2011

COMPARISON OF PLANT-ADAPTED Rhabdovirus Protein Localization and Interactions

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ABSTRACT OF DISSERTATION

Kathleen Marie Martin

The Graduate School
University of Kentucky
2011
COMPARISON OF PLANT-ADAPTED RHABDOVIRUS PROTEIN LOCALIZATION AND INTERACTIONS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Kathleen Marie Martin
Lexington, Kentucky

Director: Dr. Michael M Goodin, Associate Professor of Plant Pathology
Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

COMPARISON OF PLANT-ADAPTED RHABDOVIRUS PROTEIN LOCALIZATION AND INTERACTIONS

Sonchus yellow net virus (SYNV), Potato yellow dwarf virus (PYDV) and Lettuce Necrotic yellows virus (LNYV) are members of the Rhabdoviridae family that infect plants. SYNV and PYDV are Nucleorhabdoviruses that replicate in the nuclei of infected cells and LNYV is a Cytorhabdovirus that replicates in the cytoplasm. LNYV and SYNV share a similar genome organization with a gene order of Nucleoprotein (N), Phosphoprotein (P), putative movement protein (Mv), Matrix protein (M), Glycoprotein (G) and Polymerase protein (L). PYDV contains an additional predicted gene between N and P, denoted as X, that has an unknown function. In order to gain insight into the associations of viral proteins and the mechanisms by which they may function, we constructed protein localization and interaction maps using novel plant expression vectors. Sub-cellular localization was determined by expressing the viral proteins fused to green fluorescent protein in leaf epidermal cells of Nicotiana benthamiana. Protein interactions were tested in planta using bimolecular fluorescence complementation (BiFC). All three viruses showed Mv to be localized to the cell periphery and the G protein to be membrane associated. Comparing the interaction maps revealed that only the N-P and M-M interactions are common to all three viruses. Associations unique to only one virus include G-Mv for SYNV, M-Mv, M-G, and N-M for PYDV and P-M for LNYV. The cognate N-P proteins of all three viruses exhibit changes in localization when co-expressed. To complement the mapping data, we also mapped the functional domains in the glycoproteins of SYNV and LNYV. The truncation of the carboxyterminus has no effect on localization compared to the full-length protein; the nuclear localization signals (NLSs) present in SYNV-G do not interact with known importins. These data suggest that although the interactions of the three viruses differ, each protein may have similar functional domains.

KEYWORDS: rhabdovirus, Nicotiana benthamiana, BiFC, interaction, localization
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April 28, 2011
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COMPARISON OF PLANT-ADAPTED RHABDOVIRUS PROTEIN LOCALIZATION AND INTERACTIONS

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</tr>
</tbody>
</table>
DISSERTATION

Kathleen Marie Martin

The Graduate School
University of Kentucky
2011
# TABLE OF CONTENTS

<p>| LIST OF TABLES | ........................................................................................................................................ | v |
| LIST OF FIGURES | .......................................................................................................................................... | vi |
| CHAPTER I | ........................................................................................................................................ | 1 |
| A review of <em>Agrobacterium</em>-mediated plant vector technologies | ........................................................................................................................................ | 1 |
| History of <em>Agrobacterium</em> | ........................................................................................................................................ | 1 |
| History of the development of <em>Agrobacterium</em> Vectors | ........................................................................................................................................ | 2 |
| EXPRESSION VECTORS | ........................................................................................................................................ | 4 |
| SILENCING VECTORS | ........................................................................................................................................ | 11 |
| CONCLUDING REMARKS | ........................................................................................................................................ | 13 |
| CHAPTER II* | .......................................................................................................................................... | 40 |
| Transient expression in <em>Nicotiana benthamiana</em> fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta | ........................................................................................................................................ | 40 |
| METHODS | ........................................................................................................................................ | 41 |
| RESULTS | ........................................................................................................................................ | 44 |
| DISCUSSION | ......................................................................................................................................... | 48 |
| CHAPTER III* | .......................................................................................................................................... | 70 |
| Membrane and protein dynamics in live plant nuclei infected with <em>Sonchus yellow net virus</em>, a plant-adapted rhabdovirus | ........................................................................................................................................ | 70 |
| METHODS | ........................................................................................................................................ | 72 |
| RESULTS | ........................................................................................................................................ | 74 |
| DISCUSSION | ......................................................................................................................................... | 77 |
| CHAPTER IV | .......................................................................................................................................... | 90 |
| Comparison of the functional domains of <em>Sonchus yellow net virus</em> and <em>Lettuce necrotic yellows virus</em> glycoproteins | ........................................................................................................................................ | 90 |
| METHODS | ........................................................................................................................................ | 91 |
| RESULTS | ........................................................................................................................................ | 92 |
| DISCUSSION | ......................................................................................................................................... | 94 |
| CHAPTER V | .......................................................................................................................................... | 105 |
| Lettuce necrotic yellows virus protein localization and interaction map and comparison to two nucleorhabdoviruses, <em>Sonchus yellow net virus</em> and <em>Potato yellow dwarf virus</em> | ........................................................................................................................................ | 105 |
| METHODS | ........................................................................................................................................ | 106 |
| RESULTS | ........................................................................................................................................ | 106 |
| DISCUSSION | ......................................................................................................................................... | 108 |
| CHAPTER VI* | .......................................................................................................................................... | 126 |
| A host-factor interaction and localization map for a plant-adapted rhabdovirus implicates cytoplasm-tethered transcription activators in cell-to-cell movement | ........................................................................................................................................ | 126 |</p>
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>METHODS</td>
<td>127</td>
</tr>
<tr>
<td>RESULTS</td>
<td>130</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>134</td>
</tr>
<tr>
<td>CHAPTER VII</td>
<td>152</td>
</tr>
<tr>
<td>Concluding Remarks</td>
<td>152</td>
</tr>
<tr>
<td>APPENDIX I*</td>
<td>158</td>
</tr>
<tr>
<td>Characterization of the Sonchus yellow net virus polymerase protein domains</td>
<td>158</td>
</tr>
<tr>
<td>METHODS</td>
<td>159</td>
</tr>
<tr>
<td>RESULTS</td>
<td>160</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>161</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>168</td>
</tr>
<tr>
<td>VITA</td>
<td>195</td>
</tr>
</tbody>
</table>
LIST OF TABLES

CHAPTER I

Table 1.1. Recombination-Mediated T-DNA vectors ............................................................. 14
Table 1.2. Binary Vector Series ........................................................................................... 16
Table 1.3. Viruses developed for VIGS............................................................................ 29

CHAPTER II

Table 2.1. Characteristics of AFPs used in the construction of pSITE and pSITEII vectors. .......................................................... 51

CHAPTER IV

Table 4.1. Primers for the amplification of fragments of the Glycoproteins of SYNV and LNYV ..................................................................................................................... 96
Table 4.2. Comparison of the Glycoproteins for all sequenced Plant-adapted Rhabdoviruses in Genbank........................................................................................................... 97

CHAPTER VI

Table 6.1. Sonchus yellow net virus (SYNV) host factors characterized in this studya ........................................................................................................................................................................... 137
Table 6.2. Subcellular loci at which binary complexes of host factors and the nucleocapsid (N), matrix (M), and sc4 proteins were found. ........................................................................................................................................ 138
LIST OF FIGURES

CHAPTER I

Figure 1.1. Schematic diagram of the Agrobacterium tumefaciens Ti plasmid. .......... 31
Figure 1.2. Schematic diagram of A. tumefaciens transfer of DNA. ......................... 32
Figure 1.3. Schematic diagram of the vector providing the first expression of a transgene in plants. ........................................................................................................... 33
Figure 1.4. Schematic diagram of pBIN19................................................................. 34
Figure 1.5. Schematic diagram of pBINPLUS......................................................... 34
Figure 1.6. Schematic diagram of pED53:pED37 and pBIN19:pED23 Cre-lox system... ......................................................................................................................... 35
Figure 1.7. Schematic diagram of pBI. ................................................................. 36
Figure 1.8. Schematic diagram of pCIB770......................................................... 36
Figure 1.9. A schematic diagram of pER8............................................................... 37
Figure 1.10. Schematic diagram of pSITEII-7C1...................................................... 37
Figure 1.11. Schematic diagram of pEarleyGate 301. .............................................. 38
Figure 1.12. Schematic diagram of pPZP-RCS2 and pSAT vectors and their use to make a multi-gene cassette T-DNA. ................................................................. 38
Figure 1.13. Schematic diagram of pHELLSGATE, a Gateway-compatible silencing vector. ............................................................................................................................. 39
Figure 1.14. Schematic diagram of Tobacco rattle virus (TRV) Gateway compatible vectors. .......................................................................................................................... 39

CHAPTER II

Figure 2.1. Construction of pSAT6-AFP-DEST, a Gateway-compatible derivative of pSAT6-DEST-FS in which an EGFP ‘stuffer’ sequence was engineered with flanking FseI and SpeI restriction sites. ................................................................. 52
Figure 2.2. Construction of pSITEII vectors. ......................................................... 53
Figure 2.3. pSITE-BiFC-C1 vectors were constructed by subcloning the Agel/BglII fragment of pSAT4-BiFC (a) into pSAT6-DEST (b). ......................................................... 54
Figure 2.4. Photoactivation of DRONPA (a) and DRONPA-SYNV-N (b) in agroinfiltrated N. benthamiana leaf epidermal cells. ................................................................. 55
Figure 2.5. Photoconversion of Dendra2 fusions expressed from pSITEII-C1 (a1–c5) or pSITEII-N1 (d1–f5) vectors. ................................................................. 56
Figure 2.6. Validation of the pSITE-BiFC vectors for detecting interactions between soluble (a–d) or integral (e–h) membrane protein complexes............................................. 58
Figure 2.7. Single-section confocal micrographs of bimolecular fluorescence complementation (BiFC), showing differential interactions of NblImpα1 and NblImpα2 with SYNV-N................................................................................................................................. 59
Figure 2.8. Single-section confocal micrographs of bimolecular fluorescence complementation (BiFC), showing differential interactions of NblImpα1 and NblImpα2 with PYDV-N. ................................................................................................................................. 61

Kathleen Martin is responsible for previously published figures labeled in RED.
Figure 2.9. (a–l) Single-section confocal micrographs showing localization of AtWIP1:GFP in transgenic N. benthamiana plants expressing RFP:H2B.......................... 62
Figure 2.10. Transient expression of RFP-H2B (a) in a leaf epidermal cell of transgenic N. benthamiana expressing GFP-Talin, an actin binding protein (b). ....... 63
Figure 2.11. Photosensitivity of AFPs expressed in leaf epidermal cells of N. benthamiana. ........................................................................................................ 64
Figure 2.12. Comparative brightness of spectrally-related groups of AFPs as a function of PMT-voltage from 300-700 V in 100 V increments.......................... 66
Figure 2.13. Comparative brightness of spectrally-related groups of AFPs. ............... 68
Figure 2.14. Relative fluorescence intensity of the green and red forms of SYNV-P:Dendra2 photoconverted in panels A1-A5 in Figure 2.5........................................... 69

CHAPTER III

Figure 3.1. (a, b) TIRFM micrographs showing the distribution of ER tubules in protoplasts derived from leaves of mock-inoculated (a) or SYNV-infected (b) mGFP5-ER N. benthamiana plants. ................................................................. 80
Figure 3.2. (a) Normalized FRAP data following photobleaching of GFP in the nuclear envelope (N.E.) or three different regions of interest (ROIs 1–3) in the same nucleus........................................................................................................................................... 81
Figure 3.3. Localization of a nuclear-envelope marker in SYNV-infected nuclei....... 82
Figure 3.4. Confocal micrographs of RFP fusions of SYNV proteins expressed in SYNV-infected and mock-inoculated mGFP5-ER plants........................................... 83
Figure 3.5. Confocal micrographs showing the coexpression of CFP (cyan) and RFP (red) fusions in SYNV-infected nuclei.......................................................... 85
Figure 3.6. (a) Normalized FRAP data following photobleaching of RFP–N localized on intranuclear membranes (mem.) or nucleoplasm (nucleo.) in nuclei of virus-infected cells, or in nuclei of mock-inoculated cells in which RFP–N and CFP–P were coexpressed (N/P mock)... ........................................................................................................... 87
Figure 3.7. Model for the spatial relationship between sites of SYNV replication and morphogenesis...................................................................................... 88
Figure 3.8. Localization of sc4 in SYNV-infected (a–c) and mock-inoculated (d) cells.. .................................................................................................................. 89

CHAPTER IV

Figure 4.1. Comparison of the domains of the glycoproteins of PYDV, SYNV, and LNYV................................................................................................................. 98
Figure 4.2. Confocal micrographs showing the coexpression of SYNV glycoprotein fragments (GFP) and endoplasmic reticulum (RFP) in N. benthamiana leaf epidermal cells. ................................................................. 99
Figure 4.3. Confocal micrographs showing the coexpression of LNYV glycoprotein fragments (GFP) and endoplasmic reticulum (RFP) in N. benthamiana leaf epidermal cells. ................................................................. 101

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CHAPTER V

Figure 5.1. Confocal micrographs of the localization of LNYV proteins in relation to a red nuclear marker, histone 2B (RFP-H2B), in transgenic N. benthamiana.................................112
Figure 5.2. Confocal micrographs showing the localization of LNYV protein fusions in relation to a green fluorescent protein endoplasmic reticulum marker (GFP-ER) transgenic N. benthamiana......................................................................................................................114
Figure 5.3. Confocal micrographs showing the coexpression of GFP (green) and RFP (red) LNYV protein fusions in uninfected N. benthamiana. .................................................................116
Figure 5.4. Confocal micrographs showing the coexpression of GFP (green) and RFP (red) LNYV protein fusions in uninfected N. benthamiana. .................................................................117
Figure 5.5. Confocal micrographs showing LNYV protein interactions determined by bimolecular fluorescence complementation (BiFC)..................................................................118
Figure 5.6. Confocal micrographs showing protein interactions shared between LNYV, SYNV and PYDV determined by bimolecular fluorescence complementation (BiFC)..................................................................120
Figure 5.7. Confocal micrographs showing protein interactions only detected in two viruses..............................................................................................................................................................122
Figure 5.8. Confocal micrographs showing protein interactions only detected in one virus, LNYV, SYNV or PYDV.................................................................124
Figure 5.9. Schematic diagram of the three viral interaction maps for SYNV, LNYV and PYDV................................................................................................................................................125

CHAPTER VI

Figure 6.1. Single-section confocal micrographs showing SYNV protein interactions determined by bimolecular fluorescence complementation (BiFC).................................139
Figure 6.2. A, Single-plane confocal micrographs showing the relationship between the localization patterns of A1, green fluorescent protein–endoplasmic reticulum (GFP-ER) and A2, sc4i21........................................................................................................................................141
Figure 6.3. Single-plane confocal micrographs showing the relationship between the localization patterns of A1, green fluorescent protein–endoplasmic reticulum (GFP-ER) and A2, Ni67 and A3, the merger of these two panels (overlay).................................142
Figure 6.4. Single-plane confocal micrographs showing the relationship between the localization patterns of A, Mi7 in B, green fluorescent protein–endoplasmic reticulum plants (GFP-ER)......................................................................................................................................143
Figure 6.5. Single-plane confocal micrographs of bimolecular fluorescence complementation assays conducted in leaf epidermal cells of transgenic N. benthamiana plants expressing a nuclear marker protein (CFP: H2B).................................................................144
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CHAPTER I
A review of Agrobacterium-mediated plant vector technologies.

Agrobacterium tumefaciens is a plant pathogen capable of transforming a wide variety of dicotyledonous plants with its own deoxyribonucleic acid (DNA). In nature, Agrobacterium uses this process to drive the expression of its genes in plants to produce tumors and provide nutrients. However, in the last thirty years, scientists have determined how to manipulate A. tumefaciens to transfer any DNA to the plant cell nucleus for the expression of a gene of interest (GOI) in both dicotyledonous and monocotyledonous plants. This is done through the use of specialized plasmid vector systems that provide the tools to study gene expression, localization, protein interaction and movement in plants. This review will focus on technologies associated with Agrobacterium-mediated gene expression in plants.

History of Agrobacterium
Agrobacterium tumefaciens was first identified in 1907 as a tumor-causing bacterium infecting Argyranthemum frutescens (Paris daisy). It is a Gram-negative, rod-shaped polar bacterium with 1-3 flagella. This pathogen is also responsible for gall formation previously described on woody plants (Smith & Townsend, 1907). A. tumefaciens is capable of infecting at least 40 different species of dicotyledonous plants in 18 different families causing gall formations. In the forties and fifties, it was discovered that gall tissue from the plants infected with Agrobacterium could be cultured artificially and eventually freed from infection. However, the cultured tissue from plants initially infected did not require the hormones that were needed by tissues that had never been infected (Braun & Mandle, 1948; White & Braun, 1941; White, 1942). Due to this observation, Braun postulated that the plant tissues that had been infected underwent a permanent change due to the bacterium. Braun called the agent that caused this permanent change the “tumor inducing principle” or TIP (Braun & Mandle, 1948; White & Braun, 1941; White, 1942).

TIP was identified later as being DNA that was transferred from the bacterium plasmid into the plant cells to enact this permanent change. This DNA became known as T-DNA or “transfer” DNA (Chilton et al., 1980; Chilton et al., 1977; Willmitzer et al., 1980). After this discovery, it then became important to determine the factors associated with this transfer of DNA into the plant cell. Two main regions of the Agrobacterium plasmid (also called the tumor inducing (Ti) plasmid) were identified as being the transfer region (or T-DNA), which was also found to be present in the nucleus of plant cells, and the virulence region (Vir region), which was shown to be involved in the formation of tumor cells in the plant. The T-DNA region contains four genes called tmr, tms, tml (tumor morphology roots, shoots, large respectively) and ocs (octopine synthase) (Garfinkel & Nester, 1980; Garfinkel et al., 1981; Ooms et al., 1981). Ocs is replaced by nopaline synthase (nos) in nopaline strains of Agrobacterium (Depicker et al., 1982). Left and right borders surround the T-DNA, are recognized by the Agrobacterium proteins, and are necessary for the transfer of the DNA (Figure 1.1) (Yadav et al., 1982; Zambryski et al., 1982).
In contrast to the T-DNA which is also found in the nucleus of infected cells, the vir genes have not been found to be incorporated in plant DNA (Chilton et al., 1977). The transfer of DNA begins with acetosyringone, a plant hormone, which is released from the wounded plant and is recognized by virA (Stachel et al., 1985). This membrane bound receptor molecule then phosphorylates virG, which activates the remaining Vir operons (Stachel & Zambryski, 1986; Winans et al., 1986). VirD2 knicks the right border of the T-DNA region and binds to the 5’ end (Ward & Barnes, 1988; Yanofsky et al., 1986). This complex is transported into the plant cell through a pilus of virB proteins, coated with virE and transferred into the nucleus through interactions with host proteins. It is then incorporated into the nuclear DNA of the plant and synthesis of opines begins (Figure 1.2)(Citovsky et al., 1989; Citovsky et al., 1992; Gelvin, 2000; Thompson et al., 1988).

One of the main observations which had the greatest impact on plant science is that the left and right borders are necessary for the transfer of DNA into the plant nucleus, as are the vir genes, however, the genes found within the T-DNA region, tmr, tms, tml and ocs are not needed for transfer. Once deleted, the remaining T-DNA region is still transferred into the plant nucleus (Leemans et al., 1981). The genes present between the left and right borders were then removed and replaced by the kanamycin resistance gene. When the Agrobacterium transferred this gene into plants, the plants were then also kanamycin resistant (Figure 1.3) (Hernalsteens et al., 1980). This quickly led to the hypothesis that A. tumefaciens could be used to transform plants with any DNA between the left and right borders.

**History of the development of Agrobacterium Vectors**

During the initial development of Agrobacterium as a vehicle to facilitate plant transformation, two main types of vectors were constructed. Both types of vector systems have some general aspects to consider. The vectors have to contain the signals necessary for transfer of the sequences between the border regions, no interference with the normal development of the plant via the development of gall formations or stunting, there must be a means for selection of positive colonies containing the GOI, and lastly, there must be a way of introducing the GOI into the system (Bevan, 1984; Zambryski et al., 1983).

One of the first two vector systems developed utilized bacterial recombination and tri-parental mating as a means of introducing the GOI into the transformation vector. pGV3850 was the first vector that utilized this method (Zambryski et al., 1983). pGV3850 is used as the “acceptor vector” which contains an area of homology to pBR322 between the left and right borders. Another pBR vector present in Escherichia coli contains the GOI. E. coli and A. tumefaciens are allowed to go through bacterial conjugation through tri-parental mating and colonies are selected using ampicillin. Since the pBR vector cannot replicate in Agrobacterium, the only way for the GOI to be present is if a cross-over event occurs between pBR and pGV3850. A. tumefaciens is used for plant transformation and the plants are screened for the presence of the GOI. This vector system is useful, but it has limitations, such as the inability to properly function if there are regions of homology to either pBR or pGV3850 present in the GOI, this results in additional recombination between the bacterial plasmids to occur. Also, the cross-over event
is unspecific and would often result in excess sequences from pBR between the left and right borders. The vector itself is large, containing both the Vir genes and the T-DNA region on the same plasmid. This makes it very difficult to insert the GOI into the T-DNA region unless recombination is used. The pGA and the SEV vectors introduced in 1985 also utilize recombination to insert the GOI (An et al., 1985; Fraley et al., 1985). A table of the recombination-mediated vectors is included as Table 1.1.

