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Design and Validation of Plasmid Vectors for Characterizing Protein–Protein Interactions in *Spodoptera frugiperda* Insect Cells

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Abstract

There are limited molecular biology resources for interrogating protein–protein interactions (PPIs) in insect cells. To address this deficiency, we developed plasmid vectors for localization, bimolecular fluorescence complementation (BiFC), and co-immunoprecipitation (co-IP) assays in Sf9 insect cells. Plasmids were designed to express a protein of interest as a fusion with epitope tags and autofluorescent proteins using the Gateway cloning system. Two robust interactors were utilized to validate this system: the nucleoprotein (N) and the phosphoprotein (P) of maize mosaic virus. The viral N was fused with the carboxy-terminal portion of eYFP and a FLAG epitope tag, and P was fused with the amino-terminal portion of eYFP and a myc epitope tag. The two expression plasmids were cotransfected into Sf9 cells, fluorescence microscopy was used to visualize BiFC, and co-IP was performed to confirm that this system was sensitive enough to detect PPI between the two proteins. BiFC was seen in cells cotransfected with N and P, and co-IP validated the interaction. This plasmid-based system can be used to investigate a variety of PPIs that occur in insects. We validated viral protein interactions that occur in the insect vector, which provides further insights into the biology of rhabdoviruses that are transmitted by insects. The ability to express viral and insect proteins in insect cells for studying PPIs with this streamlined system represents an advancement for protein research in insects. Future work will focus on identifying interacting viral and host proteins and discovery of targets for control of viruses and insect vectors.

Keywords: cell biology, host parasite interactions, insects, rhabdovirus, virology

Protein–protein interaction (PPI) assays are important tools for investigating the relationship between viruses and their host organisms. Investigating PPIs between viral and host proteins enables identification and validation of novel interactions and functions and is important for understanding the virus infection cycle in insects. Molecular assays such as co-immunoprecipitation (co-IP) or yeast two-hybrid assays have been used extensively in the past to study PPIs, and as new technologies continue to be developed and refined, more sophisticated assays such as bimolecular

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fluorescence complementation (BiFC) or fluorescence resonance energy transfer have become commonplace in the molecular biology toolbox (Bailer and Haas 2009; Kerppola 2008; Kodama and Hu 2012).

Studying PPIs in live insect cells can help to elucidate the function and localization of proteins within them, but the lack of insect cell culture lines for numerous insect species, especially vectors of plant pathogens, has hindered this work. Researchers are hence reliant on commercially available S2 (*Drosophila melanogaster*) and Sf9 and Sf21 (*Spodoptera frugiperda*) insect cell lines to answer research questions in similar but divergent systems. Although there are tools currently available for use in insect cell culture research, many of them rely on baculovirus expression vector systems, which introduce additional variables into the system because of the effect of the virus on the cell, thus complicating interpretation of the study.

Maize mosaic alphanucleorhabdovirus is a member of the family *Rhabdoviridae*, which contains viruses that infect animals, plants, and insects (Whitfield et al. 2018). Maize mosaic virus (MMV) is transmitted by the corn planthopper *Peregrinus maidis* in a persistent, propagative manner. The biology of MMV transmission by *P. maidis* is well characterized, making it a model system for plant rhabdovirus-vector interactions (Barandoc-Alviar et al. 2016; Klobasa et al. 2021; Martin et al. 2017; Whitfield et al. 2011; Yao et al. 2013, 2019). Understanding the interactions between viral and host proteins in plant and insect cells will yield valuable information about how MMV functions in vivo, providing a more complete understanding of the viral life cycle and thus paving the way for implementation of unique management options for MMV. Methods for studying PPIs in plant cells are well established, but the ability to make direct comparisons to the insect phase of the infection cycle has been hampered by a lack of molecular biology tools in insects.

In this study, we have developed and validated a novel set of plasmids to investigate PPIs in Sf9 insect cells. This system utilizes Gateway technology, which enables the use of a single entry clone that can be moved into multiple destination vectors within the same system, as well as other Gateway-compatible systems, allowing for efficient screening of many virus–virus or virus–host protein interactions within insect cells using localization, co-IP, and BiFC. Two expression plasmids encoding proteins of interest fused to either the N- or C-terminal half of enhanced yellow fluorescent protein (YFP) and an epitope tag are cotransfected into Sf9 cells, then co-IP and BiFC experiments are performed to confirm the presence or absence of an interaction between the two proteins. Separate expression plasmids encoding proteins of interest fused to either enhanced green fluorescent protein (GFP) or monomeric red fluorescent protein (RFP) were also used to corroborate the localization patterns observed with the proteins fused to split YFP halves. We chose to use the nucleoprotein (N) and the phosphoprotein (P) of MMV as our test proteins because the interaction between N and P is robust and highly conserved among all rhabdoviruses (Goodin et al. 2001; Gupta et al. 2003; Tsai et al. 2005). Previous studies have also shown that the N and P proteins colocalize in *Nicotiana benthamiana* and *Drosophila melanogaster* S2 cells (Martin and Whitfield 2018).

