPR and PLA2G15 are shown in orange and pink, respectively. EGFP is shown in green and mCherry is in red. Colored arrows indicate the direction (sense primers in the forward direction and antisense primers in the reverse direction) and location of gene-specific primers (the color of a primer indicates the source of the primer sequence). See Table 1 for primer details. Please click here to view a larger version of this figure.

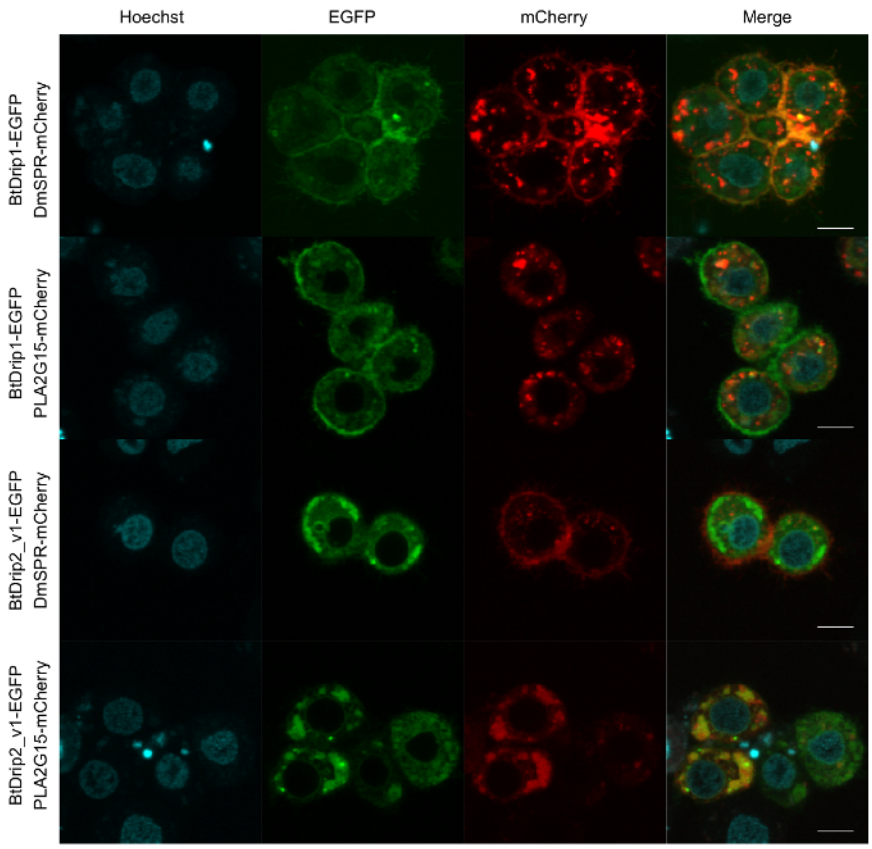

Figure 2: Live Imaging of Double-transfected Tni Cells Expressing Recombinant BtDrip1-EGFP, BtDrip2_v1-EGFP, DmSPR-mCherry, and PLA2G15-mCherry proteins. *Trichoplusia ni* (Tni) cells were double-transfected with pIB/BtDrip1-EGFP and pIB/DmSPR-mCherry (**A-D**), pIB/BtDrip1-EGFP and pIB/PLA2G15-mCherry (**E-H**), pIB/BtDrip2_v1-EGFP and pIB/DmSPR-mCherry (**I-L**), or pIB/BtDrip2_v1-EGFP and pIB/PLA2G15-mCherry (**M-P**). Images were captured with a laser scanning confocal microscope using a 60X phase contrast water-immersion objective (NA 1.2) at 90X amplification. The Hoechst panels show representative live cell images of stained cell nuclei (ex/em = 359/461 nm). The EGFP panels show the fluorescence expression of cells transfected with EGFP chimeras (ex/em = 489/510 nm). The mCherry panels show the fluorescence expression of cells transfected with mCherry chimeras (ex/em = 580/610 nm). The merge panel shows the overlay of all three fluorescent channels (*i.e.* Hoechst, EGFP, and mCherry). Scale bar = 10 µm. Please click here to view a larger version of this figure.