The second type of vectors are "binary vectors", the first of these vectors being pBIN19 (Bevan, 1984). Binary vectors separate the Vir genes and the border regions necessary for transfer onto two separate plasmids. This separation was based on previous data that the Vir genes can act in trans to the T-DNA region of A. tumefaciens (Hoekema et al., 1983). The plasmid containing the Vir regions is referred to as the helper plasmid. The T-DNA plasmid contains a multiple cloning site (MCS) in between the left and right border sequences to insert the GOI. This MCS interrupts a lac site that allows for blue/white selection of the positive colonies in E. coli. pBIN19 also contains an origin of replication for both E. coli and A. tumefaciens (Figure 1.4) (Bevan, 1984).

The binary vector system became more popular because it negated the need for bacterial recombination and relied on the use of the MCS for the insertion of GOI into the vector. With the correct restriction sites added to the ends of the GOI sequence, the gene is then cloned into a T-DNA plasmid. This plasmid is transformed into a line of A. tumefaciens that contains a helper plasmid expressing all of the Vir genes necessary for the movement of the DNA into the plant cells. Not all strains of Agrobacterium are suitable, as it is desired that plants do not develop any other symptoms associated with infection. These "disarmed" strains have been identified in both octopine and nopaline types (Hellens et al., 2000a).

Since the development of the first vector system, there has been a continued effort to improve the vectors available. The features that make an optimal vector system include the following:

1. **Ease of Use**- is able to be manipulated by a wide variety of users.
2. **Broad Range of Applications**- the same vector system can be used for a wide variety of different experiments.
3. **High level of expression**- allows for easy identification of GOI expression.
4. **High-throughput**- to facilitate the analysis of a large number of genes in a short amount of time.

Each of the newer vector systems seek to improve on one or more of the above features to arrive at the optimal system to use. Although the basic features of a binary vector system are the same, variations of one or more of the elements can improve the user friendliness of the system. For example, the conversion of the multiple cloning site to a Gateway® cassette for recombination mediated cloning can improve the ability to use the vector for high-throughput analyses of genes. In the next section, the basic features of both expression vector systems and silencing vectors will be detailed. Many of the improvements and/or adjustments to increase the utility of Agrobacterium vectors so that they are better suited to the needs of the research community will be illustrated.
EXPRESSION VECTORS

Expression vectors are used to express a GOI either stably, meaning transformed tissue is cultured into a new plant that can pass the GOI to the next generation, or transiently, where the GOI is present only in the transformed plant tissues for a short period of time. Expression vectors can be used for the study of foreign genes, alteration of the transcription levels of native genes, and protein localization, interaction or function in cells, tissues or whole plants. The features of a T-DNA plasmid include the left and right borders for Agrobacterium transformation, an antibiotic resistance gene to select for positive bacterial colonies, and origins of replication for both E. coli and A. tumefaciens. Other features included in many vector systems are a plant selectable marker (usually an antibiotic or chemical resistance gene to select for positive plant transformants), a promoter sequence to drive expression of the GOI, a multiple cloning site or other means of inserting the GOI, a terminator sequence, a gene expression reporter and epitope tags. The features of vector systems are included as part of Table 1.2.

The Left and Right Borders

The borders of the T-DNA region were first identified in 1982 and were characterized as imperfect direct repeats of 25 bp at both the left and right borders (Yadav et al., 1982; Zambryski et al., 1982). The right border of the T-DNA region is needed for the transfer of the DNA sequence in a directional manner into the nucleus of plant cells, whereas the left border determines the ending point for the sequence transfer into the nucleus (Wang et al., 1984). When the left border is repeated, it further reduces the incorporation of vector backbone sequences into the host plant nucleus (Kuraya et al., 2004; Wang et al., 1984). If one of the two borders is deleted, transfer can still take place, but the efficiency of transfer is drastically compromised if the left border alone is present (Gardner & Knauf, 1986; Joos et al., 1983). In addition, “overdrive”, a sequence flanking the right border, greatly increases the transfer of the T-DNA, so it has been included in some vectors to increase the number of transformed plants (see Table 1.2) (Peralta et al., 1986; van Haaren et al., 1987).

Bacterial Selectable Marker Genes

Genes conferring antibiotic resistance are present in all T-DNA binary vector systems to provide positive selection of transformed colonies. Kanamycin, gentamicin, spectinomycin, chloramphenicol and tetracycline are used in different vector systems. As some Agrobacterium strains have inherent antibiotic resistance, the resistance conferred by the vector must be different to ensure positive colonies for the vector can be selected (Hellens et al., 2000a; Hood et al., 1986).

Origins of Replication

Origins of replication provide maintenance functions in E. coli and A. tumefaciens. Plasmids in the same cell which share the same origin of replication directly compete for stable inheritance, thus multiple plasmids containing the same origin of replication are incompatible and classified into the same "incompatibility group" (Thomas & Smith, 1987). Many of the early binary vectors include a wide
host range origin of replication that allowed for replication in both *E. coli* and *A. tumefaciens*. This is called the RK2 origin of replication and was originally derived from a wide host range *E. coli* vector called pRK (Bevan, 1984). Other vectors also include two origins of replication, one specific for *E. coli*, called ColE1, which leads to a high copy number of plasmids (Klee *et al.*, 1985). Vectors that have ColE1 contain a second origin of replication that works in *Agrobacterium*, either from an *Agrobacterium tumefaciens* strain (Ti) or an *Agrobacterium rhizogenes* strain (Ri). Although, combinations of ColE1 with RK2 (Klee *et al.*, 1985) or pVS1 (Hajdukiewicz *et al.*, 1994) are common, origins of replication are included in the features section of Table 1.2 when possible.

**Plant Selectable Markers**

Plant selection markers are used to find positively transformed plants during tissue culture. These markers are included with their own promoters and terminators and are separate from the GOI. The first plant selection marker was neomycin phosphotransferase II (*nptII*) that conferred resistance to kanamycin (Bevan *et al.*, 1983). This was included close to the right border of pBIN19 with the site for insertion of the GOI near the left border (Figure 1.4). Later, this order would be reversed, with the GOI near the right border and the kanamycin resistance gene near the left border, the first example of this is pBINPLUS (Figure 1.5). As genes that are near the right border are transferred first, when the plant selection marker is present near the right border, kanamycin resistant plants do not always have the GOI. However, if the plant selection marker is present near the left border and is transferred last, this increases the probability that the GOI is present in the transformed plants (Bevan, 1984; van Engelen *et al.*, 1995; Wang *et al.*, 1984). Kanamycin resistance is still a popular choice as a plant selection marker, present in many of the vector systems. However, this presents a problem when trying to transform multiple genes into the same plant, or using a plant with some background resistance to kanamycin. Therefore, a number of vector systems also have hygromycin, bleomycin, methotrexate, phosphinothricin or gentamicin resistance (see Table 1.2). In monocot systems like rice, hygromycin is preferred (Hiei *et al.*, 1994), however, in maize, phosphinothricin is considered the most effective (Ishida *et al.*, 1996). There are a few vector systems that use green fluorescent protein (GFP) or β-glucuronidase as selection markers as well, see Table 1.2 for examples.

The removal of the plant selection marker after transformation may be desired in some experiments. There are two main strategies to accomplish this, one is to use sexual recombination to remove the selectable marker and the second is to use recombinase to excise the selectable marker from the plant genome. Sexual recombination involves using two vectors for transformation, one containing the GOI and a second containing the plant selectable marker. The plants expressing both after transformation are crossed and the unlinked T-DNA regions segregate. Only the transformants containing the GOI alone are selected for further study (Ishida *et al.*, 1996; Komari *et al.*, 1996).

The second means of removing the plant selectable marker is to use recombination. There are three recombination systems described in the Table 1.2,
one is the Cre-lox system. This system is from bacteriophage PI, and when loxP sites flank a gene, in the presence of a recombinase (Cre), the chromosome where the loxP sites are located meet and site-specific recombination occurs (Dale & Ow, 1990; Sternberg & Hamilton, 1981; Sternberg et al., 1981). This recombination excises the sequences between the sites. In the selectable marker system, loxP sites flank the plant selectable marker and Cre is transformed into the system on another plasmid. The first reported Cre/lox vectors were the pED vectors, (Table 1.2 Figure 1.6) (Dale & Ow, 1991). The initial vector has the selection marker flanked by recombination sites and is used to make a transgenic plant. Then the recombinase is introduced on a T-DNA in a second transformation event and excises the fragment when they are present in the same cell. Once the selection marker has been excised, the plants are crossed and sexual recombination causes segregation of the recombinase T-DNA. Unlike using sexual recombination alone, this method ensures that the GOI is present because the selection marker is on the same T-DNA. However, the end result is the same, the selection marker is removed before the plant is selected for further study. Two other systems utilize a similar mechanism, the R/Rs system, with Rs sites and an R recombinase (Sugita et al., 1999; Sugita et al., 2000), and the FRT/FLP system, with FRT sites and FLP recombinase (Coutu et al., 2007).

The Promoter and Terminator Sequences

The promoter used in the first binary vector systems was the nopaline synthase promoter (nos) which was already present in the T-DNA region of the Agrobacterium genome (Depicker et al., 1982). This promoter is used in systems such as pBIN19 to drive the expression of the kanamycin gene for selection in plant cells. There are no other promoters present in pBIN19, so genes have to be inserted with their own regulatory elements (Figure 1.4) (Bevan, 1984). Later vector systems would incorporate promoters into the system so regulatory elements for the GOI would not be required.

The first vector system to do this was pBI, which used both the Cauliflower mosaic virus (CaMV) 35S promoter and the ribulose bisphosphate carboxylase (rbsC) promoter to express the reporter β-glucuronidase (GUS). However, this vector is used to determine other promoter sequences and is not designed to use CaMV 35S or rbsC with a GOI (Figure 1.7) (Jefferson et al., 1987; Odell et al., 1985). This changed when the pCIB vectors were developed. These contain either the CaMV 35S promoter or the CaMV 19S promoter. pCIB770 was designed with a CaMV35S before a single BamHI site for use with a GOI (Figure 1.8) (Odell et al., 1985; Rothstein et al., 1987). This was an advantage because there is no longer a need to insert additional regulatory signals. Since that time, the CaMV 35S promoter has become a popular choice for vector construction as it expresses well in all tissues including roots, shoots and leaves of dicotyledons plants (Odell et al., 1985). Examples of vector systems with the 35S promoter are the pART vectors, the pMJD, pPZP, pRT, pCB to name a few (See Table 1.2). For monocot systems, the promoter of choice varies depending on the plant to be transformed. They include the maize ubiquitin promoter sequence (Christensen et al., 1992) and the rice actin promoter sequence (Zhang et al., 1991).
All of the promoters previously mentioned are constitutive promoters for the continuous expression of the GOI. This may be problematic in some cases if the GOI is toxic to the system. Thus, there are inducible promoters that promote transcription only when the correct conditions are met. There are two main types of inducible promoters, those that are chemical or environmental.

Requirements necessary for the successful use of chemically inducible promoters include: the chemical inducer should not be present in the host, should not be toxic to the plant, only affects the expression of the GOI, easy to apply or remove and is easy to detect a difference in expression when compared to wildtype (Zuo & Chua, 2000). One such chemically inducible promoter is included in pER8, an estrogen inducible vector. The estrogen receptor (XVE) transcribes sequences for a LexA transcription factor. The LexA transcription factor then binds to a LexA binding site further in the T-DNA region and the GOI is expressed (Figure 1.9). The XVE receptor is utilized in the pMDC vectors as well (Curtis & Grossniklaus, 2003; Zuo et al., 2000). (Gatz, 1996; Gatz et al., 1992; Zuo & Chua, 2000) Other chemically inducible promoters include tetracycline, dexamethasone and alcohol based regulatory elements (Gatz, 1996; Gatz et al., 1992; Zuo & Chua, 2000).

Only one environmentally regulated promoter is utilized in the plant vector systems assayed, this is in the pMDC vectors. It is the heat shock protein promoter and is activated only when the plants are kept above 37°C Celsius. Once that condition is met, the GOI is transcribed (Curtis & Grossniklaus, 2003). To accurately control the transcription of a gene under this type of promoter, growth under controlled environmental conditions is required.

The terminator sequence is a regulatory element required by the RNA polymerase to add a poly A track to the mRNA and disassociate from the DNA template (Gil & Proudfoot, 1984; Hunt, 1994). There are three main terminator sequences used in the majority of vector systems, these are the nopaline synthase (nos) poly A sequence, the CaMV poly A sequence and the octopine synthase (ocs) poly A sequence.

Insertion of the GOI

In the earliest Agrobacterium binary vector systems, the GOIs were inserted into the plasmid vector via a MCS. This characterized by the presence of several endonuclease restriction sites present in a small area of sequence. Restriction sites in the MCS of binary vectors typically do not interrupt any essential function either for replication or antibiotic selection in the bacterium. In contrast, in the E. coli plasmid vector pBR322, insertion of a gene knocks out one of the antibiotic resistance genes and requires double plating to determine the colonies positive for the insert (Bolivar et al., 1977). pBR322 forms the basis for the recombination-mediated Agrobacterium vectors. The MCS of pBIN19 was derived from m13mp19 (a DNA bacteriophage sequence) and contains a variety of restriction enzyme sites within a lacZ gene sequence to allow for blue/white selection of positive colonies (Figure 1.4; Bevan, 1984). The restriction enzyme sites differ between vectors and the sequence must be consulted to determine the best way to clone the GOI into the MCS.
In 2000, a new technology called Gateway®, was introduced to clone genes into vector systems. Gateway was co-opted from bacterial phage lambda and relies on recombination mediated cloning to generate the final vector. The DNA sequence for the GOI is first amplified via polymerase chain reaction (PCR) with primers that also include the sequences for attB1 on the forward primer and attB2 on the reverse primer. These sequences mediate specific, directional cloning into a vector that contains attP1 and attP2 sites. The reaction is attB x attP → attL x attR, with attB1 interacting with attP1 and attB2 interacting with attP2. Once the insert has undergone this recombination mediated by bacteriophage host proteins integrase (Int) and integration host factor (IHF) enzymes, the new attL1-GOI-attL2 vector can then be used in any other vector which contains attR sites in the reaction: attL x attR → attB x attP and is mediated by Int, IHF and excisionase enzymes. Depending on the desired result, the specific addition of enzymes and vectors containing the right sequences for recombination can result in the creation of a specific T-DNA vector with the GOI. This is further enhanced by the addition of a lethal ccdb gene that recombines into the incorrect vector allowing only the vector containing the GOI to survive. This allows for the quick identification of the correct vector for the next experiment (Hartley et al., 2000).

Once the attL1-GOI-attL2 vector is generated, it can be cloned into any vector with appropriate attR sites to create a variety of final clones. This negates the need for repeat PCRs to generate new ends for the GOI to clone into a multitude of different MCSs, and is useful for more high-throughput analysis of each GOI. As the generation of a single vector for use in recombination reactions, the same GOI can be used in vectors with a variety of different reporter fusions for a more complete analyses of the GOI (Hartley et al., 2000). One example of a Gateway compatible vector is provided as Figure 1.10.

**Reporter gene fusions**

Early vector systems did not include reporter genes, however, the advantage of using a reporter fusion means one can track the expression of the GOI by a visual or biochemical means. Optimal reporters can be fused to the amino or carboxy terminus of the GOI as some genes may not express in one of the two orientations. Reporters should not cause any undo artifacts, for example, dsRed forms a tetramer and can cause aggregations of protein fusions (Goodin et al., 2002). If it is an enzymatic reporter, being able to use multiple substrates that are not ordinarily found in plants is an advantage and to avoid background, the reaction between the substrate and enzyme must be specific (Jefferson et al., 1987; Ziemienowicz, 2001).

There are several types of reporter genes that can be used to determine various aspects of the GOI. These types include those that provide expression information but non-specific localization, specific localization reporters, protein-protein interaction and finally protein-tracking reporters.

Chloramphenicol acetyl transferase (cat) is an enzymatic reporter that attaches an acetyl group to chloramphenicol, and this acetylated chloramphenicol is non-toxic in plants. The expression levels of the GOI can be quantified by adding a known amount of radioactive chloramphenicol and acetyl coenzyme A to cat-
expressing plant cell extracts. After a set amount of time determined by the researcher, the reaction is stopped with the extraction of chloramphenicol, and the forms of acetylated radioactive chloramphenicol are measured (Gorman et al., 1982; Herrera-Estrella et al., 1983). The higher the levels of acetylated chloramphenicol detected, the higher the expression level of the GOI. CAT assays have also been used to determine the number of times a transgene was inserted into the plant genome. Those with fewer insertions have less acetylated forms of chloramphenicol than those with more insertions of the T-DNA in the same amount of time (Gendloff et al., 1990). However, this assay does not provide specific localization information (Herrera-Estrella et al., 1983).

pBI in 1987 utilized β-glucuronidase as the first gene fusion reporter (Figure 1.7). This is an enzymatic reporter that can be used to cleave a wide variety of glucuronides, such as 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) which is cleaved and forms a blue precipitate after exposure to air in the tissues where expression of the GOI occurs (Jefferson, 1987). β-glucuronidase as a reporter is a popular choice in many of the early vector systems (Helmer et al., 1984). Another enzymatic reporter is firefly luciferase that catalyzes the light-producing oxidation of luciferin. The luciferin can be introduced into the plant during watering, and the light given off can be read by a luminometer to determine expression (Ow et al., 1986). These two reporters can be either specific for localization or non-specific depending on how they are assayed.

Autofluorescent proteins (AFP) came into use as localization reporters in 1997 with the removal of a cryptic intron in green fluorescent protein (GFP) that made expression in plants poor (Haseloff et al., 1997). GFP was first characterized in 1961 as a protein from the jellyfish Aequorea victoria, which in nature, fluoresces upon exposure to blue light provided by a second fluorescent protein, aequorin (Shimomura et al., 1962). The first vector system to use GFP was the pCB vectors is 1999 (Xiang et al., 1999). A second fluor was identified in a coral Discosoma sp. in 1999 that fluoresces red (DsRed)(Matz et al., 1999). The first vector system to incorporate DsRed is the pGD vectors (Goodin et al., 2002). However, DsRed forms a tetramer, so a monomeric form called mRFP was developed to decrease artifacts caused by DsRed (Campbell et al., 2002). From there, mutations in these proteins would lead to a full range of fluoros which cover the entire spectrum of visible light, a full list is available (Shaner et al., 2005; Shaner et al., 2007; Snapp, 2009). If the excitation and emission spectrum are far enough apart, multiple fluoros can be utilized at the same time to localize two proteins. Colocalization of two proteins to the same cellular location can provide evidence that the two proteins interact, however, it is necessary to use fluoros specifically tailored to determine protein-protein interactions.

There are three methods to determine protein-protein interactions using fluoros, these are Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) and bimolecular fluorescence complementation (BiFC). In FRET, two suspected interacting proteins are expressed as fusions to two fluoros such as Venus (a yellow fluorescent protein or YFP) and Cerulean (a cyan fluorescent protein or CFP). When the two fluoros are within 100 Angstroms of each other, the specific excitation of the donor, Cerulean, leads to the transfer of energy
to the acceptor, Venus, triggering fluorescence. The energy transfer can be measured because the donor decreases in fluorescence as the acceptor increases, the extent of this is related to the distance the two proteins are to each other (Kenworthy, 2001; Nagai et al., 2002; Rizzo et al., 2004; Selvin, 1995). BRET is a modified form of FRET with the donor fluorophore (Cerulean in above example) replaced by luciferase. In the presence of a substrate, bioluminescence from luciferase excites the acceptor fluorophore (Venus in above example) in the same relationship that is shared with FRET, the closer together, the higher the intensity of fluorescence of the acceptor fluor (Xu et al., 2007a; Xu et al., 1999).

Bimolecular fluorescence complementation (BiFC) is also used to determine protein-protein interactions. In this method, an AFP is expressed as two halves, an amino and a carboxy terminal half, and no fluorescence is observed. If two proteins are fused to the AFP halves than two possible outcomes occur: the proteins do not interact, and no fluorescence is detected; or the two proteins do interact, and the interaction of the two proteins reconstitutes the AFP halves and restores the autofluorescent character of the AFP. Originally, BiFC was done with halves of YFP, but the technique has been adapted to a variety of fluoros (Hu et al., 2002; Lee et al., 2008; Shyu et al., 2006).

Protein tracking can be accomplished with different techniques and or with specific fluoros. Fluorescence recovery after photobleaching (FRAP) and Fluorescence loss in photobleaching (FLIP) are two techniques that can be used to measure protein movement. When an autofluorescent protein is permanently no longer able to fluoresce, this is called photobleaching. FRAP is the one time bleaching of a defined region and determining if fluorescence returns to the bleached area. Determining how quickly the fluorescence returns to the bleached area can give clues as to the mobility of the protein fusion (Axelrod et al., 1976; Köster et al., 2005). FLIP is the repetitive bleaching of an area and determination if the overall cellular fluorescence decreases; this indicates that the fluor is mobile or is isolated in a specific cellular compartment. If the protein fusion is mobile the entire cell will photobleach (Cole et al., 1996; Köster et al., 2005).