MATERIALS AND METHODS

Construction of the GFP and RFP vectors

To add autofluorescent proteins to the original plasmid, pIB/V5-His-DEST (Thermo Fisher Scientific, Waltham, MA, U.S.A.), we used a combination of gene synthesis and standard restriction enzyme cloning to generate C-terminal tag constructs. GFP was modified to be placed before the Gateway cassette in pIB/V5-His-DEST by the addition of sequences to the 5′ and 3′ ends and synthesis by GenScript (Piscataway, NJ, U.S.A.). The 5′ end of GFP was modified to contain KasI and NdeI restriction sites, and sequences from pIB including the 3′ end of the pOpIE2 promoter were added to the KasI restriction site. The NdeI contains the ATG site used to initiate translation at the beginning of GFP and sets the frame for other insertions. The 3′ end of GFP was modified to remove the stop codon and to add a HindIII restriction site, and this synthesized intermediate construct was renamed GFP-N1. Subsequent digestion with KasI and HindIII was performed to ligate it into the pIB/V5-His-DEST vector. This vector was renamed pIGW. A full description of the plasmid naming scheme is provided in Table 1. RFP was added to pIGW with PCR amplification using Phusion polymerase (Thermo Fisher Scientific) with each of the fragments with NdeI and HindIII restriction sites added to the 5′ and 3′ ends, respectively. The insect vector pHRW (emb.carnegiescience.edu/drosophila-gateway-vector-collection, Murphy, unpublished; Drosophila Resource Center, Indiana State University, Bloomington, IA) was used as the template for RFP amplification, and the amino-terminal YFP half (nYFP) and carboxy-terminal YFP half (cYFP) were amplified from the pSITE-BiFC vectors, a collection of plasmids used for expression in plants (Martin et al. 2009). All primer sequences are provided in Table 2. The RFP-N1, nYFP-N1, and cYFP-N1 fragments were all amplified with their respective primer pairs. Digestion of the fragments and ligation into a similarly digested pIGW resulted in the construction of pIRW, pInYW, and pIcYW.

To generate the vectors for C-terminal autofluorescent protein fusions, the fluorescent proteins were added with a combination of synthesis, PCR amplification, and restriction digestion. The GFP fragment was synthesized by GenScript with flanking SalI and AgeI sites, with a small fragment of the Gateway cassette added after the native SalI site to complete the attR2 site for Gateway recombination. The 3′ end of GFP was modified to remove the stop codon and add an XbaI site. To preserve the reading frame with an added AscI site at the 5′ end, an extra nucleotide was added to the sequence. The fragment described above was subsequently digested with SalI and AgeI before ligation into a similarly digested pIB/V5-His-DEST, resulting in the construction of pIWG. The RFP-C1, nYFP-C1, and cYFP-C1 intermediate pieces were amplified from the vectors stated above using Phusion polymerase (Thermo Fisher Scientific). The fragments were digested with AscI and XbaI and ligated into a similarly

TABLE 1

Names and features of the newly constructed pI vectors[a]

Vector	Orientation	Features
pIWG	GW-GOI	Gateway cassette, GFP, V5, 6xHis
pIWR	GW-GOI	Gateway cassette, RFP, V5, 6xHis
pIWnY	GW-GOI	Gateway cassette, nYFP, V5, 6xHis
pIWcY	GW-GOI	Gateway cassette, cYFP, V5, 6xHis
pIWnYM	GW-GOI	Gateway cassette, nYFP, 6x myc, 6xHis
pIWcYF	GW-GOI	Gateway cassette, cYFP, 6x FLAG, 6xHis
pIGW	GOI-GW	GFP, Gateway cassette, V5, 6xHis
pIRW	GOI-GW	RFP, Gateway cassette, V5, 6xHis
pInYW	GOI-GW	nYFP, Gateway cassette, V5, 6xHis
pIcYW	GOI-GW	cYFP, Gateway cassette, V5, 6xHis
pInYWM	GOI-GW	nYFP, Gateway cassette, 6x myc, 6xHis
pIcYWF	GOI-GW	cYFP, Gateway cassette, 6x FLAG, 6xHis

[a] GW = Gateway cassette; GOI = gene of interest; GFP = green fluorescent protein; RFP = red fluorescent protein; and n/cYFP = amino or carboxy terminal half of yellow fluorescent protein, respectively.

digested pIWG vector to construct the pIWR, pIWnY, and pIWcY vectors, respectively.

Modification of the epitopes in the BiFC vectors for pull-down studies

Epitope tags 6x myc or 6x FLAG were added to the pInYW, pIcYW, pIWnY, and pIWcY vectors by synthesizing the entire nYFP and cYFP sequence (GenScript) to be in frame with either the 6x myc epitope or the 6x FLAG epitope. Addition of nYFP and cYFP was done with restriction cloning. The AscI and AgeI sites were used for cloning the amino terminal fusions to the Gateway cassette, and the SalI and AgeI sites were used for the carboxy terminal fusions to the Gateway cassette. The addition of epitope tags allowed for pull-down assays to be conducted with the different fusion proteins after BiFC imaging to confirm the expression of the proteins if no interaction was observed, as well as to show a secondary method of interaction. There are two sets of BiFC vectors: those containing the 6x myc (nYFP) and 6x FLAG tags (cYFP) and the versions containing the original V5 epitope. This was done to ensure that each YFP half was associated with only one epitope tag, as multiple orientations of the fluorescent protein halves may be required to detect an interaction. A 6x myc or 6x FLAG sequence flanked by XbaI and AgeI restriction sites was synthesized and then digested with XbaI and AgeI before being ligated with the similarly digested pInYW and pIcYW vectors to produce pInYWM and pIcYWF. The same cloning steps and restriction digests used to make the plasmids above were also used to generate pIWnYM or pIWcYF.