Discussion

Heterologous protein expression systems are important tools for the production of recombinant proteins used in numerous downstream applications[4]. Choosing from the diverse expression systems available depends on the end goal for the protein of interest. Several insect cell expression systems are available that offer flexible alternatives to prokaryotic and eukaryotic cell expression systems[5,6]. Insect systems requiring baculovirus infection to drive protein expression are by far the most popular and widely used insect system, with many such proteins used in research and therapeutic applications[1,2,3]. Baculovirus expression does, however, have limitations, including that: 1) the viral lifecycle involves the lysis of the host cell; 2) the host cells that are lysed typically release high amounts of degradative enzymes; 3) the generation of stable cell lines is time consuming and may not be feasible for cytotoxic proteins; 4) discontinuous expression requires frequent maintenance; 5) vector generation frequently requires multiple cloning steps; 6) insect cell densities are often inversely proportional to viral infection; and 7) recombinant proteins are primarily trafficked at high levels to the cell surface or are secreted[3,7,9,10]. Nonlytic transient gene expression based on insect cell transfection with plasmid DNA offers an alternative technique for the production of recombinant proteins, without some of the unique challenges associated with baculoviruses[7,8]. Namely, transient insect cell expression offers shorter turn-around on vector construction and protein synthesis, avoids challenges associated with viral infection and cell lysis, and provides a robust means to observe the cellular trafficking of proteins.

Here, two validated expression constructs were used to determine the subcellular localization of the *B. tabaci* aquaporin proteins BtDrip1 and BtDrip2_v1. DmSPR-mCherry is a marker for the plasma cell membrane[16], and PLA2G15-mCherry localizes within cellular lysosomes[17]. **Figure 2** shows the value of such markers for assessing the locations of proteins of interest—in this case, the co-localization of BtDrip1-EGFP with DmSPR-mCherry at the cell surface and BtDrip2_v1-EGFP with PLA2G15-mCherry within lysosomes. Therefore, as shown here and in previous investigations[14,15,16,18,19,20], this methodology can be used to rapidly ascertain protein trafficking within insect cells. Although protein expression and trafficking within Tni cells is shown here (**Figure 2**), it is important to note that the protocol may be optimized and applied equally well to other insect cell lines (Sf9, Sf21, BM-N, S2, *etc.*).

Like all heterologous protein expression systems, there are limitations to transient protein production within cultured insect cells. Namely, the percentage of cells positive for plasmid-derived expression is typically lower than that achieved from baculovirus or from using stable cell lines. Thus, the amount of recombinant protein(s) produced can be lower in a transient system; however, the use of different promoters and the

incorporation of genetic enhancers can overcome this potential limitation[7,21,22]. This protocol demonstrates the co-transfection of Tni cells with two pIB vectors, each harboring different protein-coding sequences (**Figure 2**). Although similar transfection efficiencies were observed for all four expression vectors used to transfect the Tni cells (data not shown), this is not always possible. Hence, experiments with such transient expression vectors may require extensive empirical optimization (*e.g.,* variation in the amount of each vector, transfection time, transfection reagent chemistry, *etc.*) to achieve equivalent transfection efficiencies and/or levels of recombinant protein expression. Furthermore, it is possible that the addition of a fluorescent protein may influence or restrict subcellular trafficking of protein targets[23,24]. The identification of the subcellular co-localization of two proteins by the observation of fluorescence with marker proteins does not necessarily indicate direct protein-protein interactions between the proteins of interest[24,25]. Hence, caution is required when interpreting such co-localization results.

Transient gene expression does offer several advantages over currently available baculovirus expression systems. Transient expression requires less time and labor for the construction of vectors and for the synthesis of recombinant proteins, transient expression does not involve cell lysis associated with the pathogenic life cycle of baculovirus, and transient expression can provide a better study system for the subcellular trafficking of proteins[3,7,9]. This protocol highlights the ease and speed of building constructs through the direct incorporation of a gene of interest into the pIB expression vector using overlap extension PCR (**Figure 1**). OE-PCR allows for the rapid amplification of gene targets of interest, as well as a wide selection of possible fluorescent protein markers[26]. Furthermore, although EGFP[27] and mCherry[28] were incorporated here at the carboxyl termini of two *B. tabaci* aquaporin proteins, OE-PCR could also be used to place many other variant fluorescent marker proteins[26] at the amino termini of these proteins. It is notable that, in addition to numerous expression plasmids built using OE-PCR, many traditional expression cloning cassettes were prepared and can be used to produce virtually any protein of interest in-frame with the fluorescent proteins EGFP, Venus, and mCherry, fused at either the amino or terminal ends. Such expression cassettes are available upon request.

The era of "genomics" has provided an unprecedented volume of and access to meaningful data. However, the gap continues to widen between gene discovery and the assignment of function to gene products; thus, additional empirical tools are desperately needed to accelerate functional genomics. Whereas, gene-editing and other *in vivo* gene manipulation systems may ultimately provide quintessential approaches for assigning gene function, heterologous protein expression systems will likely remain important for the biomanufacture of valuable proteins and for deciphering protein structure and function. This protocol describes a method for the enhanced construction of expression plasmids and the transient expression of recombinant proteins in insect cells.

Disclosures

The authors declare that they have no competing financial interests.

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