Unlike FRAP and FLIP which can be done with any AFP, there are specific fluoros which allow for photo-tracking functions, these include PS-CFP, PA-GFP, DRONPA, dendra2 and EosFP. PA-GFP and DRONPA are photo-activatable, i.e. they are not fluorescent until exposed to a 405 nm laser, and then they become fluorescent (Ando et al., 2004; Patterson & Lippincott-Schwartz, 2002). PS-CFP, Dendra2 and EosFP are photo-convertible, they are fluorescent with one emission spectrum until exposed to a 405nm laser, then they change their emission spectrum to fluoresce in a different color (Chudakov et al., 2004; Gurskaya et al., 2006; Wiedenmann et al., 2004). These AFPs offer the advantage over FRAP and FLIP because they directly track the protein movement versus making inferences based on bleaching. This technique has been used recently with EosFP to track proteins targeted to various organelles including the Golgi, endoplasmic reticulum, mitochondria, peroxisomes, and others (Mathur et al., 2010). Dendra2 and DRONPA were incorporated into the pSITEII series of vectors for plant transformation, see Figure 1.10 (Martin et al., 2009).
Epitope Tags

Epitope tagging is the attachment of a short amino acid sequence to a protein to make it immunoreactive to a specific pre-existing antibody (Munro & Pelham, 1984). Adding an epitope tag to a protein offers the advantage of no longer needing a specialized antibody for the GOI. These tags can be used for protein purification, studies on protein-protein interactions and immuno-localization (the specific tags and their use are reviewed in (Fritze & Anderson, 2000; Jarvik & Telmer, 1998)). The pEarleyGate vectors are available for adding an epitope tag to the GOI in plants (Figure 2.11). These vectors offer a variety of epitope tags including HA, FLAG, and cMyc for use with a Gateway cassette for insertion of the GOI (Earley et al., 2006).

Multi-Gene Expression Vectors

To express multiple genes in the same cell, one can infiltrate multiple T-DNAs at the same time, but this does not guarantee that all of the genes will express in the same cell. To ensure that they are all present, one must use a multi-gene expression vector that has multiple T-DNAs on the same plasmid, or multiple sites of insertion for the GOI in the T-DNA region. pPZP-RCS was designed with a series of homing endonuclease sites in an MCS region. These unique sites facilitate the insertion of multiple pSAT expression cassettes in between the T-DNA left and right borders. There are six sites for insertion of up to six expression cassettes that would allow for multiple gene expression, see Figure 1.12 (Goderis et al., 2002; Tzfira et al., 2005).

Another means of insertion of multiple genes is the use of multi-site gateway vectors that would allow for multiple gateway reactions to place several GOI in the backbone vector at the same time. Intermediary vectors can accomplish conversion of a single gateway cassette to a multi-site gateway vector in one step. However, this system has all of the gateway cassettes in fusion to each other and is promoted as a means of inserting the optimal promoter, GOI and reporter all in frame in one gateway recombination event (Magnani et al., 2006). To utilize this technology for multiple genes, one would have to use each gateway cassette to insert complete expression cassettes with their own regulatory elements.

SILENCING VECTORS

One method of studying gene function is to knock out endogenous gene expression and determine the effect on the plant. This can be accomplished by using a pathway in plants referred to as the ribonucleic acid (RNA) silencing pathway or post-transcriptional gene silencing (PTGS). This pathway was discovered when overexpressing a chalcone synthase (chs) gene in petunia led not to the expected darker pigmented flowers, but to white and purple flowers instead. It was determined that overexpressing the gene led to its suppression (Napoli et al., 1990; van der Krol et al., 1990).

At this time, the observation was made that if a plant was expressing virus-encoded sequences through Agrobacterium transformation, the plant was resistant to infection to the virus. One example of this is the expression of Tobacco etch virus (TEV) coat protein (CP) sequences that were used to induce resistance to TEV (Lindbo et al., 1993). It was postulated that the expression of the TEV CP causes
high levels of expression in the cytoplasm that are recognized by a RNA-dependent-RNA polymerase which can identify and hybridize with the invading viral RNA to destroy the infection (Lindbo et al., 1993).

Further studies on chs expression in transgenic plants lead to the conclusion that the suppression (also called silencing) was sequence specific. Areas where suppression was not occurring had very high levels of chs, areas where silencing was occurring had very low levels of chs and contained truncated transcripts. These aberrant messenger RNAs (mRNAs) were thought to be the target of endonucleic cleavage triggered by the high levels of expression. The conclusion the authors reached was that the aberrant mRNAs triggered silencing by binding with the endogenous gene in a complementary fashion making the native gene a target for endonucleic cleavage as well (Metzlaff et al., 1997).

Double-stranded RNA (dsRNA) was then tested with Potato virus Y (PVY) as a trigger for silencing. When PVY sequences in sense and antisense orientation were brought together, there was resistance to the virus (Waterhouse et al., 1998a). This demonstrated that the trigger was double-stranded RNA. Furthermore, a hairpin structure was more efficient at silencing than the expression of a sense or anti-sense transcript (Wang & Waterhouse, 2000; Waterhouse et al., 1998b). There are many excellent reviews on the exact mechanisms by which silencing occurs, I have listed a few for consideration (Brodersen & Voinnet, 2006; Chen, 2010; Lu et al., 2003).

Small interfering RNA mediated silencing

Artificially created small interfering RNAs (siRNAs, 21-24nt) corresponding to the sequence of the gene to be silenced can also be used as a trigger. Although there are vector systems that are specifically created with Gateway to make this easy (pHELLSGATE, Figure 1.13) (Helliwell & Waterhouse, 2003; Wesley et al., 2001), any vector system can be converted into a silencing vector if the construction is correct. To do this one uses a region of the gene that is specific for either that gene or if silencing an entire family is desired, a sequence that is specifically shared by members of the same family. For the most efficient silencing, the sequence must be transcribed in sense orientation followed by a spacer and then the same sequence in anti-sense orientation to form a hairpin structure (Smith et al., 2000; Wang & Waterhouse, 2000). That construction is placed under the control of a promoter in a binary vector that can be used to create a transgenic plant to silence the GOI. Once silenced, the function of the gene can be determined by looking at the effect of the mutation on the growth, phenotype and/or chemical composition of the plant.

Virus Induced Gene Silencing (VIGS)

As many viruses generate a replication intermediate (for example dsRNA) that can induce silencing, a GOI sequence incorporated into the virus, can silence the GOI as well. The plant recognizes the replication intermediate and silencing occurs. There are a number of viral vectors that have been created for VIGS. A list of these is included as Table 1.3, and an asterisk indicates those that have been adapted for Agrobacterium-mediated transformation.
Similar to siRNA pathways, this method can also silence individual genes or gene families. However, unlike siRNA constructs, VIGS can be done rapidly, usually taking between 2-3 weeks to see a discernable phenotype. This method does not require a stable transformed plant and can be used to allow comparison of silencing in different genetic backgrounds of the same species or even different plant species. The major disadvantage of this system is that the virus must continue to be infectious in order to generate a silencing phenotype, so the virus is capable of producing symptoms of infection on the host. These symptoms must be disregarded in order to determine the effect of the silencing of the GOI. Also, the virus must be infectious, so where the GOI sequence is placed in the virus is crucial, as well as, the size of the insert in the virus genome (Lu et al., 2003). An example of the Tobacco rattle virus-silencing vector is presented in Figure 1.14.

CONCLUDING REMARKS

Vectors based on Agrobacterium tumefaciens have become essential tools in plant molecular biology labs. From the early days with pBIN19, vectors have continually evolved to meet the needs of the research community. Compared to the first vectors, the more modern vector systems utilize a variety of promoters to allow for various levels of expression in both monocot and dicot plant systems. Super binary vectors with additional genes for transformation of monocots has provided the means to transform plants such as rice and corn. Also the use of a two-vector system or a recombination system enables the removal of the plant selectable markers so that multiple genes can be stacked in the same plant or worries that the plant selectable marker will cross into wild species can be allayed. For each function that is desired, there is a vector system that addresses this, from promoter analyses, using a Gateway cassette or using a photo-activatable fluor to track protein movement. Not only is there a virtually limitless supply of vectors that can provide any function that is desired, but also these vectors have become extremely user friendly to allow even a novice to explore systems in planta.
<table>
<thead>
<tr>
<th>Vector Series</th>
<th>Important Features</th>
<th>MCS</th>
<th>Bacterial Selection</th>
<th>Plant Selection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRK290</td>
<td>The left and right borders are placed in a wide host range vector with the RK origin of replication, ColE1 origin of replication also included, Kan used to determine positives of recombination, the borders are used to force recombination with pTi T37 Agrobacterium strain</td>
<td>No</td>
<td>Kan/Gent</td>
<td>None</td>
<td>(Barton &amp; Chilton, 1983)</td>
</tr>
<tr>
<td>pMON100s</td>
<td>Sequences from the Ti region present in pMON, borders provided by the mutated Agrobacterium vector pTiB6S3tra, recombination places GOI from pMON into Ti region, RK2 origin of replication, Ti replication of origin on pTiB6S3tra</td>
<td>No</td>
<td>Spect/Strep/Chl</td>
<td>Kan</td>
<td>(Fraley et al., 1983)</td>
</tr>
<tr>
<td>pGV3850</td>
<td>Agrobacterium plasmid modified with pBR322 sequences present in Ti region (borders present) and insert placed in pBR322 vector, recombination between pBR322 causes GOI to be between the left and right borders</td>
<td>No</td>
<td>Amp</td>
<td>Nos</td>
<td>(Zambryski et al., 1983)</td>
</tr>
<tr>
<td>pGV831, pGV2260</td>
<td>pGV2260 is the acceptor plasmid with borders and intermediary sequences removed and replaced with pBR322 sequences, pGV831 contains the border regions and kan resistance in plants. pGV831 contains BamH1 to clone a GOI.</td>
<td>No</td>
<td>Spect/Strep</td>
<td>Kan</td>
<td>(Deblaere et al., 1985)</td>
</tr>
<tr>
<td>SEV</td>
<td>SEV- Split End Vector: Modification of pMON system described above with right border present in pMON, with pTiB6S3 (a octopine type Agrobacterium plasmid) modified to create pTiB6S3-SE which contains a left border, once recombination occurs via a LIH (left inside homology) region, both borders are present with GOI from pMON.</td>
<td>No</td>
<td>Kan</td>
<td>Kan/Nos</td>
<td>(Fraley et al., 1985)</td>
</tr>
</tbody>
</table>

Continued on the following page.
<p>| pMON200+ | Improvement of pMON vectors of Rogers et al. (1987), CoIE1 origin of replication, RK2 origin of replication, MCS with β-glactosidase for blue/white selection or T7/SP6 promoters. Cos region from phage λ. Some members contain the LIH present in the pMON from the SEV vectors. Recombination through either the pBR sequences with pGV3850 or with the LIH in pTiB6S3-SE. | Yes | Kan/Spect/Strep | Kan/Hyg | Kan/Hyg/dhfr/nos/bar/β-glucuronidase | (Hinchee et al., 1988; Rogers et al., 1986; Rogers et al., 1987) |
| --- | --- | --- | --- | --- | --- |
| pCIT | Improvement of pMON vectors of Rogers et al. (1987), CoIE1 origin of replication, RK2 origin of replication, MCS with β-glactosidase for blue/white selection or T7/SP6 promoters. Cos region from phage λ. Some members contain the LIH present in the pMON from the SEV vectors. Recombination through either the pBR sequences with pGV3850 or with the LIH in pTiB6S3-SE. | Yes | Spect/Strep | Kan/Hyg | (Ma et al., 1992) |</p>
<table>
<thead>
<tr>
<th>Vector Series</th>
<th>Important Features</th>
<th>Gateway or MCS</th>
<th>Bacterial Selection</th>
<th>Plant Selection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBIN</td>
<td>T-DNA borders from pTiT7 ligated into pRK252, MCS from bacteriophage m13mp19 with β-glucosidase for blue/white selection, RK2 origin of replication</td>
<td>MCS</td>
<td>Kan</td>
<td>Kan</td>
<td>(Bevan, 1984)</td>
</tr>
<tr>
<td>pGA</td>
<td>Designed for larger DNA sequence transfer with cos region from phage λ, ColE1 origin of replication, RK2 origin of replication.</td>
<td>Neither</td>
<td>Kan</td>
<td>Kan</td>
<td>(An, 1987; An et al., 1985)</td>
</tr>
<tr>
<td>pEND4K</td>
<td>Designed for larger DNA sequence transfer with cos site, colE1 origin of replication, RK2 origin of replication, MCS from pUC19 with blue/white selection.</td>
<td>MCS</td>
<td>Kan/Tet</td>
<td>Kan</td>
<td>(Klee et al., 1985)</td>
</tr>
<tr>
<td>pAGS</td>
<td>Designed for larger DNA sequence transfer, cos region from phage λ, MCS from pUC18 for blue/white selection, RK2 origin of replication.</td>
<td>MCS</td>
<td>Tet</td>
<td>Kan</td>
<td>(van den Elzen, 1985)</td>
</tr>
<tr>
<td>pPCV</td>
<td>Large vector family. ColE1 origin of replication in E. coli present in some members, RK2 origin of replication, cos region from phage λ present in some members, and bacterial selection differs depending on vector. Vector system designed to test different promoters driving either octopine synthase production or neomycin phosphotransferase activity.</td>
<td>MCS</td>
<td>Amp/Chl/Carb/Tet</td>
<td>Kan</td>
<td>(Koncz, 1986)</td>
</tr>
<tr>
<td>pC22</td>
<td>Designed for larger DNA sequence transfer, cos region from phage λ, Ri origin of replication for Agrobacterium, ColE1 origin of replication for E. coli.</td>
<td>MCS</td>
<td>Spec/Strep/Carb</td>
<td>Kan</td>
<td>(Simoens et al., 1986)</td>
</tr>
<tr>
<td>pBI</td>
<td>Promoterless with MCS before a β-glucuronidase gene for use in fusion studies and a nos poly A site, contains the RK2 origin of replication</td>
<td>MCS</td>
<td>Kan</td>
<td>Kan</td>
<td>(Jefferson, 1987)</td>
</tr>
<tr>
<td>pCIB</td>
<td>Chimeric antibiotic resistance gene, contains either CaMV 19S or 35S promoters, contains a CaMV poly A site, contains the RK2 origin of replication</td>
<td>MCS</td>
<td>Kan</td>
<td>Chimeric Kan/Hyg</td>
<td>(Rothstein et al., 1987)</td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Replicon</td>
<td>Selection Markers</td>
<td>References</td>
<td></td>
</tr>
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<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>pMRK</td>
<td>pRi- based vector (borders from <em>Agrobacterium rhizogenes</em>), ColE1 from pUC19 for <em>E. coli</em> replication, pArA4a ori for replication in Agrobacterium, contains the 19S CaMV poly A site after the KanR gene</td>
<td>MCS, Kan/Amp, Kan</td>
<td>(Vilaine &amp; Casse-Delbart, 1987)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pOCA18</td>
<td>Designed for transferring plant genomic libraries, right border contains &quot;overdrive&quot; sequences, cos region from phage λ, ColE1 origin of replication for <em>E. coli</em>, RK2 origin of replication, supF gene for <em>E. coli</em> selection</td>
<td>MCS, Tet, Kan</td>
<td>(Olszewski <em>et al.</em>, 1988)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBIB/pBIG</td>
<td>Derivatives of pBIN and pBI vectors, contains pUC19 polylinker, nos promoter before MCS, pBIG contains the GUS gene as marker for selection of the GOI, contains the RK2 origin of replication</td>
<td>MCS, Kan, Kan/Hyg</td>
<td>(Becker, 1990)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTOK</td>
<td>&quot;Super-binary&quot; vector. Contains the genes for virB, virC and virG on the plasmid to increase the virulence of some <em>Agrobacterium</em> strains. Recombination required to introduce the GOI into the vector. Derived from pGA vector mentioned above, RK2 origin of replication, and cos region from phage λ.</td>
<td>Neither, Tet, Kan</td>
<td>(Komari, 1990)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCGN</td>
<td>ColE1 origin of replication in <em>E. coli</em>, pRiHRI origin for replication in <em>Agrobacterium</em>, MCS with blue/white selection, nptII expressed from either a CaMV35S or mannopine synthase promoters</td>
<td>MCS, Gent, Kan</td>
<td>(McBride &amp; Summerfelt, 1990)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTRA</td>
<td>Contains a CaMV 35S promoter with the nptII gene and a nos poly A signal. RK2 origin of replication.</td>
<td>Neither, Tet, Kan</td>
<td>(Ohshima <em>et al.</em>, 1990)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIG121</td>
<td>Derived from pBI, this vector contains two plant selectable markers on the left and right borders. There is a CaMV 35S promoter, β-glucuronidase gene with an intron to enhance expression followed by a nos poly A signal. RK2 origin of replication.</td>
<td>Neither, Kan, Kan/Hyg</td>
<td>(Hiei <em>et al.</em>, 1994; Ohta, 1990)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pED</td>
<td>Derived from pBIN described above. Lox sites surround the plant selectable markers with the firefly luciferase gene on the outside of these sites. Once Cre recombinase is present, the selectable markers are removed and only the luciferase remains. The Cre-recombinase is on a T-DNA also carrying Kan selection, genetic segregation in the next generation leads to plants with only Luciferase expressed. RK2 origin of replication.</td>
<td>Neither, Kan, Kan/Hyg</td>
<td>(Dale &amp; Ow, 1991)</td>
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<td></td>
</tr>
</tbody>
</table>