Construction of MMV N and P expression clones

For validation of the functionality of the pIB vectors, we used the MMV N and P genes. In previous work, pENTR_MMV-N and pENTR_MMV-P entry vectors were constructed and used to test expression, BiFC in plants, and localization in insect cells (Martin and Whitfield 2018). The negative control protein used in these PPI assays was the maltose-binding protein (MBP) from *E. coli*, and MBP was amplified from pMAL-c2x. The MBP forward and reverse primers in Table 2 were used to clone MBP into pENTR. Gateway recombination using LR Clonase II (Thermo Fisher Scientific) was then performed with various combinations of entry and destination vectors. The final expression clones used for cell transfections is as follows: pING (N-GFP), pIPR (P-RFP), pIcYNF (cYFP-N), pIPnYM (P-nYFP), pIMnYM (MBP-nYFP), and pIcYMF (cYFP-MBP).

Plasmid DNA transfection into Sf9 cells

To prepare the transfection reagent, 3 μg of plasmid DNA for each construct and 8 μl of Cellfectin II Reagent (Thermo Fisher Scientific) were added to 200 μl of supplemented Grace's Insect Medium (Thermo Fisher Scientific) and incubated at room temperature for at least 30 min. An additional 800 μl of supplemented Grace's Insect Medium (Thermo Fisher Scientific) was added to the solution afterward. Sf9 cells were diluted in fresh Sf900-III serum-free medium (Thermo Fisher Scientific) to a final concentration of 4×10^5 cells/ml, and 2 ml of cell solution was seeded in each well of a 35-mm six-well plate (Thermo Fisher Scientific) before being incubated for 1 h at 27°C for cell reattachment. The medium in the six-well plates was then replaced with 1 ml of transfection reagent and incubated at 27°C for at least 3 h, after which the transfection reagent was replaced with 2.5 ml of fresh Sf900-III medium. After a 72-h incubation at 27°C, the cells either had total protein extracted from them or were used for fluorescence imaging.

Co-IP of N and P fusion proteins

Total protein was extracted from transfected Sf9 cells by first resuspending the cells in 1 ml of fresh Sf900-III serum-free medium, centrifuging them at 2,000 × *g* for 1 min, and then washing the pellet with an ice-cold solution of phosphate-buffered saline (PBS; pH 7.4). The cells were then centrifuged again at 2,000 × *g* for 1 min, and the PBS was replaced with Pierce IP Lysis Buffer with Halt Protease Inhibitor (Thermo Fisher Scientific) before being incubated on ice for 5 min. The cell lysate was then centrifuged at 2,000 × *g* for 1 min, and the supernatant was transferred to a separate 1.7-ml microcentrifuge tube containing anti-FLAG affinity gel (Bimake, Houston, TX, U.S.A.) that had been washed with a mixture of Tris-buffered saline with 1% Tween-20 (TBST). An aliquot of the supernatant was saved for direct western blot analysis to confirm successful protein expression. The cell supernatant was incubated with the washed anti-FLAG affinity gel for at least 3 h at room temperature on a Mini LabRoller Rotator (Labnet, Edison, NJ, U.S.A.), then centrifuged at 5,000 × *g* for 5 min at 4°C. The affinity gel was washed once with Pierce IP Lysis Buffer before direct addition of Laemmli buffer to the tube containing the gel. The sample was then heated at 95°C for 10 min before being loaded into a 10% SDS-PAGE protein gel for downstream western blot analysis.

TABLE 2

List of primer sequences used for cloning pI vectors

Primer name	Primer sequence (5′–3′)	Purpose
mRFP-N1_F	CGAATTTACATATGGCCTCCTCCGAGGACGTCATCAAGGAG	Movement of mRFP into vector-marked NdeI site
mRFP-N1_R	CGATATCAAGCTTGGCGCCGGTGGAGTGGCGGCCCTCGGCGC	Movement of mRFP into vector-marked HindIII site
nEYFP-N1_F	CGAATTTACATATGGTGAGCAAGGGCGAGGAGCTGTTC	Initial cloning for V5 site-marked NdeI site
nEYFP-N1_R	CGATATCAAGCTTCTTGTACAGCTCGTCCATGCCGTGAGTGATCCC	Initial cloning for V5 site-marked HindIII site
cEYFP-N1_F	GTTCGAATTTACATATGGGCGGCAGCGTGCAGCTCGCCGACCA	Initial cloning for V5 site-marked NdeI site
cEYFP-N1_R	AATTCGATATCAAGCTTCTTGTACAGCTCGTCCATGCCGAGAGTGATC	Initial cloning for V5 site-marked HindIII site
mRFP-C1_F	CGGGAGGGCGCGCCATGGCCTCCTCCGAGGACGTCATCAAGGAG	Initial cloning for V5 site-marked AscI site
mRFP-C1_R	CGGGCCCTTTCTAGAGGCGCCGGTGGAGTGGCGGCCCTCGGCGCGC	Initial cloning for V5 site-marked XbaI site
nEYFP-C1_F	CCCGGGAGGGCGCGCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGG	Initial cloning for V5 site-marked AscI site
nEYFP-C1_R	CCGCGGGCCCTTTCTAGAGTCCTCGATGTTGTGGCGGATCTTGAAG	Initial cloning for V5 site-marked XbaI site
cEYFP-C1_F	CCCGGGAGGGCGCGCCGGCAGCGTGCAGCTCGCCGACCACTACCAGC	Initial cloning for V5 site-marked AscI site
cEYFP-C1_R	ACCGCGGGCCCTTTCTAGACTTGTACAGCTCGTCCATG	Initial cloning for V5 site-marked XbaI site
MBP_F	CACCATGAAAATCGAAGAAGGTAAACTG	Cloning of MBP into pENTR d-Topo
MBP_R	AGTCTGCGCGTCTTTCAGGGC	Cloning of MBP into pENTR d-Topo

Western blot analysis of proteins

For analysis of proteins, 10% SDS-PAGE gels were cast with the TGX FastCast acrylamide kit (Bio-Rad, Hercules, CA, U.S.A.) following the manufacturer's protocol. The Mini-PROTEAN Tetra Cell (Bio-Rad) was used to run the gel at 150 V for approximately 30 to 45 min, and the Trans-Blot Turbo Transfer System (Bio-Rad) was used to transfer the proteins in the gel onto a PVDF membrane. The membrane was blocked with 5% dry milk in TBST before probing with anti-myc (1:5,000 dilution) (Thermo Fisher Scientific) or anti-FLAG (1:5,000 dilution) (Thermo Fisher Scientific) antibodies. The membrane was then washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) against the corresponding primary antibodies. In the case of the membrane containing protein samples from co-IP experiments with contamination from the mouse anti-FLAG antibodies from the affinity gel, mouse TrueBlot HRP-conjugated secondary antibodies (Rockland Immunochemicals, Pottstown, PA, U.S.A.) were used for staining. Another TBST wash was performed on the membrane after secondary antibody incubation, and Clarity Western ECL Substrate (Bio-Rad) was used to visualize the bands on the membrane using the iBright Western Blot Imaging System (Thermo Fisher Scientific).

Fluorescence imaging of Sf9 cells

Each well of plasmid-transfected Sf9 cells was diluted fourfold with Sf900-III SFM in a new six-well plate after the initial 72-h incubation period. The cells were incubated at 27°C for 1 h for reattachment, and then the medium was replaced with PBS 7.4 and 105 ng/µl of 4′,6-diamidino-2-phenylindole, dihydrochloride (Thermo Fisher Scientific) to stain the cell nuclei. After 10 min, the PBS medium was removed, and the cells were washed with fresh PBS. The Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) was then used to assay for fluorescent protein expression in the cells.

RESULTS

Vectors for expression of fusion proteins in insect cells

A collection of plasmid vectors for expressing proteins and studying PPIs in insect cells was created. All plasmids use the baculovirus immediate early promoter (OpIE2) from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV) for expression, which has been shown to function in diverse insect cell lines (Theilmann and Stewart 1992). Insect expression plasmids were constructed for expression of fusions with autofluorescent