Continued on the following page.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>MCS</th>
<th>Tet/Gent</th>
<th>Kan/Strep</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>pLZO3</td>
<td>Modified from pOCA, designed to transfer large DNA sequences, cos region, CoIE1 origin of replication for <em>E. coli</em>, supF gene for <em>E. coli</em> selection, RK2 origin of replication</td>
<td>MCS</td>
<td>Tet/Gent</td>
<td>Kan/Strep</td>
<td>(Lazo et al., 1991)</td>
</tr>
<tr>
<td>pGPTV</td>
<td>Promoterless with MCS before a β-glucuronidase gene for use in fusion studies, contains a nos poly A site after the β-glucuronidase gene, derivatives of pBIN19, pBI and pBIB-Hyg, RK2 origin of replication</td>
<td>MCS</td>
<td>Kan</td>
<td>Kan/Hyg/Bar/bleo/Dhfr</td>
<td>(Becker et al., 1992)</td>
</tr>
<tr>
<td>pRD</td>
<td>Modified from pBin19 and pBI vectors to improve the nptII gene to increase expression. Contains the RK2 origin of replication, nos promoter and terminator with nptII gene. β-glucuronidase gene present with nos terminator either with or without a CaMV 35S promoter.</td>
<td>MCS</td>
<td>Kan</td>
<td>Kan</td>
<td>(Datla et al., 1992)</td>
</tr>
<tr>
<td>pART</td>
<td>CoIE1 replication of origin for <em>E. coli</em>, RK2 replication of origin for Agrobacterium, β-galactosidase for blue/white selection of binary, utilizes a <em>E. coli</em> vector (pART7) for cloning of GOI into MCS, with CaMV 35S promoter and poly A site. pART7 must be digested with Not1 to place in the pART binary vector</td>
<td>MCS in <em>E. coli</em> vector pART7</td>
<td>Spec/Strep</td>
<td>Kan</td>
<td>(Gleave, 1992)</td>
</tr>
<tr>
<td>pJJ or pSLJ</td>
<td>Modified from a pAGS vector and pRK290 described above, designed in tandem with a series of pUC plasmids for insertion of the GOI into the <em>E. coli</em> vectors, RK2 origin of replication.</td>
<td>MCS</td>
<td>Tet</td>
<td>Kan/Hyg/Bar/Strep/β-glucuronidase</td>
<td>(Jones et al., 1992)</td>
</tr>
<tr>
<td>pYS143</td>
<td>Derived from a pTOK vector. &quot;Super-binary&quot; vector, contains the genes for virB, virC and virG on the plasmid to increase the virulence of some Agrobacterium strains. CoIE1 origin of replication in <em>E. coli</em>, cos region of phage λ, and GOI introduced from recombination. RK2 origin of replication.</td>
<td>Neither</td>
<td>Spec/Tet</td>
<td>Kan</td>
<td>(Saito, 1992)</td>
</tr>
<tr>
<td>pGBK5</td>
<td>Designed primarily for T-DNA tagging, promoterless gusA gene for promoter studies, origin of replication is pRiA4, contains a nos poly A site after the gusA gene</td>
<td>Neither</td>
<td>Kan</td>
<td>Kan/Bar</td>
<td>(Bouchez, 1993)</td>
</tr>
<tr>
<td>pCLD04541</td>
<td>Derived from pSLJ vector listed above. Cos site from phage λ, as well as, the pBluescript polylinker. RK2 origin of replication.</td>
<td>MCS</td>
<td>Tet</td>
<td>Kan</td>
<td>(Bent et al., 1994)</td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Selection Markers</td>
<td>REFERENCES</td>
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<tr>
<td>pNFHK1</td>
<td>Derived from pBI, this vector contains plant selectable markers at both ends of the T-DNA so double selection assures complete T-DNA is incorporated, RK2 origin of replication, CaMV 35S promoter driving β-glucuronidase with rbcS-E9 terminator used to test the vector.</td>
<td>Neither</td>
<td>Kan/Kan/Hyg</td>
<td>(Bhattacharyya et al., 1994)</td>
<td></td>
</tr>
<tr>
<td>pMJD</td>
<td>35S promoter, TMV leader sequence as enhancer, nos poly A site, RK2 replication of origin</td>
<td>MCS</td>
<td>Kan/Kan</td>
<td>(Day, 1994)</td>
<td></td>
</tr>
<tr>
<td>pSR</td>
<td>Derivative of pPCV. Minimal vector meant to eliminate any unnecessary sequences present in the T-DNA region. RK2 origin of replication and ColE1 origin of replication in E. coli.</td>
<td>MCS</td>
<td>Amp/Kan</td>
<td>(During, 1994)</td>
<td></td>
</tr>
<tr>
<td>pPZP</td>
<td>CoIE1 origin of replication in E. coli, pVS1 origin of replication for Agrobacterium. E. coli vector is used to clone genes of interest (may vary depending on needs) into the binary vector which contains no promoter before the MCS</td>
<td>MCS</td>
<td>Spec/Chl/Kan/Gent</td>
<td>(Hajdukiewicz et al., 1994)</td>
<td></td>
</tr>
<tr>
<td>pTAB</td>
<td>Derived from pGA and pSLJ. Contains a single EcoRI site for cloning in expression cassettes from other vectors. RK2 origin of replication.</td>
<td>Neither</td>
<td>Tet/Bar</td>
<td>(Tabe et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>pBINPLUS</td>
<td>Based on pBIN, MCS moved to right border, β-galactosidase for blue/white screening, addition of PacI and AscI sites for cloning with E. coli vector pUCAP. ColE1 origin of replication in E. coli, RK2 origin of replication in Agrobacterium.</td>
<td>MCS</td>
<td>Kan/Kan</td>
<td>(van Engelen et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>pSB, pNB, pGA, pTOK</td>
<td>Called &quot;super-binary vectors&quot;. Pair-wise vectors, one containing a drug resistance and the other containing the GOI. The two must be transformed into plants together to have both. Cos site from phage λ, CoIE1 origin of replication in E. coli, RK2 origin of replication, Vir genes present on the vector to promote transformation, β-glucuronidase was used as a test GOI. pTOK series modified from earlier work cited above.</td>
<td>MCS</td>
<td>Spec/Tet/Hyg/Kan/β-glucuronidase</td>
<td>(Komari et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>pGPTV- Asc</td>
<td>pGPTV is modified to include a AscI site in the MCS. This site corresponds to the E. coli vectors pRT-Ω/Not/Asc or pHis-Ω/Not/Asc to use as intermediary vectors ligated into pGPTV vector, all other features the same, see entry above.</td>
<td>MCS</td>
<td>Kan/Kan/Hyg/Bar/bleo/Dhfr</td>
<td>(Uberlacker, 1996)</td>
<td></td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Selectable Markers</td>
<td>References</td>
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<tr>
<td>pJD303</td>
<td>Modified from pTAB10, designed to work with a series of specially designed expression vector cassettes ligated into the backbone at specific EcoR1 or Not1 sites. RK2 origin of replication.</td>
<td>Neither</td>
<td>Tet, Bar</td>
<td>(de Majnik, 1997)</td>
<td></td>
</tr>
<tr>
<td>MAT</td>
<td>Derived from pBl, this vector contains a isopentenyl transferase (ipt) within a maize transposable element Ac. Once the plant is selected after transformation, the Ac element is utilized to remove the ipt gene. As the Ac element does not re-insert all the time, only those were it is absent, are selected further. This is another means of removing a selectable marker from the transgenic plants. Contains the RK2 origin of replication.</td>
<td>Neither</td>
<td>Kan</td>
<td>Ipt/Kan/β-glucuronidase</td>
<td>(Ebinuma et al., 1997)</td>
</tr>
<tr>
<td>BIBAC</td>
<td>Designed to transfer large DNA fragments, F origin of replication from the F plasmid of E. coli, Ri origin of replication for Agrobacterium, Origin of transfer from the RK2 plasmid, double CaMV 35S promoters, Alfalfa mosaic virus Enhancer, contains sacB as a marker for selection of inserts.</td>
<td>MCS</td>
<td>Kan</td>
<td>Hyg</td>
<td>(Hamilton, 1997)</td>
</tr>
<tr>
<td>pBECKS</td>
<td>Based on pBIN, Members of this series contain either a 35S promoter or β-galactosidase for blue/white screening, AMV leader as an enhancer by 35S promoter, poly-A terminator sequences, variable bacterial selection, variable plant selection, TMV leader sequence incorporated into the nos cassette from pBIN19, RK2 origin of replication, some contain the ColE1 origin of replication for E. coli.</td>
<td>Neither</td>
<td>Kan/Amp/Gent/Spect</td>
<td>Kan/Bar/Hyg or β-glucuronidase gene/Anthocyanin accumulation</td>
<td>(McCormac et al., 1997)</td>
</tr>
<tr>
<td>pDM805</td>
<td>Derived from pTAB described above. Described as a transformation vector for cereals, this plant uses two selectable markers, β-glucuronidase driven by a rice actin promoter with a rice rubisco poly A sequence and Bar gene driven by a maize ubiquitin promoter and followed by a nos poly A sequence. ColE1 origin of replication in E. coli and RK2 origin of replication.</td>
<td>Neither</td>
<td>Amp/Tet</td>
<td>Bar/β-glucuronidase</td>
<td>(Tingay et al., 1997)</td>
</tr>
<tr>
<td>pWBVec1</td>
<td>Basic system with only left and right border sequences. Meant to be used with a series of expression cassettes which are digested and ligated into the T-DNA region. Expression cassettes contain either CaMV 35S or maize ubiquitin promoter to drive the plant selectable markers. ColE1 origin of replication in E. coli and RK2 origin of replication.</td>
<td>MCS</td>
<td>Spec/Strep</td>
<td>Hyg/Bar</td>
<td>(Wang, 1998, 1997)</td>
</tr>
</tbody>
</table>

Continued on the following page.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>MCS</th>
<th>Selection Markers</th>
<th>Reporters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pART54/ pCre1</strong></td>
<td>Derived from pART described above, for use in Cre-recombinase systems. pART54 expresses the plant selectable marker with a conditional lethal gene (codA) surrounded by lox P sites. β-glucuronidase is expressed on the same T-DNA. A second vector, pCre1 contains cre-recombinase and a hygromycin resistance gene. These vectors in tandem are meant to select a progeny plant containing a GOI which is missing the plant selectable marker without sexual recombination. ColE1 origin of replication in <em>E. coli</em> and RK2 origin of replication.</td>
<td>Neither</td>
<td>Spec/Strep</td>
<td>Kan/Hyg/β-glucuronidase</td>
</tr>
<tr>
<td><strong>pSK1</strong></td>
<td>Left and right borders from pGA vector mentioned above, Ri origin of replication, ColE1 origin of replication in <em>E. coli</em>, contains sites for gene insertion between a CaMV 35S promoter and a nopaline synthase poly A signal.</td>
<td>MCS</td>
<td>Kan</td>
<td>Hyg</td>
</tr>
<tr>
<td><strong>pYLTAC</strong></td>
<td>Designed for large DNA sequence transfer, derivative of pOCA and pGA. Contains right border &quot;overdrive&quot; sequences. Contains sacB as a marker for selection of inserts pRiA4 origin of replication in Agrobacterium. PI bacteriophage replicon for replication in <em>E. coli</em>.</td>
<td>MCS</td>
<td>Kan</td>
<td>Hyg</td>
</tr>
<tr>
<td><strong>MAT version2</strong></td>
<td>Derived from the MAT system described above, utilizing the ipt gene as a selectable marker. Instead of Ac system in previous system, this vector system uses the R/RS system of recombination to remove the selectable marker ipt. In pRP1132, Rs sites flank the R recombinase and the ipt gene. In pRZK1MTPTGSTUS, RS sites flank the ipt and Kan genes and the R recombinase is added on a separate T-DNA. A second improvement later included a chemically inducible GST-II-27 promoter from maize to regulate the R recombinase on T-DNA vector pMAT8.</td>
<td>MCS</td>
<td>Kan</td>
<td>Ipt/Kan/β-glucuronidase</td>
</tr>
<tr>
<td><strong>pCB</strong></td>
<td>Redesigned pBIN19 to be smaller, most contain multiple MCSs, GFP or β-glucuronidase fusions of GOI, double or single CaMV 35S promoter, Maize ubiquitin promoter, TMV leader sequence, mitochondrial or plastid targeting sequences in some, RK2 origin or replication, either CaMV poly A terminator or nos poly A terminator.</td>
<td>MCS</td>
<td>Kan</td>
<td>Kan/Bar</td>
</tr>
</tbody>
</table>

Continued on the following page.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>MCS</th>
<th>Selectable Marker</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGREEN</td>
<td>Replicates in Agrobacterium only in the presence of a helper plasmid pSOUP. β-galactosidase gene around MCS for blue/white selection, pSa origin of replication. Also constructed a number of cassettes intended to be inserted into the MCS to allow for different plant selection/reporter gene (β-glucuronidase, luciferase or GFP)/and promoter constructions (CaMV 35S or nos) depending on needs.</td>
<td>Kan/Tet</td>
<td>Kan/Hyg/Sul/Bar</td>
<td>(Hellens et al., 2000b) <a href="http://www.pgreen.ac.uk">http://www.pgreen.ac.uk</a></td>
</tr>
<tr>
<td>pTN</td>
<td>Derived from pPZP described above. Dual binary system, vector contains two T-DNA regions for transfer of two genes from the same backbone. One T-DNA region contains the plant selectable marker and the other region contains β-glucuronidase. Both driven by a CaMV 35S promoter with either a TMV or TEV leader as an enhancer. ColE1 origin of replication in E. coli, pSa origin of replication in Agrobacterium.</td>
<td>Neither</td>
<td>Spec/Strep</td>
<td>Bar/β-glucuronidase</td>
</tr>
<tr>
<td>pER8</td>
<td>Derivative of pPZP described above. This vector has a promoter that is estrogen-inducible. The promoter causes the transcription of a LexA DNA binding domain, an acidic transactivating domain of VP16 and the carboxyl region of the human estrogen receptor. Once activated the regulatory elements bind to the LexA operator sequence which before a MCS an a rubisco small subunit poly A sequence. ColE1 origin of replication in E. coli, pVS1 origin of replication in Agrobacterium.</td>
<td>MCS</td>
<td>Spec</td>
<td>Hyg</td>
</tr>
<tr>
<td>pHELLSGATE</td>
<td>Derived from pART described above. For use in RNAi experiments, contains a gateway cassette in two orientations with an intron in between. Gateway cassettes are driven by a CaMV 35S promoter. ColE1 replication of origin for E. coli, RK2 replication of origin for Agrobacterium.</td>
<td>Gateway</td>
<td>Spec</td>
<td>Kan</td>
</tr>
<tr>
<td>pPZP-RCS2</td>
<td>Derived from pPZP mentioned above. Between left and right borders are a series of 24 unique restriction sites including 5 homing endonuclease sites. Designed to be used with expression cassette vectors that have these sites. ColE1 origin of replication in E. coli, pVS1 origin of replication in Agrobacterium.</td>
<td>MCS</td>
<td>Spec</td>
<td>Kan/Gent</td>
</tr>
<tr>
<td>pGD</td>
<td>Derivatives of pCAMBIA1301. Vectors for tagging proteins with GFP or DsRed2. CaMV 35S promoter before fluor and MCS. Meant to be used in transient infiltration assays. pVS1 origin of replication in Agrobacterium, ColE1 origin of replication in E. coli.</td>
<td>MCS</td>
<td>Kan</td>
<td>None</td>
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</tbody>
</table>

Continued on the following page.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Promoters</th>
<th>Replication Origins</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATEWAY</td>
<td>Derived from pPZP mentioned above, ColE1 origin of replication in <em>E. coli</em>, pVSI origin of replication in <em>Agrobacterium</em>. Designed to fuse the Gateway cassette with either β-glucuronidase or GFP. Also designed were silencing constructs.</td>
<td>Gateway</td>
<td>Spec</td>
<td>Kan/Hyg/Bar</td>
</tr>
<tr>
<td>pCAMBIA</td>
<td>Derived from pPZP mentioned above. Large series of vectors under continual development, many include a double CaMV 35S promoter driving a β-glucuronidase gene or GFP, some have no promoter to use in promoter studies. ColE1 origin of replication in <em>E. coli</em>, pVSI origin of replication in <em>Agrobacterium</em>.</td>
<td>MCS</td>
<td>Kan/Chl</td>
<td>Kan/Hyg/Bar</td>
</tr>
<tr>
<td>pMDC</td>
<td>Derivatives of pCAMBIA series and pER8. Includes vectors for promoter studies, fusion studies with GFP and β-glucuronidase, and studies with alternative promoters, heat shock protein (hsp) and G10-90 the estrogen-inducible promoter. A double CaMV 35S promoter with a nos poly A sequence is present in other members of this vector series. Many of the vector constructs are available in three reading frames. ColE1 origin of replication in <em>E. coli</em>, pVSI origin of replication in <em>Agrobacterium</em>.</td>
<td>Gateway</td>
<td>Kan/Chl</td>
<td>Kan/Hyg/Bar</td>
</tr>
<tr>
<td>pYLTAC747</td>
<td>Derivative of pYLTAC described above. Works with donor vectors pYLSV and pYLVS. This system uses a Cre-lox recombination system in order to stack multiple DNA sequences into the same vector backbone. Vector backbones are removed after each addition of genes with rare endonucleases I-SceI and PI-Sce-I. P1 origin of replication in <em>E. coli</em> and Ri origin of replication in <em>Agrobacterium</em>.</td>
<td>MCS</td>
<td>Kan</td>
<td>None</td>
</tr>
<tr>
<td>pAGRIKOLA</td>
<td>Derivative of pGREEN. Silencing vector with double gateway cassette for the creation of a hairpin construct with two intron sequences between the cassettes. Driven by a CaMV 35S promoter and contains an octopine synthase poly A sequence. ColE1 origin of replication in <em>E. coli</em> and pSA origin of replication in <em>Agrobacterium</em>.</td>
<td>Gateway</td>
<td>Kan</td>
<td>Bar</td>
</tr>
</tbody>
</table>