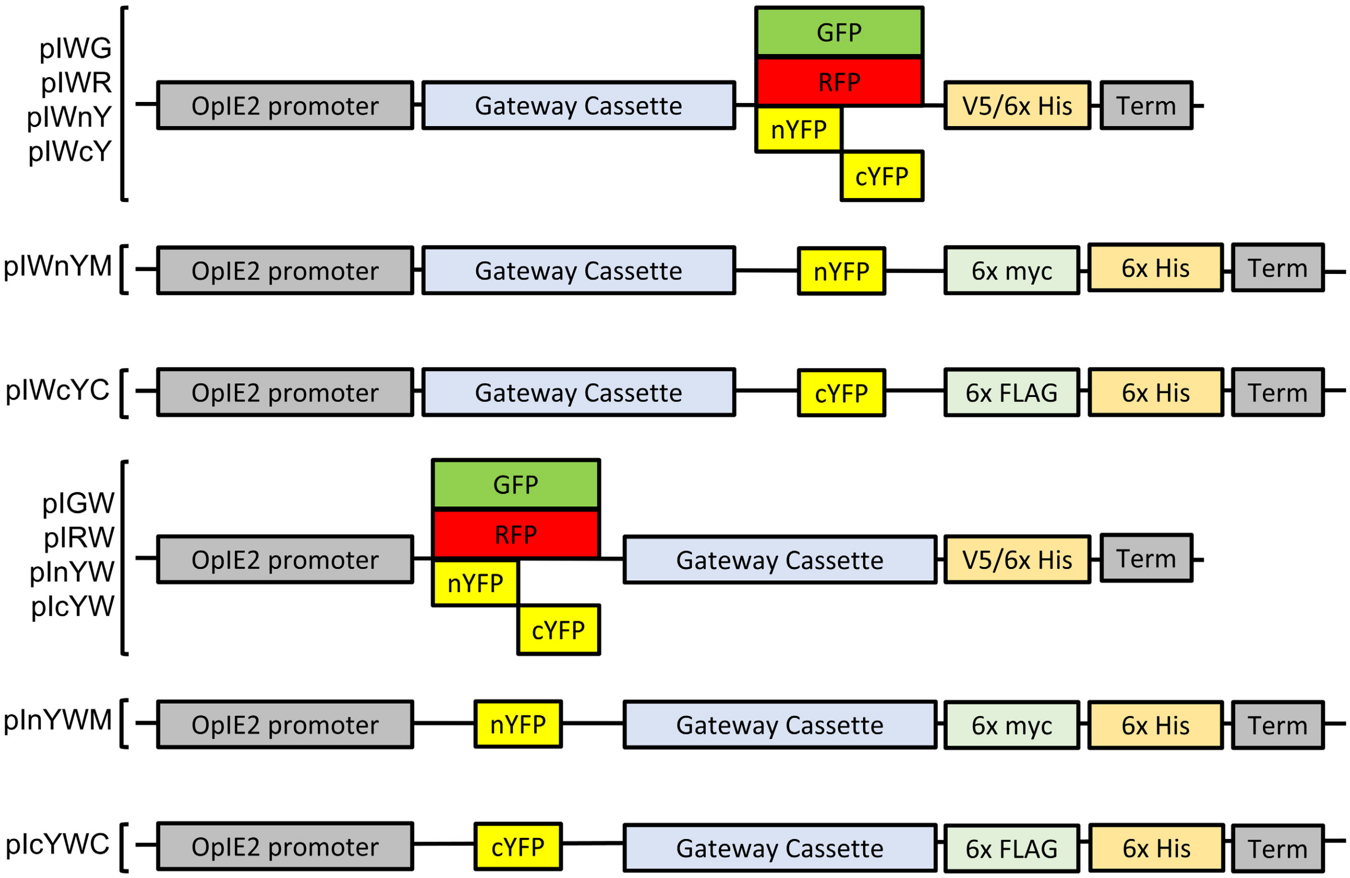

FIGURE 1

Schematic of the pI plasmid vector system showing the orientation of constructs within each plasmid. The plasmid naming scheme is as follows: W refers to the Gateway cassette, G refers to green fluorescent protein, R refers to red fluorescent protein, and nY and cY refer to the amino-terminal or the carboxy-terminal portion of yellow fluorescent protein. OpIE2 is the immediate early promoter of the *Orgyia pseudotsugata* multiple nucleopolyhedrovirus. The Gateway cassette is located either before or after the autofluorescent proteins, depending on the construct, to enable creation of N- or C-terminal fusion proteins. The original plasmid backbone contains V5 and 6x His epitope tags on the C-terminus region just before the OpIE2 polyadenylation signal/terminator (Term). Additional vectors were constructed for dual bimolecular fluorescence complementation and co-immunoprecipitation by replacing the V5 tag with either a 6x myc or a 6x FLAG epitope tag.

(RFP and GFP) proteins at the N- or C- terminus. These plasmids also contain the V5 and 6xHis tags for detection and purification. Plasmids were also designed for BiFC experiments in insect cells, and the split YFP halves were inserted into the vectors for in-frame expression as N- or C-terminal fusions. In these vectors, we replaced the V5 tag with myc or FLAG tags for co-IP experiments to validate BiFC assays and to confirm protein expression using western blots. The MMV N and P and the negative control MBP proteins were transferred from entry clones into expression vectors for validation of the system using standard Gateway recombination reactions. The resulting plasmid vectors are shown in Figure 1.

Imaging of autofluorescent fusion proteins

Strong fluorescence signals were observed in insect cells transfected with plasmid constructs of MMV N-GFP and P-RFP. GFP fluorescence was seen in the nuclear region of N-GFP-transfected cells, with weak fluorescence seen in the cytoplasm. RFP fluorescence was strictly nuclear-localized in P-RFP-transfected cells (Fig. 2B). To determine if the N and P fusion proteins would influence the localization of the other, as seen previously (Martin and Whitfield 2018), the two expression plasmids were cotransfected into Sf9 cells. Small, punctate green and yellow foci, as well as red fluorescence, were seen in the nuclear region of the cells, with green fluorescence seen in the cytoplasm as well (Fig. 2C). These results suggest that the interaction between the viral proteins was not disrupted by the presence of the fused fluorescent proteins. The colocalization of the N and P fusion proteins in the nucleus Sf9 cells is consistent with that in S2 insect cells and plant cells (Martin and Whitfield 2018).