Continued on the following page.
<table>
<thead>
<tr>
<th>Vector Name</th>
<th>Description</th>
<th>Mendel</th>
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<tr>
<td>pJCGLOX</td>
<td>Derived from pCAMBIA. Utilizes the Cre-lox recombination system, with a heat inducible promoter driving a ligand-inducible CRE recombinase and lox sites surrounding the EGFP. The CRE recombinase coding region includes an intron to prevent expression in bacteria. Once recombination occurs the GOI in the gateway cassette region has a CaMV 35S promoter and a octopine synthase poly A sequence. CoLE1 origin of replication in <em>E. coli</em>, pVS1 origin of replication in <em>Agrobacterium</em>.</td>
<td>Gateway</td>
<td>Chl</td>
<td>Kan</td>
<td>(Joubes et al., 2004)</td>
</tr>
<tr>
<td>pSTARGATE/pWATERGATE</td>
<td>Derived from pHELLSGATE. For use in RNAi experiments, contains a gateway cassette in two orientations with an intron in between. Gateway cassettes are driven by either a Ubiquitin promoter and intron or Arabidopsis rubisco promoter (ARbcS). The Ubiquitin promoter is meant to be used in monocot systems. CoLE1 replication of origin for <em>E. coli</em>, RK2 replication of origin for <em>Agrobacterium</em>.</td>
<td>Gateway</td>
<td>Spec</td>
<td>Hyg/Kan</td>
<td><a href="http://www.pi.csiro.au/rnai/vectors.htm">http://www.pi.csiro.au/rnai/vectors.htm</a></td>
</tr>
<tr>
<td>pRCS2-ocs</td>
<td>Derived from pPZP-RCS2, designed to work with a series of expression cassette vectors named pSAT. <em>E. coli</em> pSAT vectors have a variety of promoter sequences including actin (act), manopine synthase (mas), nopaline synthase (nos), rubisco small subunit (rbc), and a tandem CaMV 35S promoter. There are also a variety of terminator sequences including the the poly A sequences from ocs, nos, mas, 35S, rbc and agropine synthase (ags). TEV leader sequences present only with 35S promoter. Fusion to flours CFP, GFP, YFP, citrine-YFP and RFP is available in two orientations. Also available are a series of silencing vectors with the same promoters listed above. CoLE1 origin of replication in <em>E. coli</em>, pVS1 origin of replication in <em>Agrobacterium</em>.</td>
<td>MCS</td>
<td>Spec/Strep</td>
<td>Kan/Hyg/Bar</td>
<td>(Chung et al., 2005; Dafny-Yelin et al., 2007; Tzfira et al., 2005)</td>
</tr>
<tr>
<td>p*</td>
<td>Derived from pPZP described above. A series of Gateway Binary Vectors to allow for Multi-site gateway recombination. One set has a CaMV 35S promoter before and fluor either before or after the gateway site. The flours for fusion include GFP, CFP, YFP and RFP. CoLE1 origin of replication in <em>E. coli</em>, pVS1 origin of replication in <em>Agrobacterium</em>.</td>
<td>Gateway</td>
<td>Spec</td>
<td>Kan/Hyg/Bar</td>
<td>(Karimi et al., 2005)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Origin of Replication</th>
<th>Selectable Marker</th>
<th>Additional Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pFGC</strong>/ <strong>pGSA</strong>/ <strong>pMCG</strong></td>
<td>Derived from pCAMBIA. These vectors are designed for silencing applications. Inverted repeat sequences are driven by a CaMV 35S promoter and a Chalcone syntase A intron to stabilize the inverted repeat and Octopine synthase poly A sequence. <strong>pMCG</strong> was designed for monocots and contains a maize ubiquitin promoter. <strong>pVS1</strong> origin of replication in Agrobacterium, CoIE1 origin of replication in <em>E. coli</em>.</td>
<td>MCS</td>
<td>Chl/Kan</td>
<td>Hyg/Bar</td>
</tr>
<tr>
<td><strong>pMOA</strong></td>
<td>Derivatives of pSR, these are minimal T-DNA region vectors designed to eliminate any unnecessary sequences. The selectable marker is under control of the nos or CaMV 35S promoter and contains nos poly-A terminator site. There is a MCS present towards the right border of the vectors to facilitate cloning of GOI. <strong>RK2</strong> origin of replication and CoIE1 origin of replication in <em>E. coli</em>.</td>
<td>MCS</td>
<td>Spec/Amp</td>
<td>Kan/Bar/Bleo/ dhfr/Hyg</td>
</tr>
<tr>
<td><strong>pGWTAC</strong></td>
<td>Derived from pYLTAC747. Contains a gateway cassette and a loxP site for recombination between the left and right borders to facilitate multiple genes cloned into the T-DNA region. Also contains a I-SceI site beside the loxP site for the removal of contaminating sequences. <strong>P1</strong> origin of replication in <em>E. coli</em> and Ri origin of replication in <em>Agrobacterium</em>.</td>
<td>Gateway</td>
<td>Kan</td>
<td>Chl</td>
</tr>
<tr>
<td><strong>pEarleyGate</strong></td>
<td>Derived from pFGC. Gateway compatible vectors for gene fusions to YFP, CFP, GFP and epitope tags HA, 6xHis, Flag, c-Myc, AcV5 and Tap. Fusions are driven by a CaMV 35S promoter and finish with a octopine synthase poly A sequence. <strong>pVS1</strong> origin of replication in <em>Agrobacterium</em>, CoIE1 origin of replication in <em>E. coli</em>.</td>
<td>Gateway</td>
<td>Kan</td>
<td>Bar</td>
</tr>
<tr>
<td><strong>pGPro</strong></td>
<td>Derived from pGreen and pCAMBIA described above. This vector is designed to test novel promoter sequences in monocots. Hyg resistance is driven by a rice actin promoter surrounded by loxP sites for Cre-lox recombination to remove the selectable marker. GFP/β-glucuronidase dual fusion to the MCS for the determination of the promoter function. Two left borders present to decrease vector backbone contamination. CoIE1 origin of replication in <em>E. coli</em>, pSa origin of replication.</td>
<td>MCS</td>
<td>Kan</td>
<td>Hyg</td>
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Table 1.2 Continued
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<th>Vector</th>
<th>Description</th>
<th>Gateway</th>
<th>Spec/Strep/Chl</th>
<th>Kan</th>
<th>Reference</th>
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<tr>
<td>pSITE</td>
<td>Derived from pPZP and pSAT series described above. The pSAT was converted to Gateway and placed in the pPZP background. Fluors available for fusion are GFP, RFP, YFP and CFP in two orientations relative to the gateway cassette. The fusion is driven from a tandem CaMV 35S promoter with a TEV leader sequence. There is a 35S poly A sequence after the fusion. CoE1 origin of replication in E. coli and a pVS1 origin of replication in Agrobacterium.</td>
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<td></td>
<td>(Chakrabarty et al., 2007)</td>
</tr>
<tr>
<td>pORE</td>
<td>Derived from pCB described above. A set of binary vectors which have various combinations of the promoters (tobacco cryptic constitutive promoter, Arabidopsis thaliana hydroperoxide lyase promoter, and Triticum aestivum lipid transfer promoter fused to an alcohol dehydrogenase intron), selectable markers (Kan or Basta) or reporter genes (β-glucuronidase or GFP). FRT recombination sites flank the selectable marker cassette for excision by FLP recombinase. CoE1 origin of replication in E. coli, RK2 origin of replication.</td>
<td></td>
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<td></td>
<td>(Coutu et al., 2007)</td>
</tr>
<tr>
<td>pMSP</td>
<td>Derived from pGPTV. Contains a trimer of octopine synthase transcriptional activating element and a mnnopine synthase2' activator-promoter region to form a &quot;super-promoter&quot;. A maize adh1 intron or a TEV leader as an enhancer may be present, also an agropine or nopaline synthase poly A signal. RK2 origin of replication.</td>
<td></td>
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<td></td>
<td>(Lee et al., 2007)</td>
</tr>
<tr>
<td>pCB20--/ pCB308</td>
<td>Derived from pCAMBIA. CaMV 35S promoter, Rice actin promoter, two stress-inducible rice promoters, SalT and Hav22, Arabidopsis gamma-glutamyltransferase 2 gene promoter, Arabidopsis RD29A promoter, and Arabidopsis glutamate dehydrogenase gene promoter are all available for use before the Gateway cassette. EGFP and β-glucuronidase available as fusion markers to the GOI. Some vectors designed to create an inverted repeat after Gateway reaction for silencing applications. CoE1 origin of replication in E. coli, pVS1 origin of replication.</td>
<td>Gateway</td>
<td></td>
<td></td>
<td>(Lei et al., 2007)</td>
</tr>
<tr>
<td>pGWB</td>
<td>Derived from pBI, contains a gateway cassette and a variety of available tags: β-glucuronidase, luciferase, GFP, YFP, CFP, 6xHis, FLAG, 3xHA, 4xMyc, 10xMyc, GST, and TAP. Vectors contain either no promoter or a CaMV 35S. Contains the RK2 origin of replication.</td>
<td>Gateway</td>
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<td>(Nakagawa et al., 2007)</td>
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Table 1.2 Continued
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<th>Vector</th>
<th>Description</th>
<th>MCS/TAG</th>
<th>Selection Markers</th>
<th>Reference</th>
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</thead>
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<tr>
<td>pCLEAN</td>
<td>Derived from pGreen/pSoup system described above. pSa origin of replication. Part of a dual vector system where two vectors are required for transformation. Both of the pCLEAN vectors work with the pGreen/pSoup vectors as well. The left border has been doubled in some vectors to promote precise integration. Some vectors include virG gene to increase transformation efficiency.</td>
<td></td>
<td>Hyg/GFP/ β-glucuronidase</td>
<td>(Thole et al., 2007)</td>
</tr>
<tr>
<td>pBINPLUS/ARS</td>
<td>Derived from pBINPLUS, this vector is modified to be usable to include different regulatory sequences flanked by rare cutting sites for removal and replacement if necessary. Potato Ubi3 gene sequences used as promoter and poly A sequences B-galactosidase for blue/white selection in bacteria. CoIE1 origin of replication in E. coli, RK2 origin of replication.</td>
<td></td>
<td>Kan/Kan</td>
<td>(Belknap et al., 2008)</td>
</tr>
<tr>
<td>pG</td>
<td>Derived from pGreen and modified to include Gateway® and and the tags CFP, YFP and GFP. System also includes a number of expression vectors that are not binary vectors. Either a CaMV 35 S or no promoter available. pSa origin of replication.</td>
<td>Gateway</td>
<td>Bar/Hyg/ Kan</td>
<td>(Zhong et al., 2008)</td>
</tr>
<tr>
<td>pCX, pX</td>
<td>Derived from pCAMBIA. This is a set of expression vectors which utilize TA cloning to insert the GOI into the vector. CaMV 3SS and maize ubiquitin-1 promoter drive the TA region with fusions to FLAG, HA, Myc, GFP, DsRed and β-glucuronidase. Promoter-less cassettes for promoter studies. pVS1 origin of replication in Agrobacterium, CoIE1 origin of replication in E. coli.</td>
<td>Neither</td>
<td></td>
<td>(Chen et al., 2009)</td>
</tr>
<tr>
<td>pSITEII</td>
<td>Modification of the pSITE vectors described above. These vectors contain a variety of newer flours for fusion to a GOI. These include Cerulean, Venus, TagRFP, Dendra2, Dronpa, miCy and mKO. There are unique restriction sites surrounding the flours to facilitate their removal and replacement with any other fluor or GOI in the pSAT background. There is a tandem CaMV 3SS promoter with a TEV leader sequence and CaMV poly A sequence. pVS1 origin of replication in Agrobacterium, CoIE1 origin of replication in E. coli.</td>
<td>Gateway</td>
<td>Spec/Strep/ Chl Kan</td>
<td>(Martin et al., 2009)</td>
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<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Promoters</th>
<th>MCS</th>
<th>Spec/Tet</th>
<th>Kan/Hyg</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pZK, pZH</td>
<td>Derived from pPZP or pTRA described above. Promoters included in the vector set are rice polyubiquitin1, 10kD prolamin and the 13kDa prolamin clone RM1. The 3rd intron of aspartic protease (RAP int) is also included in many constructions. Poly A sequences corresponding to the promoters used to form cassettes around GOI. Cassettes containing GFP or β-glucuronidase are also included. Set designed to incorporate a series of expression cassettes into the same T-DNA region. pPZP based vectors have a pVS1 origin of replication in Agrobacterium and a ColE1 origin of replication in E. coli, pTRA based vectors have a RK2 origin of replication.</td>
<td>pPZP</td>
<td>MCS</td>
<td>Spec/Tet</td>
<td>Kan/Hyg</td>
<td>(Kuroda et al., 2010)</td>
</tr>
<tr>
<td>pEAQ</td>
<td>Derived from pBINPLUS mentioned above, these vectors are modified to delete any unnecessary sequences. Vectors contain the CaMV 35S promoter, CPMV RNA-2 5'UTR, CPMV RNA-2 3'UTR and nos poly A signal. Some vectors come with an incorporated P19 as a silencing suppressor against the high levels of expression. Fusions to His also allow for protein tagging. Some members have an MCS, others have a gateway cassette. RK2 origin of replication, ColE1 origin of replication in E. coli.</td>
<td>pEAQ</td>
<td>Both</td>
<td>Kan</td>
<td>Kan</td>
<td>(Sainsbury et al., 2010)</td>
</tr>
<tr>
<td>RNA Viruses</td>
<td>Host Range (Family)*</td>
<td>References</td>
<td></td>
<td></td>
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<tr>
<td>*Alternanthera mosaic virus (AltMV)</td>
<td><em>Potexvirus</em> Portulacaceae, Polemoniaceae, Lamiaceae, Acanthaceae, Apocynaceae, Solanaceae</td>
<td>(Lim &lt;i&gt;et al.&lt;/i&gt;, 2010)</td>
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<tr>
<td>Apple spherical latent virus (ASLV)</td>
<td><em>Cheravirus</em> Brassicaceae, Cucurbitaceae, Fabaceae, Solanaceae</td>
<td>(Igarashi &lt;i&gt;et al.&lt;/i&gt;, 2009)</td>
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<tr>
<td>Barley stripe mosaic virus (BSMV)</td>
<td><em>Hordeivirus</em> Amaranthaceae, Poaceae, Solanaceae</td>
<td>(Holzberg &lt;i&gt;et al.&lt;/i&gt;, 2002)</td>
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<tr>
<td>Bean pod mottle virus (BPMV)</td>
<td><em>Comovirus</em> Fabaceae</td>
<td>(Zhang &amp; Ghabrial, 2006; Zhang &lt;i&gt;et al.&lt;/i&gt;, 2009)</td>
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<tr>
<td>Brome mosaic virus (BMV)</td>
<td><em>Bromovirus</em> Poaceae</td>
<td>(Ding &lt;i&gt;et al.&lt;/i&gt;, 2006)</td>
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<tr>
<td>Cucumber mosaic virus (CMV)</td>
<td><em>Cucumovirus</em> Amaranthaceae, Apocynaceae, Chenopodiaceae, Compositae, Convolvulaceae, Cruciferae, Cucurbitaceae, Fabaceae, Malvaceae, Phytolaccaceae, Polygonaceae, Scrophulariaceae, Solanaceae, Tetragniaceae, Tropaeolaceae, Umbelliferae. (ICTVdB_Management, 2006c; Nagamatsu &lt;i&gt;et al.&lt;/i&gt;, 2007)</td>
<td></td>
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<td>*Grapevine virus A (GVA)</td>
<td><em>Vitivirus</em> Vitaceae, Solanaceae</td>
<td>(Muruganantham &lt;i&gt;et al.&lt;/i&gt;, 2009)</td>
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<td>*Pea early browning virus (PEBV)</td>
<td><em>Tobravirus</em> Fabaceae, Solanaceae</td>
<td>(Constantin &lt;i&gt;et al.&lt;/i&gt;, 2004)</td>
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<td>*Poplar mosaic virus (PopMV)</td>
<td><em>Carlavirus</em> Fabaceae, Salicaceae, Solanaceae</td>
<td>(Naylor &lt;i&gt;et al.&lt;/i&gt;, 2005)</td>
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<tr>
<td>*Potato virus X (PVX)</td>
<td><em>Potexvirus</em> Amaranthaceae, Cruciferae, Solanaceae</td>
<td>(Ruiz &lt;i&gt;et al.&lt;/i&gt;, 1998)</td>
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<tr>
<td>*Sunn hemp mosaic virus (SHMV)</td>
<td><em>Tobamovirus</em> Fabaceae, Solanaceae</td>
<td>(Liu &amp; Kearney, 2010)</td>
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<tr>
<td>*Tobacco mosaic virus (TMV)</td>
<td><em>Tobamovirus</em> Chenopodiaceae, Compositae, Cucurbitaceae, Fabaceae, Papaveraceae, Solanaceae</td>
<td>(ICTVdB_Management, 2006a; Kumagai &lt;i&gt;et al.&lt;/i&gt;, 1995)</td>
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<tr>
<td>Virus Name</td>
<td>Genus</td>
<td>Host Range</td>
<td>Reference(s)</td>
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<tr>
<td>*Tobacco rattle virus (TRV)</td>
<td>Tobraivirus</td>
<td>Alliaceae, Amaranthaceae, Amaryllidaceae, Apocynaceae, Boraginaceae, Campanulaceae, Caryophyllaceae, Chenopodiaceae, Compositae, Cruciferae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Gramineae, Hyacinthaceae, Labiatae, Liliaceae, Linaceae, Phytolaccaceae, Scrophulariaceae, Solanaceae, Tropaeolaceae, Umbelliferae, Violaceae</td>
<td>(ICTVdB_Management, 2006b; Liu et al., 2002a; Liu et al., 2002b; Ratcliff et al., 2001)</td>
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<td>Turnip yellow mosaic virus (TYMV)</td>
<td>Tymovirus</td>
<td>Brassicaceae, Fabaceae</td>
<td>(Pflieger et al., 2008)</td>
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<td><strong>Satellite Viruses</strong></td>
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<td>*Satellite tobacco mosaic virus</td>
<td>RNA satellite</td>
<td>Solanaceae</td>
<td>(Gossele et al., 2002)</td>
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<tr>
<td>*Tobacco curly shoot virus</td>
<td>DNA satellite</td>
<td>Solanaceae</td>
<td>(Huang et al., 2009)</td>
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<td>*Tomato yellow leaf curl China virus</td>
<td>DNA satellite</td>
<td>Solanaceae</td>
<td>(Tao &amp; Zhou, 2004)</td>
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<tr>
<td><strong>DNA Viruses</strong></td>
<td></td>
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<tr>
<td>*Abutilon mosaic virus (AbMV)</td>
<td>Geminivirus</td>
<td>Cucurbitaceae, Fabaceae, Malvaceae, Solanaceae</td>
<td>(Krenz et al., 2010)</td>
<td></td>
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<tr>
<td>African cassava mosaic virus (ACMV)</td>
<td>Geminivirus</td>
<td>Euphorbiaceae, Solanaceae</td>
<td>(Fofana et al., 2004)</td>
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<td>Cabbage leaf curl virus (CaLCuV)</td>
<td>Geminivirus</td>
<td>Brassicaceae</td>
<td>(Muangsan et al., 2004)</td>
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<td>Cotton leaf crumple virus (CLCrV)</td>
<td>Geminivirus</td>
<td>Malvaceae, Solanaceae</td>
<td>(Tuttle et al., 2008)</td>
<td></td>
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<td></td>
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<tr>
<td>*Rice tungro bacilliform virus (RTBV)</td>
<td>Tungrovirus</td>
<td>Poaceae</td>
<td>(Purkayastha et al., 2010)</td>
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<td>Tomato golden mosaic virus (TGMV)</td>
<td>Geminivirus</td>
<td>Solanaceae</td>
<td>(Kjemtrup et al., 1998; Peele et al., 2001)</td>
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<tr>
<td>*Tomato leaf curl virus (TLCV)</td>
<td>Geminivirus</td>
<td>Solanaceae</td>
<td>(Pandey et al., 2009)</td>
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</table>

*-indicates this virus has been developed for Agrobacterium transformation.
Figure 1.1. Schematic diagram of the *Agrobacterium tumefaciens* Ti plasmid. Included are the virulence region containing the Vir genes, the origin of replication, the opine catabolism genes, the left and right borders and the native genes found between the left and right borders. The abbreviations are as follows: tml, tumor morphology large; tmr, tumor morphology roots; tms, tumor morphology shoots; and ocs, the octopine synthase gene.
Figure 1.2. Schematic diagram of A. tumefaciens transfer of DNA. The process begins with wounding and the release of phenolics. These compounds are recognized by virA, which then phosphorylates virG which binds to the Vir genes and synthesis of the remaining vir genes begins. VirD1 and D2 bind to the right border of the T-DNA region, D2 knicks the T-DNA and binds to this end. This complex binds VirE2 after entering the plant cell and then is imported into the nucleus of the plant cell. Once in the nucleus, the T-DNA is incorporated into the plant cell chromosome.
Figure 1.3. Schematic diagram of the vector providing the first expression of a transgene in plants. Kanamycin resistance replaces the native Agrobacterium genes between the left and right borders, but the remainder of the plasmid is wildtype as shown in Figure 1.1.
Figure 1.4. Schematic diagram of pBIN19. Between the left and right borders, kanamycin resistance in plants is conferred by nptII, blue/white selection is provided by the interruption of the lacZ gene with the insertion of the GOI into the provided sites. There is also a bacterial kanamycin resistance gene and an origin of replication (not shown). P= promoter, T= terminator, both originally regulating the nopaline synthase gene (Bevan, 1984).

Figure 1.5. Schematic diagram of pBINPLUS. Between the left and right borders, kanamycin resistance in plants is conferred by nptII, blue/white selection is provided by the interruption of the lacZ gene with the insertion of the GOI into the provided sites. There is also a bacterial kanamycin resistance gene and an origin of replication (not shown). P= promoter, T= terminator, both originally regulating the nos gene (van Engelen et al., 1995).
Figure 1.6. Schematic diagram of pED53:pED37 and pBIN19:pED23 Cre-lox system. This is a two plasmid system, the top plasmid contains from left to right, a lox P site before a hygromycin resistance gene cassette driven by a CaMV 35S promoter, a bacterial ampicillin resistance gene, a second loxP site, a luciferase cassette also driven by the CaMV 35S promoter. T= nos poly-A site. The bottom plasmid was modified from pBIN19 containing a Cre recombinase gene driven by a CaMV 35S promoter. There is also a kanamycin resistance gene for selection in plants. P= nos promoter, T= nos poly A site. Both vectors utilize kanamycin resistance in bacteria and have an origin of replication for replication in E. coli and Agrobacterium (not shown)(Dale & Ow, 1990, 1991).
Figure 1.7. Schematic diagram of pBI. Between the left and right borders: kanamycin resistance in plants is conferred by nptII, the insertion of the GOI into the MCS, and the β-glucuronidase gene. There is also a bacterial kanamycin resistance gene and an origin of replication (not shown). Pictured above the MCS are the CaMV 35S and ribulose bisphosphate carboxylase (rbcS) promoters used in the original study that replace the MCS. P = nos promoter, T = nos poly A site (Jefferson, 1987).

Figure 1.8. Schematic diagram of pCIB770. Between the left and right borders: kanamycin resistance in plants is conferred by nptII, a CaMV 35S before a terminator sequence. There is also a bacterial kanamycin resistance gene and an origin of replication. P = nos promoter, T = nos poly A site (Rothstein et al., 1987).
Figure 1.9. A schematic diagram of pER8. G10-90, a synthetic promoter drives the expression of a chimeric transcription factor composed of a LexA DNA-binding domain, the transcription activation domain of VP16, and the regulatory region of the human estrogen receptor. There is a hygromycin plant selectable marker. The GOI is inserted after a LexA binding site and a CaMV 35S promoter. T= terminator sequence. The terminator sequences are as follows: hygromycin resistance, nopaline synthase poly-A sequence, XVE, ribulose bisphosphate carboxylase E9 poly-A sequence and the GOI, a ribulose bisphosphate carboxylase 3A poly-A sequence. This vector contains spectinomycin resistance in bacteria and origins of replication for E. coli and A. tumefaciens (not shown)(Zuo et al., 2000).

Figure 1.10. Schematic diagram of pSITEII-7C1. From the left to the right border, a kanamycin resistance gene (nptII) gene flanked by a octopine synthase promoter (P) and terminator (T), two CaMV 35S promoters and a Tobacco etch virus enhancer driving the expression of a green/red photoactivable fluor, dendra, a Gateway® cassette (attR1, chloramphenicol resistance gene (chl), the ccdB gene, and an attR2 site) and a CaMV 35S poly A sequence (T) (Martin et al., 2009). This vector also contains spectinomycin resistance and origins of replication (not shown).
Figure 1.11. Schematic diagram of pEarleyGate 301. From the left to right border, a Basta resistance gene (Bar) flanked by its own promoter and terminator (not specified in original publication), a single CaMV 35S promoter driving the expression of a Gateway cassette and a HA epitope tag followed by an octopine synthase terminator (Earley et al. 2006).

Figure 1.12. Schematic diagram of pPZP-RCS2 and pSAT vectors and their use to make a multi-gene cassette T-DNA. Top diagram is the minimal Agrobacterium vector pPZP-RCS2 (Goderis et al., 2002) with six homing endonuclease sites between the left and right borders. The middle row is four out of the seven possible expression cassettes that are present in the pSAT series of vectors (Tzfira et al., 2005) for insertion into pPZP-RCS2. These expression cassettes include a variety of promoters, five possible autofluorescent proteins (AFPs) and a variety of terminators (see (Tzfira et al., 2005) for details). The bottom row is an example of a possible multi-gene expression vector.
Figure 1.13. Schematic diagram of pHELLSGATE, a Gateway-compatible silencing vector. Beginning from the right border, pHELLSGATE contains a CaMV promoter driving a Gateway® cassette, two introns, a PDK intron, and a catalase-1 intron, followed by a second inverted Gateway cassette and an octopine synthase terminator. Plant selection is conferred by a kanamycin resistance gene (nptII) under the control of a nopaline synthase promoter and terminator followed by the left border (Helliwell & Waterhouse, 2003; Wesley et al., 2001).

Figure 1.14. Schematic diagram of Tobacco rattle virus (TRV) Gateway compatible vectors. Genome of TRV placed between the left and right borders of a pBIN19 derivative vector pYL44 (Liu et al., 2002b) with two CaMV 35S promoters and a nopaline synthase terminator. RdRp, RNA-dependent RNA-polymerase; MP, movement protein; 16K, 16 kDa cysteine-rich protein; Rz, self-cleaving ribozyme; and T, nos terminator. The second plasmid is also cloned into pBIN19 with two CaMV 35S promoters, the coat protein (CP) and Gateway® cassette containing chloramphenicol resistance (chl) and ccdB gene (Liu et al., 2002a).

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CHAPTER II*

Transient expression in *Nicotiana benthamiana* fluorescent marker lines provides enhanced definition of protein localization, movement and interactions *in planta*.

Defining plant protein interaction networks and accurate determination of the subcellular localization of the proteome are fundamental requirements for plant cellular biology research in the post genomics era. To assist such studies, *N. benthamiana* is increasingly being used to conduct protein localization and bimolecular fluorescence complementation (BiFC) assays in live plant cells (Citovsky *et al.*, 2006; Goodin *et al.*, 2007a; Goodin *et al.*, 2007b; Ohad *et al.*, 2007; Waadt *et al.*, 2008). In order to enhance the utility of this plant in cell biology studies we, and others, have developed enhanced vector systems primarily for the expression of autofluorescent protein (AFP) fusions derived from the modular pSAT vectors (Citovsky *et al.*, 2006; Goodin *et al.*, 2007b; Lee *et al.*, 2008; Tzfira *et al.*, 2005). While these vectors have been of great utility for steady-state localization experiments, we report here an assessment of novel AFPs that can be used for monitoring protein movement, or which are brighter or more photostable than AFPs used in currently available Gateway-compatible plant binary vectors (Chakrabarty *et al.*, 2007; Dubin *et al.*, 2008; Earley *et al.*, 2006; Goodin *et al.*, 2007a; Nelson *et al.*, 2007; Tzfira *et al.*, 2005; Zhong *et al.*, 2008).

In addition to the requirement for appropriate vectors, generating accurate protein localization data often necessitates the use of marker dyes or proteins in order to provide a subcellular reference in micrographs. For example, one of the most commonly used markers is the DNA-selective dye 4′, 6-diamidino-2-phenylindole (DAPI) used to counterstain nuclei (Goodin *et al.*, 2007b). In addition to being highly toxic or mutagenic, the use of dyes adds to the time and expense of experiments, and limits high-throughput analyses in plants tissues. To circumvent such problems, we have generated a series of transgenic plants that express fluorescent markers targeted to the endoplasmic reticulum, actin filaments or nuclei.

In order to rigorously test the utility of our new vectors, we conducted protein localization and interaction studies using soluble and membrane-associated proteins encoded by *Sonchus yellow net virus* (SYNV) or *Potato yellow dwarf virus* (PYDV) as well as two isoforms of *N. benthamina* importin-α (NbImpα1 and NbImpα2; (Kanneganti *et al.*, 2007). We demonstrate how the data content of BiFC experiments is increased when conducted in transgenic plants expressing a subcellular reference. Additionally, we have evaluated our marker lines in the context of virus-induced changes in nuclear membranes. These technically challenging experiments provide confidence that the marker lines reported here will be of utility in demanding experiments required to elucidate protein and membrane dynamics in plant cells.

METHODS

Construction of Modified pSAT6 vectors
This section was performed by Kathleen Martin

In order to permit expression of fusions proteins with AFPs at the carboxy-termini of proteins of interest, a modified pSAT6 vector that included Fse1 and Spe1 sites flanking a stuffer GFP was constructed. Primers corresponding to green fluorescent protein (GFP) with the following modifications: to the 5' end, the addition of Ncol and FseI; and on the 3' end, SpeI and BgIII. These primers were designed using Vector NTI Advance v.10 (Invitrogen). Forward primer sequence: 5'-CCATGGGGCCCGCCGCTATGGTGAGCAAGGGCGAGGAGCTGTTCA-3' and the reverse primer sequence: AGATCTACTAGTCCGCGCGCGGACGACTCCAGCAGGACCATG-3'. Using these primers, polymerase chain reaction (PCR) for amplification of GFP yielded a band on agarose gel electrophoresis of the predicted size that was cloned into pGEM-T (Promega) following kit directions. This fragment is hereafter referred to as the "AFP" fragment.

The pSAT6-MCS (Tzfira et al., 2005) and pGEM-T-AFP clones were digested with Ncol and BgIII. The corresponding backbone from pSAT6-MCS and the inserted fragment from the pGEM-T clone were gel-purified and the AFP insert was ligated into pSAT6-MCS via T4 ligase (New England Biolabs).

The pSAT-AFP construct was modified to include the DEST fragment from the Gateway system (Invitrogen) via digestion of pSAT-AFP and pSAT6-EYFPC1-DEST (Chakrabarty et al., 2007), with BgIII and Ndel. The backbone from pSAT6-EYFPC1-DEST and the insert from pSAT-AFP were gel-purified and ligated together via T4 ligase. The pSAT6-AFP-DEST (Figure 2.1) constructs were confirmed via PCR using the same primers designed for cloning and enzyme digestion with FseI and SpeI (New England Biolabs).

The construct pSAT6-AFP-DEST was digested with Pl-PspI (New England Biolabs) to release the expression cassette insert. This fragment was ligated to a similarly digested binary vector RCS2-nptII (Goderis et al., 2002). This created the vector pSITEII-AFP (Figure 2.2). The vector was checked for accuracy using PCR with the specific primers described above and enzyme digestion with FseI and SpeI.

In order to facilitate expression of fusions proteins with AFPs at the C-termini of proteins of interest, a modified pSAT6 vector was constructed using a strategy similar to that reported for pSAT6-AFP-DEST. The forward and reverse primers used for this construction were 5' GGGCCCGGGCGGCCATGGTGAGCAAGGGCGAGGAGCTGTT and 5'GGATCCACTAGTTTGTCAGCTGATCCATGCGAGGAGCTGTT 3', respectively. The modified GFP stuffer was mobilized into two variants of pSAT6 to create pSAT6-AFP-N1A and pSAT6-AFP-N1B, which contain or lack an Nco1 sites upstream of the AFP, respectively. The DEST cassette was added to the pSAT-AFP-N1 vectors using an Age1/Apa1 fragment from pSAT-DEST-GFP-NA and pSAT-DEST-RFP-NB (Chakrabarty et al., 2007) to create pSAT-DEST-AFP-N1A and pSAT-DEST-AFP-N1B.
The two pSAT-DEST-AFP-N1 vectors were digested with \textit{Pl-PspI} (New England Biolabs) to release the expression cassettes, which were in turn moved into binary vector RCS2-nptII (Goderis \textit{et al.}, 2002). This created the vectors pSITEII-AFP-N1A and pSITEII-AFP-N1B. Only the pSITEII-AFP-N1B vector was used in experiments reported here.

**Construction of pSITEII vectors containing different AFPs**

This section was performed by Kathleen Martin

In this study, we present data for nine different AFPs, including: Cerulean (Rizzo \textit{et al.}, 2004); Midori-ishi cyan (MiCy; (Karasawa \textit{et al.}, 2004); Venus (Nagai \textit{et al.}, 2002); monomeric Kusabira-Orange (mKO; (Karasawa \textit{et al.}, 2004); TagRFP (Merzlyak \textit{et al.}, 2007); DRONPA (Ando \textit{et al.}, 2004); and Dendra2 (Chudakov \textit{et al.}, 2007). We also examined photoswitchable CFP (PS-CFP; (Chudakov \textit{et al.}, 2004); monomeric EosFP (Wiedenmann \textit{et al.}, 2004); PA-GFP (Patterson & Lippincott-Schwartz, 2004); and Azurite (Mena \textit{et al.}, 2006). However, fluorescence from these latter three AFPs was not easily detectable in plant cells (data not shown) and will therefore not be considered further.

Primers for Cerulean, MiCy, Venus, mKO, TagRFP, DRONPA, Dendra2, PA-GFP, PS-CFP and GST were designed to add \textit{FseI} to the 5’ end and \textit{SpeI} to the 3’ end. Each fragment was PCR-amplified and ligated into either pGEM-T or pJET-T (Fermentas Life Sciences) following the kit(s) directions. The accuracy of inserts was determined by PCR amplification from pGEM-T or pJET-T. Correct plasmids were then digested with \textit{FseI} and \textit{SpeI}. The pSAT6-DEST-AFP construct was similarly digested with \textit{FseI} and \textit{SpeI} and the inserts were added to replace the AFP fragment with each of the fragments: Cerulean, MiCy, Venus, mKO, TagRFP, DRONPA, Dendra2, PA-GFP, PS-CFP and GST. These constructs were moved into RCS2-nptII as described above.

**Construction of the pSITE-BiFC vectors**

This section was performed by Kathleen Martin

Digestion of pSAT6-EYFP-C1-DEST previously constructed (Chakrabarty \textit{et al.}, 2007) with \textit{AgeI} and \textit{Bgl II} was conducted to release the EYFP fragment. pSAT4-nEYFP-C1 (DQ168994) and pSAT1-cEYFP-C1 (DQ168996; (Citovsky \textit{et al.}, 2006). were also digested with \textit{AgeI} and \textit{BglIII} to release the nEYFP and cEYFP fragments. The nEYFP and cEYFP fragments were ligated to the backbone of pSAT6-EYFP-DEST-C1 to create pSAT6-nEYFP-DEST-C1 and pSAT6-cEYFP-DEST-C1, respectively. The \textit{Pl-PspI} fragments from these vectors were mobilized into RCS2-nptII to create pSITE-BiFC-C1nec and pSITE-BiFC-C1cec vectors (Figure 2.3). In a similar manner, pSITE-BiFC-N1nen and pSITE-BiFC-N1cen vectors were constructed from pSAT4A-nEYFP-N1 (DQ169002) and pSAT4A-cEYFP-N1 (DQ169003; Figure 2.3).

**Protein fusion construction**

This section was performed by Kathleen Martin

Genes of interest were mobilized into pSITE and pSITEII vectors from pDONR intermediates by recombination-based cloning as described (Goodin \textit{et al.}, 2008).
Transient expression of proteins in leaf epidermal cells

This section was performed by Kathleen Martin

Suspensions of A. tumefaciens strain LBA4404 or C58C1 were infiltrated into leaves of N. benthamiana as previously described (Goodin et al., 2002; Tsai et al., 2005). To express proteins in SYNV-infected cells, symptomatic leaves of plants were infiltrated at the peak of symptom expression, typically ten to fourteen days post inoculation. Following a 48 h incubation of infiltrated plants under constant illumination at 25°C, water-mounted sections of leaf tissue were examined by confocal microscopy.

Confocal microscopy

All microscopy was conducted using an FV1000 point-scanning/point-detection laser scanning confocal microscope (Olympus, http://www.olympus-global.com), equipped with lasers spanning the spectral range of 405 nm-633 nm. Micrographs for dual-color imaging were acquired sequentially, as described (Goodin et al., 2007b). The objective used was an Olympus water immersion PLAPO60XWLSM (NA 1.0), unless otherwise noted. Image acquisition was conducted at a resolution of 512x512 pixels and a scan-rate of 10 ms/pixel. Olympus Fluoview software version 1.5 was used to control the microscope, image acquisition and export of TIFF files. Figures were assembled using Photoshop 7.0 (Adobe, http://www.adobe.com) and Canvas 8.0 (Deneba Software, http://www.deneba.com).

Photoactivation/photoconversion of AFPs

All photoactivation/photoconversion experiments were performed using the Olympus FV1000 described above. Briefly, 25 mm² sections of tissue were excised from agroinfiltrated leaves and mounted on glass slides in water and covered with a glass coverslip. Imaging for DRONPA experiments was conducted using a 40X objective and 488 nm laser line from a multi-line argon laser set at 0.3-1.0% of full power. Regions of interest were photoactivated for 50 ms using a 405 nm diode laser, set at 50-80% of full power, which was delivered via the FV1000 Simultaneous (SIM) scanner. Images for FRAP analyses were acquired at a resolution of 512x512 pixels and a scan-rate of 2 ms/pixel, which was necessary to monitor fast protein dynamics. Two images were acquired prior to photobleaching, followed by an additional 7 images to monitor fluorescence recovery. Quantitative fluorescence data, in Excel format (Microsoft, http://www.microsoft.com), and confocal images, in TIFF format, were exported using Olympus Fluoview software.

Photoconversion of Dendra2 was conducted in a similar manner to settings employed for DRONPA photoactivation. However, following the 405 nm pulse, the fluorescence from photoconverted Dendra2 was imaged using a 543 nm laser set at 20-40% of full power.

Where applicable, mean and standard deviations for fluorescence intensity at each timepoint were calculated and plotted using Excel.
**Agrobacterium tumefaciens-mediated plant transformation**

Transgenic plants were derived from the parental line used to generate the “16c” line of *N. benthamiana* mGFP5-ER plants (Brignet *et al.*, 1998; Haseloff *et al.*, 1997; Ruiz *et al.*, 1998). The transformation procedure was an adaptation of the methods described (Chakrabarty *et al.*, 2007; Horsch *et al.*, 1985; Kalantidis *et al.*, 2002). Briefly, *A. tumefaciens* strain LBA4404 carrying the pSITE vectors for the expression of RFP-H2B, CFP-H2B, or RFP-ER were grown overnight at 28°C. Surface sterilized leaves from greenhouse-grown *N. benthamiana* plants were inoculated with the *Agrobacterium* culture. The explants were co-cultivated for 2 days on MS media (Murashige & Skoog, 1962) supplemented with benzylaminopurine (BA) and indole-3-acetic acid (IAA), 2 mg/L and 0.5 mg/L, respectively. Putative transgenic shoots from the leaf explants were induced on the same medium supplemented with Cefotaxime (500 mg/L) and Kanamycin (150 mg/L). Regenerated shoots were transferred to rooting media that included MS, with Cefotaxime (250 mg/L) and Kanamycin (50 mg/L). After rooting, the plants were transferred to soil in pots and were kept in culture room at 25°C with 16 h photoperiod. Later, seeds were collected from T0 plants. Repeated rounds of screening resulted in the selection of plant lines for which there was no segregation of the fluorescent markers for five generations. These lines are assumed to be homozygous.

**RESULTS**

**Construction of pSAT6-DEST-FS and derivative pSITEII vectors**

As we were interested in enhancing the facility by which AFPs can be exchanged to create new vectors, we constructed pSAT6-DEST-AFP so that sites for rare cutting restriction endonucleases were included at the 5’ and 3’ termini of EGFP, respectively (Figure 2.1A), which permitted rapid subcloning to replace the EGFP “stuffer sequence” to generate a variety of pSAT6-DEST-AFP derivatives (Figure 2.1B). Using this strategy a diverse set of pSITEII vectors were constructed (Figure 2.2; Table 2.1).

**Construction of pSITE vectors for BiFC**

To provide a consistent series of Gateway-compatible vectors for both localization and protein interaction studies, we converted several of the previously reported pSAT-BiFC-N1 and -C1 vectors (Citovsky *et al.*, 2006) to their pSITE derivatives (Figure 2.3). Note that these new Gateway vectors do not have the restriction site modifications of pSITEII vectors; therefore we will refer to these vectors as pSITE-BiFC vectors, according to the established convention (Chakrabarty *et al.*, 2007).

**In planta photostability of nine different AFPs**

To our knowledge, the relative photostability of a large number of AFPs expressed from a similar vector backbone has not been compared *in planta*. However, these data are critical for evaluating the suitability of AFPs for use in various biological assays. Therefore, we examined the localization and photostability of nine AFPs expressed from either pSITE or pSITEII vectors.
Expressing the AFPs via agroinfiltration in *N. benthamiana* leaves showed that GFP spectral variants and TagRFP accumulated in nuclei, but they were excluded from nucleoli. In contrast, both MiCy and mKO appeared to accumulate in both loci, such that nucleoli could not be discerned in cells expressing these proteins (Figure 2.11). The nine AFPs examined differed greatly in their photostability, with relative order from most to least stable being: EYFP > EGFP = Venus > mKO > Cerulean > ECFP > mRFP1 > TagRFP > MiCy (Figure 2.11).

**Comparative brightness of AFP fusions in plant cells**

Based solely on published calculations of brightness (Table 2.1), the expected utility of AFPs in plants was predicted to be Venus > EYFP > EGFP > mKO > Cerulean > MiCy = TagRFP > ECFP = mRFP1. Except for mKO and MiCy, this prediction generally holds under conventional imaging conditions (Figure 2.12 and 13). For SYNV-N fusions we found that Venus fusions, more so than any other AFP, were prone to aggregation (Figure 2.12 and data not shown).

**Photostability of DRONPA fusions in plant cells**

DRONPA is a reversibly photoactivatable AFP that has been used for protein tracking experiments in a manner similar to EosFP or the more popular PA-GFP (Ando *et al.*, 2004; Lippincott-Schwartz & Patterson, 2008; Schenkel *et al.*, 2008). To extend the range of functionality of the pSITEII vector series, we tested DRONPA expression, photoactivation, and stability in *N. benthamiana* leaf epidermal cells. DRONPA and DRONPA fusions proved to be photoactivatable in plant cells (Figure 2.4A-B). We also found that fusions to DRONPA were more resistant to photobleaching than the unfused AFP (Figure 2.4C). Unexpectedly, DRONPA-SYNV-N localized exclusively to the nuclear periphery, in marked contrast to GFP- or RFP-SYNV-N fusions, which accumulated in the nucleus proper and were excluded from the nucleolus (Goodin *et al.*, 2001; Goodin *et al.*, 2007b). Although we do not know why DRONPA-SYNV-N fusions mislocalize, this result emphasizes that researchers should be aware that not all AFPs provide the same localization information when fused to the same protein.

**Dendra2 for protein tracking in plant cells**

We verified pSITEII-7-C1 and pSITEII-7-N1 vectors for the expression of Dendra2 fusions in plant cells (Figure 2.5). Dendra2-SYNV-N, in contrast to DRONPA fusions, localized exclusively to nuclei in a pattern similar to that for GFP or RFP fusions (Goodin *et al.*, 2007a; Goodin *et al.*, 2001; Goodin *et al.*, 2007b). It was possible to selectively photoconvert subnuclear regions of interest from green (Figure 2.5A1-A5) to red (Figure 2.5B1-B5, C1-C5) without affecting nuclei in adjacent cells. The Dendra2-SYNV-N fusion in these selected regions was undetectable within seconds after photoconversion (Figure 2.5C2 and C3). Further investigation is required to determine if the rapid disappearance of red Dendra2-SYNV-N is related to diffusion, degradation, or some combination thereof. Interestingly, the red form of SYNV-P-Dendra2 (Figure 2.5D1-D5; E1-E5) diffused more slowly than the SYNV-N fusion. Additionally, we were unable to photoactivate SYNV-P-Dendra2 with the precision observed for SYNV-N (compare Figure 2.5C2
and F2). Unlike the SYNV-N fusion, SYNV-P-Dendra2 was still detectable at five minutes post photoconversion (data not shown). Quantification of relative fluorescence intensities showed that the green and red forms of SYNV-P-Dendra2 equilibrated in nuclei within 15 s post photoconversion (Figure 2.14). These results suggest that each protein of interest may have unique rates of diffusion, which must be determined empirically.

**Validation of BiFC vectors**

In order to be broadly useful, BiFC vectors must permit protein associations to be assayed in a variety of cellular loci. The simplest associations to determine are those formed by proteins in soluble complexes, such as those between the phosphoprotein (P) and nucleocapsid (N) proteins of SYNV (Goodin et al., 2001). However, testing interactions of membrane-associated proteins, such as the SYNV glycoprotein (SYNV-G; Goldberg et al., 1991) is technically more challenging given the requirement for the YFP fragments to be on the same side of the membrane in order for them to associate. Additionally, conventional wisdom holds that AFPs should be fused to the carboxy-termini of membrane-associated proteins in order to prevent interference of the function of signal peptides at their amino-termini. Therefore, the pSITE-BiFC vectors were validated in the contexts of both soluble (Figure 2.6A-D) and membrane-associated (Figure 2.6E-H) viral protein complexes. Converting the pSAT-BiFC vectors to their Gateway-compatible pSITE derivatives resulted in insignificant background fluorescence when the two non-fused halves of YFP were coexpressed (Figure 2.6A and E) or when non-fused halves were coexpressed with protein fusions (Figure 2.6B, C, F and G). Thus, *bona fide* interactions could be scored easily, such as in the case of the soluble SYNV-N/P (Figure 2.6D) complex or the self-association of SYNV-G (Figure 2.6H). We note that, contrary to conventional wisdom, the SYNV-G interaction was detected only when this protein was expressed from the BiFC-C1 vectors, which places the YFP fragments in front of the SYNV-G signal peptide. When expressed from the BiFC-N1 vectors, which places the YFP fragments at the carboxy-terminus of SYNV-G, no fluorescent signal was observed, despite the fact that fusions could be detected by immunoblotting (data not shown).

A further reduction in background in BiFC experiments was achieved when nYFP or cYFP fragments were expressed as fusions to glutathione-S-transferase (GST) or maltose-binding protein (data not shown). We now routinely use these GST:YFP fragment fusions as negative controls in BiFC assays (Figure 2.7).

In addition to enhancing the utility of the pSITE-BiFC vectors, we succeeded in further increasing data content and quality of micrographs by conducting BiFC experiments in transgenic *N. benthamiana* plants that expressed CFP fused to histone 2B (CFP-H2B; Figure 2.6I-Q). To validate this approach we used BiFC to confirm the homo- and heterologous interactions of the SYNV-N and -P proteins, which have been shown previously using GST-pulldowns and yeast two-hybrid assays (Deng et al., 2007; Goodin et al., 2001). Consistent with previous reports (Goodin et al., 2002; Goodin et al., 2001), the -N protein complex localized to the nucleus exclusively (Figure 2.6I-K), whereas coexpression of -N and -P resulted in relocalization of both proteins to a subnuclear locale (Figure 2.6L-M). The SYNV-P
protein complex showed accumulation in both the nucleus and cytoplasm (Figure 2.60-Q). Given these results, it is important that researchers conducting similar experiments be aware that coexpression may alter the subcellular localization of proteins relative to patterns formed when they are expressed individually.

Differential interaction of cargo with importin-α

Prior to conducting BiFC experiments, it is important to be aware that isoforms encoded by a particular multi-gene family might interact with different subsets of proteins. To demonstrate such differential binding we examined the interactions of two isoforms of importin-α (NbImpα1 and NbImpα2), with multiple cargo proteins of viral origin.