BiFC imaging was performed on Sf9 cells co-expressing cYFP-N and P-nYFP, and yellow fluorescence was observed in the nuclear region (Fig. 3B). No yellow fluorescence was observed in the negative controls where MBP was expressed in place of N or P protein. Even in cases of low transfection efficiency, imaging of fluorescently tagged proteins using epifluorescence microscopy is possible because a single 9.6-cm² well of a six-well plate contains thousands of cells. The BiFC assay with N and P validates that the pI vectors function in insect cells.

Western blotting and co-IP analysis of N and P fusion proteins

Western blots of cell lysate from cells expressing cYFP-N and P-nYFP, stained with anti-FLAG and anti-myc primary

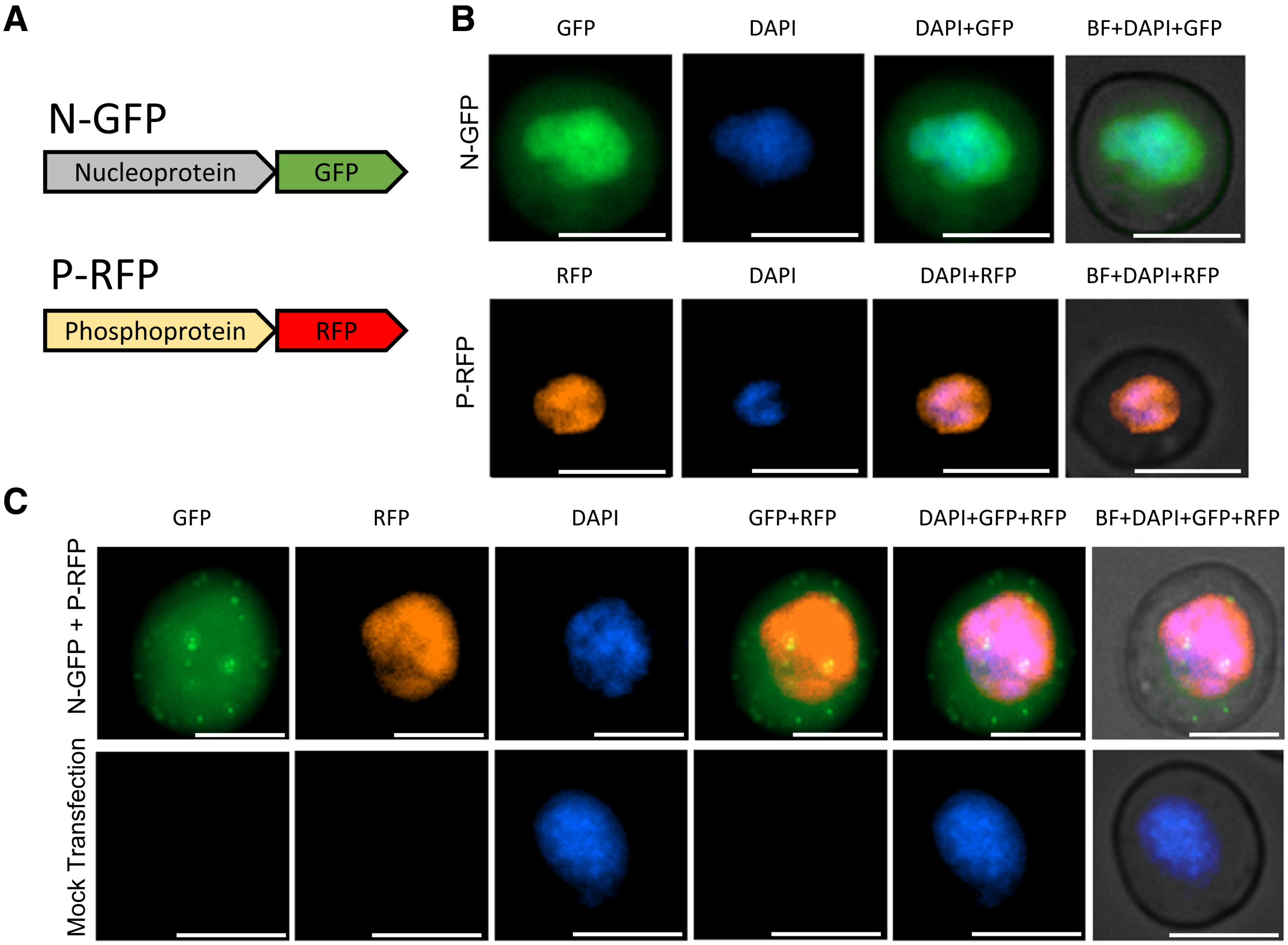

FIGURE 2

A, Pictorial representation of the fusion proteins encoded by the respective plasmids. **B,** Fluorescence microscopy imaging of Sf9 insect cells transfected with pING. Strong green fluorescent protein (GFP) fluorescence is observed in the nucleus, and faint GFP signals can be seen in the cytoplasm, N appears to be primarily nuclear-localized. Red fluorescent protein fluorescence is seen only in the nucleus of Sf9 cells transfected with pIPR, the localization of P appears to be strictly nuclear. **C,** Cotransfection of N and P fusion proteins into Sf9 cells appears to have no effect on the original localization of the N or P proteins. Small punctate green foci are also observed in the cell nucleus. DAPI = 4′,6-diamidino-2-phenylindole; BF = Brightfield; and scale bars represent 15 μM.