The import of proteins into the nucleus is commonly mediated, in part, by importin-α proteins, which form oligomeric complexes with cargo proteins and importin-β, which then translocate from the cytoplasm through nuclear pore complexes into the nucleus (Xu et al., 2007b). The Arabidopsis isoforms of importin-α have recently been shown to differ in their selectivity for particular cargo proteins that are imported into the nucleus (Bhattacharjee et al., 2008; Lee et al., 2008). To determine if this phenomenon holds for N. benthamiana, we tested the ability of NbImpα1 and NbImpα2 to interact with SYNV and PYDV proteins as test cargo (Kanneganti et al., 2007; Palma et al., 2005). The SYNV-N protein contains an arginine/lysine-rich nuclear localization signal (NLS) at its carboxy-terminus, which has been shown to mediate its interaction with importin-α in vitro (Deng et al., 2007; Goodin et al., 2001). In contrast, SYNV-P does not contain a predictable NLS, and does not bind importin-α in vitro (Deng et al., 2007). Moreover, although the cognate proteins from PYDV lack predictable NLSs, both are localized exclusively to the nucleus (Ghosh et al., 2008). However, the nuclear import of proteins lacking canonical NLSs has been shown, in some cases, to be mediated via an importin-α-dependent pathway (Wolff et al., 2002). These experiments showed that, consistent with in vitro binding data, SYNV-N interacted with both NbImpα1 and NbImpα2 (Figure 2.7). However, interactions of these proteins localized to different loci, with the SYNV-N/NbImpα1 interaction being distinctly subnuclear and SYNV-N/NbImpα2 interacting on the periphery of the nucleus. In contrast to SYNV, we observed that PYDV-N interacted with NbImpα1, but not with NbImpα2 (Figure 2.8). No detectable fluorescence was produced when PYDV-P and SYNV-P were tested for interaction with either NbImpα1 or NbImpα2 (Figure 2.8).

RFP-marker N. benthamiana plants for supporting localization projects

In addition to the CFP-H2B plants, the use of which clearly improves BiFC experiments, we have also developed N. benthamiana lines that express RFP-H2B or RFP with an ER retention signal. In order to demonstrate their utility, we took advantage of the ability of SYNV to selectivity induce intranuclear accumulation of the inner nuclear membrane (INM), as has been reported (Goodin et al., 2007b; Martins et al., 1998). We postulated that, in SYNV-infected plants, GFP fused to the human LaminB receptor (LBR-GFP), which is targeted to the INM in plants, should accumulate on the periphery of intranuclear membranes. The perinuclear space in such nuclei should then “fill in” with RFP in transgenic plants that express RFP
targeted to the ER (Collings et al., 2000; Goodin et al., 2007b; Irons et al., 2003). Conversely, GFP fused to WIP1, which is anchored to RanGAP1 on the outer nuclear membrane (ONM) in Arabidopsis (Xu et al., 2007b), should not accumulate on intranuclear membranes in SYNV-infected cells, as electron micrograph studies suggest that the ONM remains largely unaffected in SYNV-infected plants (Martins et al., 1998). Consistent with its localization in A. thaliana, WIP1-GFP localized exclusively to the nuclear rim in N. benthamiana RFP-H2B plants (Figure 2.9). In contrast to the results predicted for LBR-GFP (Figure 2.9M-O; Goodin et al., 2007b), WIP1-GFP did not accumulate on intranuclear membranes in SYNV-infected RFP-ER transgenic lines, as expected for a protein that is indeed anchored to the ONM (Figure 2.9J-L). Taken together, these experiments provide confidence that our transgenic plant lines are suitable for use in experiments where complex changes in protein or membrane localization need to be studied.

Finally, for situations where researchers may need to localize proteins to the cytoskeleton, we produced plants that express the actin-binding protein, Talin, fused to EGFP (Figure 2.10). Despite potential problems with Talin as a marker for labeling actin, which includes cytotoxicity, it remains a widely utilized and effective marker (Ketelaar et al., 2004; Schenkel et al., 2008; Yoneda et al., 2007). Plant lines transgenic for GFP-Talin, which were indistinguishable from wild-type plants under greenhouse conditions, were screened for their ability to provide high-contrast views of actin filaments (Figure 2.10A). We also produced transgenic plants expressing Talin fused to RFP, but were unsatisfied with these lines with respect to producing high-contrast micrographs (data not shown).

**DISCUSSION**

The pSITE-BiFC and pSITEII vectors described here offer a greatly expanded range of functionality of AFPS previously unavailable in Gateway-compatible binary vectors (Chakrabarty et al., 2007; Coutu et al., 2007; Dubin et al., 2008; Earley et al., 2006; Goodin et al., 2007a; Nelson et al., 2007; Tzfira et al., 2005; Zhong et al., 2008). By characterizing a wide variety of AFPS in planta, we hope to prevent the costs, frustration, and time delays often associated with acquiring and screening novel AFPS. It is abundantly clear from our experience that AFPS deemed suitable for use in animal or bacterial systems may or may not be similarly useful in plant cells (Teerawanichpan et al., 2007). Additionally, the restriction site modifications to the pSITEII vectors make it very efficient to replace and test novel AFPS, which are being reported at rates that greatly outpace the ability to rigorously validate them in plant-based assays (Merzlyak et al., 2007; Schenkel et al., 2008; Shaner et al., 2008; Subach et al., 2008; Tasdemir et al., 2008; Teerawanichpan et al., 2007).

Importantly, AFPS such as DRONPA can affect the localization of their fusion partners, as demonstrated by fusion to the SYNV-N protein. However, given that this protein is brighter than PA-GFP, and that DRONPA fusions are reasonably photostable compared to the native protein, it is worthwhile to develop DRONPA-based assays.

In contrast to DRONPA, we showed that fusion of SYNV proteins to Dendra2 resulted in localization patterns consistent with those observed for conventional
Moreover, the efficient photoconversion of these fusions from green to red suggests that the pSITEII-7-C1 and -7-N1 vectors might be of significant utility in protein tracking experiments, as has been demonstrated for mEosFP (Schenkel et al., 2008).

Another important finding relevant to selecting the appropriate AFP for localization studies was that both mKO and MiCy accumulated in nucleoli, from which GFP-variants, TagRFP, and RFP-variants were excluded. This raises the possibility that some AFPs may negatively impact subcellular localization of fusion partners. In addition to its accumulation in nucleoli, the extreme sensitivity of MiCy to photobleaching and low brightness of mKO prevent us from recommending these fluoros for use in planta. However, problems encountered here may be overcome by using variants of AFPs selected for greater photostability or enhanced spectral characteristics (Ai et al., 2008; Shaner et al., 2008).

Our BiFC results that demonstrate the differential interactions of plant nuclear importers with the -N and -P proteins of SYNV and PYDV support the contention that the various isoforms of plant importin-α proteins differ in their cargo specificities (Bhattacharjee et al., 2008; Deng et al., 2007; Jiang et al., 1998; Palma et al., 2005). Interestingly, the interaction of SYNV-N with NbImpα1 and NbImpα2 on the periphery of nuclei or in intranuclear sites, respectively, is similar to the recent finding that the VirE2 protein of Agrobacterium tumefaciens interacts with Arabidopsis importin-α isoform-1 and isoform-4 in the cytoplasm and nucleus, respectively (Lee et al., 2008). These studies, together with the discovery that MOS6, an importin-α homologue required for signaling responses related to innate immunity (Palma et al., 2005), demonstrate that importin-α isoforms cannot be entirely functionally redundant. Collectively, these data underscore the need to determine and compare the cargo specificities of nuclear import-receptor isoforms in order to fully appreciate nuclear transport in plants. More generally, differential sites of localization and interaction may reflect functional differences of protein isoforms that are involved in different physiological processes (Morsy et al., 2008).

While the CFP-H2B plants were generated to improve data quality in BiFC experiments, our RFP-H2B and RFP-ER expressing lines proved equally useful for a variety of localization studies. Both of the H2B lines offer an exceptional alternative to the use of 4′, 6-diamidino-2-phenylindole (DAPI) that is commonly used to counterstain nuclei, particularly when many infiltrations need to be conducted (Launholt et al., 2006; Goodin, unpublished data).

Importantly, we demonstrated that marker proteins for the outer and inner nuclear membranes function in N. benthamiana as predicted based upon their function in A. thaliana or N. tabacum. These experiments should help to underscore the functional conservation of proteins and that accurate data related to the localization of heterologous proteins can be obtained in N. benthamiana (Citovsky et al., 2006; Goodin et al., 2007; Ohad et al., 2007; Tardif et al., 2007).

One potential criticism of the present vectors is their utilization of a double 35S promoter to drive expression of the AFP fusions. It is often speculated that this will lead to artifacts due to protein overexpression. While this is potentially the case with some protein fusions, the data presented here strongly suggest that biologically relevant interactions can be easily scored with these vectors. One such comparison...
made here is the localization of RFP-H2B in transient assays and transgenic plants. This nuclear marker accumulated in both nuclei and nucleoli in transient assays, but was excluded from nucleoli in transgenic plants. Such differences do not obviate the use of RFP-H2B as a nuclear marker, *per se*. However, it does suggest that proteins of interest whose localization was initially determined under transient conditions may need to be further studied in transient plants. However, given the great expense and time required for generating transgenic plant lines, it is infinitely more practical to first determine protein localization in transient assays. Should weaker promoters be required, the pSAT vectors from which the present series were derived are conveniently modular, which permits facile replacement of promoters (Chung *et al.*, 2005). Users of these vectors are therefore encouraged to select promoter/AFP combinations relative to the specifics of their research objectives.

Taken together, the combination of binary vectors and transgenic plants reported here provides a novel set of tools to probe membrane and protein dynamics in potentially many areas of plant biology.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Protein</th>
<th>Structure</th>
<th>Ex. Max.(^a) (nm)</th>
<th>Em. Max.(^b) (nm)</th>
<th>Mol Ex. Coeff.(^c) (M(^-1)cm(^-1))</th>
<th>Quantum Yield</th>
<th>Brightness(^d)</th>
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<td>pSITEII-1C1</td>
<td>Mi-Cy</td>
<td>Dimer</td>
<td>472</td>
<td>495</td>
<td>27,300</td>
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<td>25</td>
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<td>Monomer*</td>
<td>439</td>
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<td>0.40</td>
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<tr>
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<td>Monomer*</td>
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<td>475</td>
<td>43,000</td>
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<td>27</td>
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<tr>
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<td>Monomer*</td>
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<td>507</td>
<td>56,000</td>
<td>0.60</td>
<td>34</td>
</tr>
<tr>
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<td>Monomer*</td>
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<td>527</td>
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<tr>
<td>pSITEII-4C1</td>
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<td>Monomer*</td>
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<td>528</td>
<td>92,200</td>
<td>0.57</td>
<td>53</td>
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<tr>
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<td>Monomer</td>
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<td>559</td>
<td>51,600</td>
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<td>mRFP1</td>
<td>Monomer</td>
<td>584</td>
<td>607</td>
<td>50,000</td>
<td>0.25</td>
<td>13</td>
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<tr>
<td>pSITEII-6C1</td>
<td>TagRFP</td>
<td>Monomer</td>
<td>555</td>
<td>584</td>
<td>52,000</td>
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<td>507/573</td>
<td>45,000/35,000</td>
<td>0.50/0.55</td>
<td>23/19</td>
</tr>
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<td>57,000</td>
<td>0.62</td>
<td>35</td>
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**Figure 2.1.** Construction of pSAT6-AFP-DEST, a Gateway-compatible derivative of pSAT6-DEST-FS in which an EGFP 'stuffer' sequence was engineered with flanking FseI and SpeI restriction sites. Replacement of the stuffer with any appropriately cloned gene (autofluorescent protein, AFP) resulted in the facile construction of intermediates required for pSITEII-C1 vectors. A similar strategy was used to generate pSAT6-DEST-AFP intermediates for constructing pSITEII-N1 vectors (see Methods).
Figure 2.2. Construction of pSITEII vectors. (a) New pSITEII vectors were constructed by subcloning pSAT6-DEST-AFP derivatives into the PI-Psp1 site of the plant gene expression vector pRCS2-nptII, which carries the nptII-selectable marker and the Ti plasmid left and right borders. (b) Seven pSITEII C1 vectors were constructed with FRET optimized autofluorescent proteins (AFPs) such as Cerulean, Midoriishi Cyan (MiCy), Venus and monomeric Kusabira Orange (mKO). Additionally, TagRFP was used as a brighter alternative to mRFP1. Finally, DRONPA was used to enable the tracking of proteins in plant cells using photoactivation or photoconversion. (c) For the construction of fusions to the N termini of AFPS we have produced and validated a series of pSITEII-N1 vectors. The AFP cloning intermediates for constructing the C1 series can also be used for the construction of N1 vectors.

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Figure 2.3. pSITE-BiFC-C1 vectors were constructed by subcloning the Agel/BglIII fragment of pSAT4-BiFC (a) into pSAT6-DEST (b). As for all pSITE vectors, the pSAT6 derivatives were subcloned into RCS2-nptII to produce Gateway cloning compatible binary vectors. (c) pSITEBiFC- N1 vectors were constructed by subcloning the Agel/ApaI fragment of pSAT6-DEST into pSAT4-BiFC. The four available vectors are designated pSITE-BiFC-C1nc, pSITE-BiFC-C1cec, pSITE-BiFC-N1nen and pSITE-BiFC-N1cen.
Figure 2.4. Photoactivation of DRONPA (a) and DRONPA-SYNV-N (b) in agroinfiltrated *N. benthamiana* leaf epidermal cells. DRONPA was photoactivated (1.1 s) with a 50-ms pulse from a 405-nm laser following the acquisition of a preactivation image (0 s). Cells were imaged continuously for another 23 s using a 488-nm laser line for excitation. (c) Normalized fluorescence intensity showing the relative photostability of DRONPA-SYNV-N and DRONPA. Each curve represents the average of three independent assays. Scale bars: 5 μm (a); 10 μm (b). For (c), the average fluorescence intensity of DRONPA prior to photoactivation was taken as the zero point.

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**Figure 2.5.** Photoconversion of Dendra2 fusions expressed from pSITEII-C1 (a1–c5) or pSITEII-N1 (d1–f5) vectors. Dendra2 fusions were transiently expressed in *N. benthamiana* leaf epidermal cells. ROIs within selected nuclei (arrowheads) were photoconverted using a 50-ms pulse from a 405-nm laser set at 78% of full power. Micrographs were acquired immediately prior to photoactivation, and were continuously acquired for 11 s thereafter. Shown are the green (a1–a5; d1–d5), red (b1–b5; e1–e5) and overlain (c1–c5; f1–f5) micrographs of Dendra2-SYNV-N and SYNV-P:Dendra2, respectively. The inserts in panels c2 and c3 highlight the photoconverted region in a nucleus in which Dendra2-SYNV-N was localized. Scale bar: 10 μm.
Figure 2.6. Validation of the pSITE-BiFC vectors for detecting interactions between soluble (a–d) or integral (e–h) membrane protein complexes. Identical detector settings were used to acquire all images. Expression was conducted in leaf epidermal cells of *N. benthamiana* using agroinfiltration. Micrographs taken 48 h post-infiltration show results of co-expressed nYFP+cYFP (a and e); nYFP+cYFP-SYNV-P (blue) (b); nYFP-SYNV-N (red)+cYFP (c); and nYFP-SYNV-N+cYFP:SYNV-P (d). (f) nYFP+cYFP:SYNV (purple); (g) nYFP-SYNV-G+cYFP. (h) nYFP-SYNV-G+cYFP:SYNV-G. (i–q) Single section confocal micrographs demonstrating the use of CFP-H2B tagged *N. benthamiana* plants for marking nuclei in bimolecular fluorescence complementation (BiFC) experiments. (i, k and o) Fluorescence from CFP localized to nuclei in CFP-H2B plants. (j) YFP fluorescence in a nucleus of a cell expressing nYFP-SYNV-N+cYFP:SYNV-N. (k) Overlay of images (i) and (j). (m) YFP fluorescence in a nucleus of a cell expressing nYFP-SYNV-N+cYFP-SYNVP. (n) Overlay of images (l) and (m). (p) YFP fluorescence in a cell expressing nYFP-SYNV-P+cYFP-SYNV-P. (q) Overlay of images (o) and (p). Scale bars: 10 μm (a–h; o–q); 5 μm (i–k); 3 μm (l–m).

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Figure 2.7. Single-section confocal micrographs of bimolecular fluorescence complementation (BiFC), showing differential interactions of NbImpα1 and NbImpα2 with SYNV-N. Importin-α proteins were expressed as fusions to the C-terminal half of YFP (Impc). SYNV-N was expressed as a fusion to the N-terminal half of YFP (Nn). (a–f) Interaction of SYNV-N with NbImpα1. (a–c) Whole-cell views showing fluorescence from CFP-H2B, BiFC interaction of Nn and NbImpα1, and the resulting overlain images, respectively. (d–f) Confocal sections of nuclei in cells expressing the same fusions shown in a–c. (g–i) Whole-cell views showing fluorescence from CFP-H2B, BiFC interaction of Nn and NbImpα2, and the resulting overlain images, respectively. (j–l) Confocal sections of nuclei in cells expressing the same fusions shown in (g–i). (m–o) Lack of interaction between SYNV-P and NbImpα1. (p–r) Lack of interaction between SYNV-P and NbImpα2. (s–x) Control reactions showing a lack of interactions between GST and NbImpα1 or NbImpα2. (y–a1) Control reactions showing a lack of interactions between GST and NbImpα1 or SYNV-N. Scale bars: 10 μm, except for (d–f) and (j–l), 5 μm.
Figure 2.8. Single-section confocal micrographs of bimolecular fluorescence complementation (BiFC), showing differential interactions of NblImpα1 and NblImpα2 with PYDV-N. Importin-α proteins were expressed as fusions to the C-terminal half of YFP (Impc). SYNV-N was expressed as a fusion to the N-terminal half of YFP (Nn). Agroinfiltration was used to express proteins in transgenic *N. benthamiana* plants expressing the nuclear marker CFPH2B. (a–c) Whole-cell views showing the interaction between PYDV-N and NblImpα1 (Nn/Imp1c). (d–f) Whole-cell views showing the lack of interaction between PYDV-N and NblImpα2 (Nn/Imp2c). (g–i) Whole-cell views showing the self-interaction of PYDV-N (Nn/Nc). Scale bars: 10 μm.

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Figure 2.9. (a–l) Single-section confocal micrographs showing localization of AtWIP1:GFP in transgenic *N. benthamiana* plants expressing RFP:H2B. (a–c) Whole-cell views showing the GFP channel (a), the RFP channel (b) and the overlay (c) of WIP1:GFP in mock-inoculated plants. (d–f) As for (a–c), showing a view of a single nucleus. (g–i) As for (a–c), except that WIP1:GFP expression was conducted in SYNV-infected plants. (j–l) Similar to (d–f), showing WIP1:GFP localization in SYNV-infected transgenic *N. benthamiana* plants expressing RFP-ER. (m–o) Expression of the inner nuclear marker LBRGFP in SYNV-infected cells. (p–r) GFP channel, RFP channel and overlay micrographs of a single nucleus in mock-inoculated transgenic plants expressing RFP-ER agroinfiltrated with the LBR-GFP marker. Scale bars: 2 μm, except (a–c) and (g–i), 10 μm.

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Figure 2.10. Transient expression of RFP-H2B (a) in a leaf epidermal cell of transgenic *N. benthamiana* expressing GFP-Talin, an actin binding protein (b). The overlain image of (a) and (b) is presented in (c).
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Figure 2.11. Photosensitivity of AFPs expressed in leaf epidermal cells of *N. benthamiana*. (A) Expression of various AFPs from pSITEII and pSITE vectors following agroinfiltration of *N. benthamiana* leaves. Single confocal micrograph sections show that all AFPs accumulated in the nucleus and that all except MiCy and mKO are excluded from the nucleolus. The dark circle located in each nucleus is the nucleolus (B) Photobleaching curves representing the relative stability of the AFPs shown in A under conventional imaging conditions. Micrographs were acquired using the minimal laser intensities required for exciting each AFP. AFPs were then imaged continuously over an 80 s time course. The relative fluorescence intensities were determined and normalized as described in the Experimental procedures section. Each curve represents the average of three independent assays conducted in equivalent areas of interest in nuclei. The relative fluorescence intensity at the start of the experiment was assigned a value of 1.00 and used to normalize the intensity values over the course of the experiment.
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Figure 2.12. Comparative brightness of spectrally-related groups of AFPs as a function of PMT-voltage from 300-700 V in 100 V increments. In all cases, the fluorescent proteins were expressed as AFP-SYNV-N fusions targeted to nuclei in transgenic plants expressing CFP or RFP fused to histone 2B. Shown are representative series of micrographs for each AFP fusion. Not shown is fluorescence from the histone markers, which served only to locate the position of nuclei in ROIs when the SYNV-N fusion was too dim to be detected visually. Power settings and scan-rates were held constant for each laser-line used to excite the different AFPs. (A1-A5) CFP-SYNV-N. (B1-B5) Cerulean-SYNV-N. (C1-C5) GFP-SYNV-N. (D1-D5) YFP-SYNV-N. (E1-E5) Venus-SYNV-N. (F1-F5) mKO-SYNV-N. (G1-G5) RFP-SYNV-N. (H1-H5) TagRFP-SYNV-N. Not included in this experiment is MiCy, which was deemed to be too photosensitive to be of practical utility. Scale bar = 50 μm.
Figure 2.13. Comparative brightness of spectrally-related groups of AFPs. In all cases, the fluorescent proteins were expressed as AFP-SYNV-N fusions targeted to nuclei in transgenic plants expressing CFP or RFP fused to histone 2B. Each averaged fluorescence intensity data point, determined from three nuclei, was plotted as a function of PMT voltage. Laser intensities and scan-rates were held constant for each laser-line used to excite different AFPs. Shown are comparisons of red (A), yellow-green/green (B) and cyan (C) AFPs, which were excited with 543 nm, 515 nm and 440 nm lasers, respectively, with the exception of EGFP (B), the sole green AFP examined, which was excited using the 488 nm laser line.