antibodies, respectively, showed the presence of bands corresponding to the predicted molecular weights of the fusion proteins, confirming stable expression of the constructs in Sf9 cells (Supplementary Fig. S1). The control fusion, cYFP-MBP and MBP-nYFP, proteins could also be detected via western blotting when total protein from transfected Sf9 cells was loaded onto SDS-PAGE gels and stained with the appropriate anti-myc or FLAG primary antibodies. Co-immunoprecipitation of N and P was performed with anti-FLAG affinity gel (Rockland), and the N-FLAG fusion protein successfully precipitated the P-myc fusion protein. The MBP-FLAG negative control did not precipitate P-myc (Fig. 3C). These results confirm that the system is sensitive enough to detect the strong, conserved PPI between the two viral proteins without detecting a signal between the control treatment of MBP and the viral P protein.

DISCUSSION

We have developed a plasmid-based system to enable medium-throughput screening of protein interactions via BiFC and co-IP and to conduct experiments without the use of another virus that could have confounding effects on cell physiology, increasing the repertoire of tools available for use in similar types of studies. The Sf9 insect cell line is a useful analogous system for studying PPIs of vector-borne plant viruses and their vectors, such as *P. maidis* and other insects that lack cell culture lines. These PPI tools for examining viral protein and virus–host interactions will further advance our understanding of viruses that replicate in their insect vectors, an area of study with a rapidly expanding array of technological resources (reviewed in German et al. 2020).

There are limited tools available for use in insect systems to study PPIs compared with methods used in plant systems. Protein localization and BiFC studies are often used in planta to assay PPIs because plant cells are easy to modify via agrobacterium-mediated delivery of plasmids and high levels of protein expression are routinely achieved. Conversely, insect cell lines can be difficult to develop and maintain, and there are numerous challenges to overcome when attempting to overexpress foreign proteins in insect cells. The shortage of non-baculovirus expression vector system-based tools for standard insect cell lines stalls functional analysis studies. Past studies have identified promoters for use in insect cells, but without a complete set of fluorescent proteins in a user-friendly and versatile system, individual design and validation of a variety of tools to study specific proteins of interest was required for each system. In addition, some PPI tools developed are highly specific to insect cell types. For example, BiFC tools have been developed to study PPI in *Bombyx mori*, but the specific promoter that the system relies on limits its use to only *B. mori* (Bao et al. 2013). To circumvent the issue of host specificity, the plasmids developed herein rely on the second immediate early regulatory gene of the baculovirus OpMNPV (Theilmann and Stewart 1992). The immediate early promoters of baculoviruses have been shown to function effectively in diverse host cells, indicating that they might be useful in other insect cells systems as they are developed (Pfeifer et al. 1997). In experiments with a cell line of *Graminella nigrifrons*, the hemipteran vector of maize fine streak

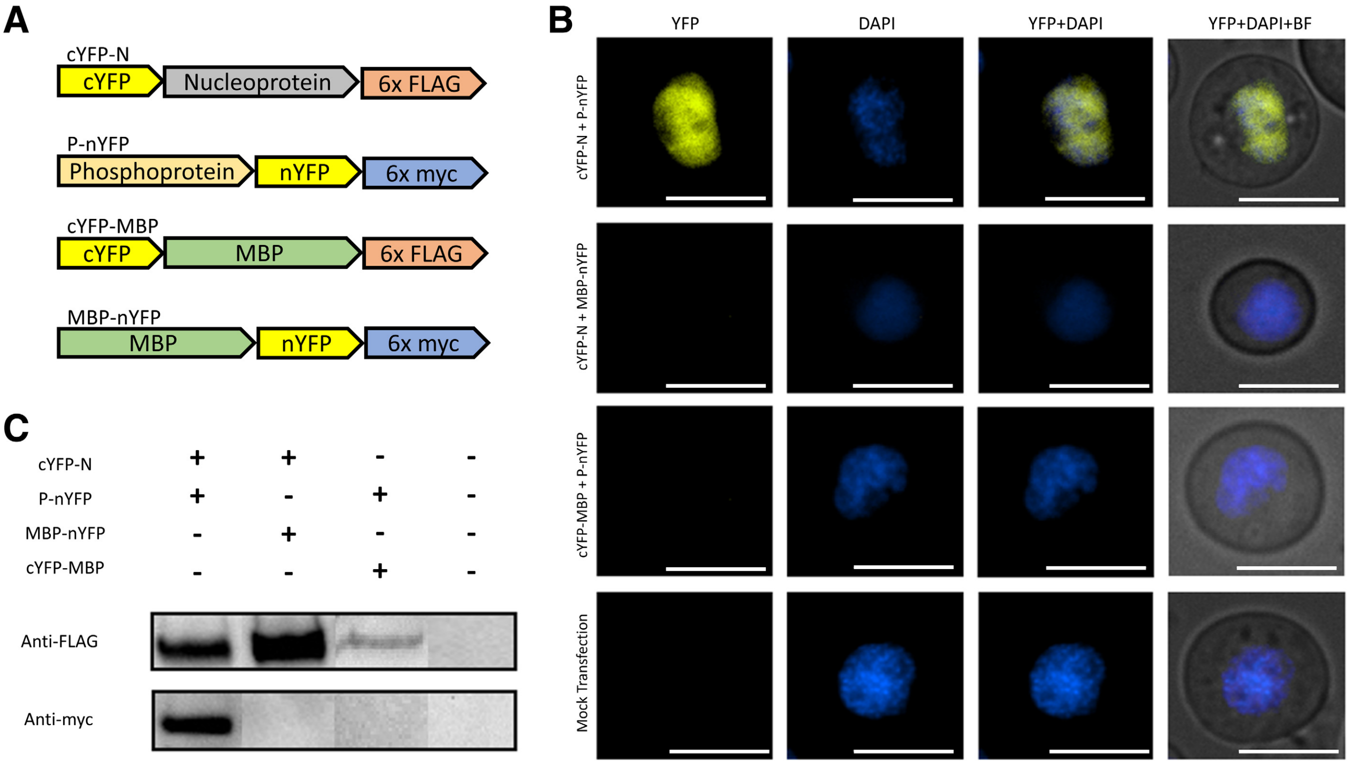

FIGURE 3

A, Pictorial representation of the different fusion protein constructs. **B,** Bimolecular fluorescence complementation (BiFC) imaging of Sf9 insect cells cotransfected with the listed combination of plasmids. Yellow fluorescence is observed in the nuclear region of cells co-expressing cYFP-N and P-nYFP fusion proteins but not in any of the negative controls expressing maltose-binding protein (MBP) in place of either N or P protein. Scale bar represents 15 μM. **C,** Western blotting results after co-immunoprecipitation of raw cell lysate from cotransfected Sf9 cells was performed with anti-FLAG affinity gel. P was able to be coprecipitated with N, but MBP was not able to be precipitated by N, and MBP was not able to precipitate P.

virus and maize rayado fino virus, we observed expression of GFP from the OpIE2 promoter (Martin, Wayadande, and Whitfield, unpublished).

Another advantage of this system is that the plasmids are generated via the Gateway system, resulting in a more streamlined and higher-throughput cloning process compared with other conventional cloning methods. Plasmid transfection to express foreign proteins in insect cells is also less time- and labor-intensive than using baculovirus expression vector systems, at the cost of comparatively lower foreign protein expression levels. For complex systems such as those involving a virus, a plant, and an insect host, investigating the biological functions of each component often requires extensive familiarity and expertise with DNA manipulation, plasmid design, and other molecular biology techniques. Our system uses the same entry clones for both plant and insect cells, improving workflow efficiency and ease of implementation. The same entry clones developed can be used in experiments with the insect system described here or in the pSITE-BiFC and pSITE-II series of plasmids for testing in plants (Martin et al. 2009). This system is useful for viruses that replicate in insects and plants for comparative analysis of viral protein interactions and can be applied to the study of virus–host interactions as well. We also expect that researchers focused on insect-specific viruses and the function of insect proteins will find these plasmid vectors useful.

A key step in the optimization of BiFC for insect cells was the selection of a negative-control protein to demonstrate the specificity of protein interactions. We generated constructs with Glutathione S-transferase (GST), a portion of Lac-Z, and MBP and tested three of them in our experiments (data not shown). We found that the Lac-Z construct was not expressed efficiently in insect cells and could not be detected in western blots. The GST protein that is commonly used as a nonbinding control in experiments in plants resulted in nonspecific interactions in insect cells. MBP was selected as the best control because it was expressed as a full-length fusion protein and did not show nonspecific interactions when tested against N and P in BiFC and co-IP assays.

Based on our results with the pI vectors described here and comparison to previous experiments with various viral and insect proteins, we propose an optimized PPI discovery and validation workflow (Badillo-Vargas et al. 2019; Martin and Whitfield 2018; Martin et al. 2009). First, we recommend that proteins be expressed as N- or C-terminal fusions with autofluorescent proteins for observing localization patterns and validation of full-length protein using western blots. It is often helpful to express target proteins as N- and C-terminal autofluorescent protein fusions to determine the optimal site for tag location. If the target protein has been studied in other systems, preliminary insect cell expression enables comparisons of localization patterns. In our validation system, MMV N and P, we found that protein localization in Sf9 cells followed a similar pattern as observed in plant and *Drosophila* cells (Martin and Whitfield 2018). The second step for exploring PPIs in insect cells would be BiFC experiments to determine if proteins interact and the subcellular localization of the protein interactions. Transfection conditions may need to be optimized for each protein target. In some cases, the transfection efficiency and inability to deliver sufficient plasmid quantities of plasmids can result in few cells expressing high levels of fluorescent fusion proteins. For this reason, we recommend secondary validation of interactions by co-IP, and the design of these plasmid vectors enables the same constructs to be used in these two PPI assays. The epitope tags present in all vectors also enable purification of proteins for other downstream applications.

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We dedicate this paper to Dr. Michael Goodin, a pioneer in the field of rhabdovirus PPI and a dear friend and colleague.

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