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Figure 2.14. Relative fluorescence intensity of the green and red forms of SYNVP:Dendra2 photoconverted in panels A1-A5 in Figure 2.5. Prior to photoconversion Dendra2 fluoresces green as was imaged using a 488 nm laser line. The photoconverted (red) form of Dendra2 was imaged using a 543 nm laser line.
CHAPTER III*
Membrane and protein dynamics in live plant nuclei infected with Sonchus yellow net virus, a plant-adapted rhabdovirus.

Plant-adapted rhabdoviruses are classified into two genera. Members of the genus Nucleorhabdovirus differ from the Cytorhabdovirus genus and their mammal-, fish- and insect-infecting relatives, in that they replicate and undergo morphogenesis in nuclei of infected cells (Dietzgen et al., 2006; Jackson et al., 2005; Reed et al., 2005; Revill et al., 2005; Tsai et al., 2005).

Nucleorhabdoviruses share many of the structural features of animal rhabdoviruses, such as Vesicular stomatitis virus (VSV; Jackson et al., 2005). They are consequently composed of an infectious nucleocapsid “core” surrounded by a phospholipid membrane. The core can be purified by density gradient centrifugation of non-ionic detergent-treated virions ((Wagner et al., 1996). In the case of SYNV, the core is a ribonucleoprotein (RNP) complex that consists of the negative-strand genomic RNA (Jackson & Christie, 1977) encapsidated by three associated proteins, namely nucleocapsid (N), phosphoprotein (P) and polymerase (L) proteins (Choi et al., 1992; Heaton et al., 1987; Zuidema et al., 1987). The membrane fraction of mature virions contains a glycoprotein (G) that protrudes from the surface of the virion (Goldberg et al., 1991). A sixth protein, sc4, which localizes to the periphery of cells, may play a role in virus cell to cell movement (Goodin et al., 2002; Huang et al., 2005; Melcher, 2000). The matrix (M) protein (Hillman et al., 1990) is believed to associate with G, presumably condensing the core during virion maturation (Jackson et al., 2005; Jayakar et al., 2004). Electron microscopy studies suggest that during morphogenesis the condensed cores acquire the G protein and a host-derived lipid envelope as they bud through the inner nuclear membrane (INM), and accumulate as mature particles in the perinuclear space (Martins et al., 1998; van Beek et al., 1985). The relationship between the sites of nucleocapsid assembly and viral morphogenesis has not been definitively determined for plant-adapted rhabdoviruses. However, biochemical characterization of purified cores and virus suggest that SYNV is structurally similar to the Indiana strain of VSV, for which the estimated numbers of molecules per infectious virus particle are, N (1,000-2,000); P (100-300); M (1,500-4,000) G (500-1,500); L (20-50), (Tordo et al., 2005). For the cognate proteins of SYNV, this represents roughly a ten to one molar ratio of N to P per particle. In contrast, purified complexes have been shown to be equimolar or in a 2:1 ratio with respect to the N and P proteins of VSV (Masters & Banerjee, 1988). Results from purification of N:P complexes of SYNV are consistent with these data (Goodin et al., 2001; unpublished data). According to current models for the assembly of VSV, the excess P is removed during maturation of the nucleocapsid (Green et al., 2006; Green et al., 2000).

In addition to coordination of the activities of viroplasm-associated proteins, infection of plants with members of the genus *Nucleorhabdovirus* results in marked alterations in nuclear membranes (Goodin *et al.*, 2005; Martins *et al.*, 1998). In the case of SYNV, there is an invagination of the INM into the nucleus. Thus, single membranes surround sites at which virions accumulate (Martins *et al.*, 1998).

These alterations of nuclear membranes can be observed by live-cell imaging of rhabdovirus-infected *Nicotiana benthamiana* “16c” plants, which express endomembrane-targeted green fluorescent protein (hereafter referred to as mGFP5-ER plants; Brigneti *et al.*, 1998; Goodin *et al.*, 2005; Haseloff *et al.*, 1997). However, it was unclear from our initial study (Goodin *et al.*, 2005), as well as previous EM studies (Martins *et al.*, 1998), whether the membrane-bound sites of virion accumulation remain contiguous with the endomembrane system. Determination of this relationship profoundly impacts proposed models for rhabdoviral morphogenesis and systemic movement (Jackson *et al.*, 2005). If the intranuclear membranes are not contiguous with the endomembrane system then virion maturation may be a terminal process in plants that does not contribute to systemic movement of these viruses. Alternatively, if the intranuclear membranes are contiguous with the endomembrane system, then mature, or partially budded virions, may participate in cell to cell movement by associating with components of the endomembrane system, which are contiguous with desmotubules that pass through plasmodesmata connecting adjacent cells (Lucas, 2006; Scholthof, 2005).

That the endomembrane system of a host cell may play a role in rhabdovirus cell to cell movement comes from studies that show the presence of mature particles of *Maize mosaic virus* (MMV) in ER tubules in cells of its insect vector (Herold & Munz, 1965). Experimental support for either of the models described above requires the characterization of the virus-induced intranuclear membranes as well as their relationship to sites of SYNV protein accumulation. Therefore, we conducted experiments using fluorescence recovery after photobleaching (FRAP) and total internal reflectance fluorescence microscopy (TIRFM). Given that mGFP5-ER plants accumulate soluble GFP in the lumen of the endoplasmic reticulum (ER) and nuclear membranes (Brigneti *et al.*, 1998; Goodin *et al.*, 2005; Ruiz *et al.*, 1998; Turner *et al.*, 2004), we reasoned that FRAP should be rapid if virus-induced nuclear membranes were contiguous with the ER. Alternatively, if the sites of virus accumulation were completely membrane-bound then no FRAP should occur since GFP is not membrane permeable (Collings *et al.*, 2000; Sbalzarini *et al.*, 2005). Moreover, the biological relevance of the rhabdovirus-induced intranuclear membranes in infected mGFP5-ER plants would be enhanced if their linkage with the viroplasm could be determined. We investigated the relationship between intranuclear membranes and viroplasm by transiently expressing autofluorescent protein (AFP) fusions of the SYNV N, P, sc4, M, and G proteins in virus-infected cells.

In addition to integrating localization data for SYNV-encoded proteins into models for rhabdovirus assembly and morphogenesis, our data underscore the importance of conducting protein localization studies in the context of viral infection.
METHODS

Plant growth, viral inoculation and detection

Virus inoculations and maintenance of non-transgenic or mGFP5-ER plants (Brigneti et al., 1998) *N. benthamiana* plants were conducted as described (Goodin et al., 2005; Senthil et al., 2005). Note that mGFP5-ER is targeted to the ER via an amino-terminal signal sequence derived from the *Arabidopsis thaliana* basic chitinase and a carboxy-terminal HDEL, ER-retention signal. Because of the contiguity of the outer nuclear membrane with the ER, GFP-HDEL also accumulates in the lumen of the nuclear envelope (Collings et al., 2000; Haseloff et al., 1997).

Construction of binary vectors for expression of fluorescent protein fusions in plant cells

**Vectors for visualization of SYNV-M, -sc4, and G were built by Kathleen Martin.**

Binary vectors employed in this study for transient expression of autofluorescent proteins (AFP) fusions in plant cells were derivatives of the pSAT series described (Chung et al., 2005; Tzfira et al., 2005). Following confirmation by PCR-screening, recombinant binary vectors were transformed into *Agrobacterium tumefaciens* as described (Goodin et al., 2002), except that strain LBA4404 was used instead of C58C1. Primers for PCR were designed according to the SYNV sequences deposited into Genbank. The accession numbers for each SYNV gene were L32603 (N); AY971951 (P); M35689 (M); L32604 (sc4); L32603 (G) and M87829 (L). The construction of correct in-frame fusions between AFPs and SYNV genes was validated by DNA sequencing and immunodetection (data not shown).

Transient expression of proteins in plant cells using agroinfiltration

**Expression of SYNV genes in plant cells was conducted by Kathleen Martin.**

Suspensions of *A. tumefaciens* were infiltrated into leaves of *N. benthamiana* as previously described (Goodin et al., 2002; Tsai et al., 2005). In order to express proteins in SYNV-infected cells symptomatic leaves of plants were infiltrated at the peak of symptom expression, typically fourteen days post inoculation. Leaves were examined by confocal microscopy after incubation for 48 h under constant illumination.

In order to mark the nuclear envelope, we expressed GFP fused to the first 238 amino acids of the human lamin B receptor, as described by Irons et al., 2003.

Preparation of protoplasts

Protoplasts were prepared from mock-inoculated or virus-infected leaves of mGFP5-ER plants essentially as described by Panaviene et al. (2003). Prior to preparation of protoplasts, leaf samples were examined by epifluorescence microscopy to confirm the presence of virus-induced intranuclear GFP (Goodin et al., 2005). Protoplasts were immediately used for microscopy or kept in 10 x 35 mm petri plates in protoplast culture medium (Panaviene et al., 2003) at room temperature in the dark for up to 24 hours.
**Total internal reflectance fluorescence microscopy (TIRFM)**

TIRFM was conducted using a Nikon Inverted Microscope TE2000E equipped with CFI Plan ApoTIRF 60X-NA1.45 and CFI Plan ApoTIRF 100X-NA1.45 oil immersion objectives. Excitation of GFP was accomplished using the 488 nm line of a multi-line argon laser. Controlling software for image acquisition was Metamorph version 6.2 (Molecular Devices Corporation, Sunnyvale, CA).

**Laser scanning confocal microscopy**

All confocal microscopy was performed on an Olympus FV1000 (Olympus America Inc., Melville, NY). CFP, GFP and RFP were excited using 440 nm and 488 nm and 543 nm laser lines, respectively. When using multiple fluorophores simultaneously, images were acquired sequentially, line-by-line, in order to reduce excitation and emission cross talk. The primary objective used was an Olympus water immersion PLAPO60XWLSTM-NA1.0 (hereafter referred to as the 60X objective). Image acquisition was conducted at a resolution of 512 x 512 pixels and a scan-rate of 10 ms/pixel, except where noted. Control of the microscope, as well as image acquisition and exportation as TIFF files, was conducted using Olympus Fluoview software version 1.5. Exposure settings that minimized oversaturated pixels in the final images were used. Figures of micrographs were assembled using Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) and Canvas 8.0 (Deneba Software, Miami FL, USA).

**Fluorescence recovery after photobleaching (FRAP)**

We conducted FRAP experiments using leaf tissue harvested 10-14 dpi from SYNV-infected mGFP5-ER plants. Mock-inoculated plants of similar ages were used as controls. FRAP experiments were performed using the Olympus FV1000 described above. Briefly, 5 mm square sections of leaf tissue were mounted on glass slides in water and covered with a glass coverslip. Imaging for FRAP experiments was conducted using a 60X objective and 488 nm laser line from a multi-line argon laser set at 0.3% power. Regions of interest (ROIs) were photobleached for 50 ms using a 405 nm diode laser, set at full power, which was delivered via the FV1000 Simultaneous (SIM) scanner. Images for fluorescence recovery after photobleaching (FRAP) analyses were acquired at a resolution of 512 x 512 pixels and a scan-rate of 2 ms/pixel, which was necessary to monitor fast protein dynamics. Two images were acquired prior to photobleaching followed by an additional 7 images to monitor fluorescence recovery. Quantitative fluorescence data, in Excel format, and confocal images, in TIFF format, were exported using Olympus Fluoview software. FRAP experiments were repeated three times for each ROI, with 2 min between bleaching events in order to allow full recovery of fluorescence. For proteins such as SYNV-M that did not show FRAP, independent ROIs were used for each experiment. Replicated fluorescence intensity data were averaged and these data were normalized across experiments. Mean and standard deviations for fluorescence intensity at each timepoint were calculated and plotted using Excel software (Microsoft Corporation, Redmond, WA).
RESULTS

mGFP5-ER in SYNV-infected cells remains in the endomembrane system

We have previously reported that SYNV induces changes in the nuclear accumulation of mGFP5-ER (Goodin et al., 2005). In order to rule out that mGFP5-ER localization was not affected per se in virus-infected cells, we confirmed the membrane-associated fluorescence in the mGFP5-ER line by examination of protoplasts, derived from mock-inoculated or SYNV-infected leaves, by both TIRFM and confocal microscopy. In both cases GFP fluorescence was restricted to ER tubules with no detectable differences of fluorescence in the cytoplasm (Figure 3.1a-b). Given that the outer nuclear membrane is contiguous with the ER, the lumen of the nuclear envelope fills with mGFP5-ER (Collings et al., 2000; Figure 3.1c). As we have reported previously, mGFP5-ER accumulates in nuclei of SYNV-infected cells (Figure 3.1d; (Goodin et al., 2005).

SYNV-induced intranuclear membranes are contiguous with the ER

The current models, based largely upon light and electron microscopy data, suggest that SYNV morphogenesis takes place on the INM, which invaginates into virus-infected nuclei (Jackson et al., 2005). Condensed nucleocapsids of SYNV bud through the INM and accumulate as mature virions in the perinuclear space (Goodin et al., 2005; Martins et al., 1998; van Beek et al., 1985). We determined the relationship between intranuclear membranes by FRAP analysis. No significant difference in the diffusion rates of GFP was observed when either the nuclear envelope (NE) or any region-of-interest (ROI) of intranuclear membranes were photobleached (Figure 3.2a-b). Fluorescence intensity for all ROIs returned to 94-100% of pre-bleach values within the course of these experiments. The mean values for 50% recovery of fluorescence (t1/2) were approximately 2.3 seconds in all cases.

Localization of LBR:GFP in SYNV-infected or mock-inoculated cells

The human lamin B receptor (LBR) is targeted to the INM (Holmer & Worman, 2001; Irons et al., 2003; Makatsori et al., 2004). It has been determined that the amino-terminal 238 amino acids of LBR are sufficient to target GFP to the nuclear envelope in plant cells (Irons et al., 2003). We therefore used this fusion to determine if the SYNV-induced intranuclear membranes were single-stranded, as suggested by electron microscopy (Martins et al., 1998). Expression of LBR:GFP in mock-inoculated leaves resulted in accumulation of this marker primarily on the nuclear envelope (Figure 3.3a-c). In contrast, expression of LBR:GFP in SYNV-infected leaves resulted in accumulation of this marker on both the nuclear envelope and intranuclear membranes (Figure 3.3d-f). The relative fluorescence intensity of GFP on the nuclear envelope, measured in a 0.196 mm² ROI, which spans the average width of the nuclear envelope, was 1416 ± 222 units. In equivalent areas of the intranuclear membranes the fluorescence intensity was found to be 757 ± 110 units, a 47% reduction compared to the nuclear envelope (Figure 3.3g).
Localization of SYNV-proteins relative to intranuclear membranes in infected cells

Localization of rhabdoviral proteins in plant cells have been conducted in virus-free cells using light microscopy-based examination of autofluorescent protein fusions (Goodin et al., 2005; Goodin et al., 2002; Goodin et al., 2001; Tsai et al., 2005) or by immunological methods using light or electron microscopy (Martins et al., 1998; van Beek et al., 1985). However, these studies, which focused primarily on the N and P proteins, did not determine the localization of SYNV proteins in the context of infected cells. Therefore, we expressed the SYNV-N, -P, -sc4, -M and -G proteins as fusions to a monomeric red fluorescent protein in virus-infected and mock-inoculated cells of mGFP5-ER leaves (Figure 3.4). The majority of single optical sections showed that the RFP:P fusion in virus-infected nuclei accumulated in discrete ring-shaped structures that did not colocalize with intranuclear membranes (Figure 3.4a-c). In contrast, expression of this fusion in mock-inoculated cells resulted in diffuse accumulation throughout the entire nucleoplasm (Figure 3.4d). RFP:N accumulated in nuclei in contiguous areas that partially overlapped with the intranuclear membranes (Figure 3.4e-g), whereas this fusion expressed in mock-inoculated cells was entirely localized in the nucleoplasm without any association with the nuclear envelope (Figure 3.4h). RFP:M was found to be primarily colocalized with membranes in virus-infected nuclei whereas expression of this fusion in mock-inoculated cells resulted in accumulation throughout the entire nucleoplasm (Figure 3.4i-l). RFP:G expressed in virus-infected nuclei was found to be associated with intranuclear membranes (Figure 3.4m-o). Expression of RFP:G outside of the context of virus infection had a profound effect on nuclear membranes (Figure 3.4p). In contrast to virus-infected cells, RFP:G did not induce nor accumulate on intranuclear membranes. However, large aggregates of this protein formed in membrane-associated bodies on the nuclear envelope (Figure 3.4p). Expression of RFP alone showed that this protein was excluded from intranuclear membranes in virus-infected nuclei and accumulated diffusely in nuclei of mock-inoculated cells (Figure 3.4q-t). Expression of RFP fused to the 240 kDa SYNV-L protein did not result in detectable fluorescence or protein (data not shown).

Protein coexpression establishes the link between nucleoplasm-localized and membrane-associated proteins

In addition to their roles as structural proteins, the SYNV-N and -P proteins have been shown by electron microscopy studies to be localized in the viroplasm, which is believed to be the site of rhabdoviral replication (Martins et al., 1998). However, the spatial organization of viral proteins within the viroplasm has not been addressed in detail. Given the reported association of rhabdoviral N and P proteins as complexes and in association with viroplasma (Goodin et al., 2001; Majumdar et al., 2004; Martins et al., 1998) we considered it curious that the localization of the P protein did not overlap that of the intranuclear membranes, whereas that of the N protein did (Figure 3.4 b and f). Therefore, in order to further define the spatial relationship between viral proteins in SYNV-infected nuclei, we coexpressed CFP and RFP fusions of the N, P, sc4, M, and G proteins in pair-wise
combinations in virus infected leaves of non-transgenic *N. benthamiana* plants.

A subset of these micrographs is shown in Figure 3.5. Coexpression of CFP:P and RFP:N revealed that these proteins colocalize in the viroplasm but that the accumulation of P protein is reduced relative to N in regions immediately adjacent to the intranuclear membranes (Figure 3.5a-c). Consistent with this finding are the results obtained when CFP:P and RFP:M were coexpressed. In this case, CFP:P localized adjacent to RFP:M, which was shown to localize exclusively on intranuclear membranes in virus-infected nuclei (Figure 3.5d-f). Likewise, coexpression of CFP:M with RFP:N demonstrated that M colocalizes with N only on intranuclear membranes (Figure 3.5g-i). Finally, RFP:N partially colocalized with CFP:G, which was found to localize exclusively on intranuclear membranes (Figure 3.5j-l).

**FRAP analysis of the SYNV-N protein**

The N proteins of monopartite negative-strand RNA viruses are believed to exist *in vivo* as complexes with their cognate P proteins (Albertini *et al.*, 2006; Goodin *et al.*, 2001; Green *et al.*, 2006; Mavrakis *et al.*, 2006). It has been shown previously that coexpression of SYNV-N and -P results in colocalization of these proteins and that a soluble N/P complex can be isolated from cells coexpressing these proteins (Goodin *et al.*, 2001). Therefore, in order to obtain insight into the nature of the N protein in virus-infected cells, we compared the FRAP kinetics of RFP:N expressed in virus-infected nuclei and mock-inoculated cells in which CFP:P was also expressed. We were able to clearly distinguish membrane- and viroplasm-associated forms of N in virus-infected nuclei (Figure 3.6a and b). Consistent with the prediction that the majority of N in the viroplasm should exist as an N/P complex is the finding that RFP:N FRAP in viroplasm of virus-infected nuclei was not significantly different than RFP:N coexpressed with CFP:P in mock-inoculated cells (Figure 3.6a and b). FRAP for N expressed in mock-inoculated cells or associated with the viroplasm ranged from 70-80%, respectively, with t_{1/2} values of approximately 2.3 seconds. FRAP kinetics for RFP:N expressed in the absence of P was faster than that for this fusion in the presence of P (data not shown).

**RFP:M is incorporated into complexes that move on ER membranes**

Our live-cell imaging data, presented above, support a model that links nucleocapsid assembly and protein localization (Figure 3.7). However, the fate of virions that accumulate in the perinuclear space, as well as the mechanism by which plant-adapted rhabdoviruses move systemically in plants are poorly understood. It has been proposed that sc4 is the putative movement protein for SYNV (Goldberg *et al.*, 1991; Melcher, 2000). Indeed, P3, the *Rice yellow stunt virus* homolog of sc4, has been shown to complement a movement deficient mutant of PVX. (Huang *et al.*, 2005) further showed that P3 interacts with the RYSV nucleocapsid protein and thus proposed that this virus moves as a protein:nucleocapsid complex. Similar data for sc4 are lacking, however this protein localizes to punctate loci on cell walls, consistent with plasmodesmatal-targeting expected for virus movement proteins (Figure 3.8a-d). Intriguingly, we found that expression of RFP:M, which is entirely nuclear localized in mock-inoculated cells (Figure 3.4l; (Goodin *et al.*, 2002) or
associated with intranuclear membranes in virus-infected cells (Figure 4.4i-k), could also be found in complexes that appeared to bud from nuclei (Figure 3.8e-i). Examination of the M-containing complexes showed that they were surrounded by, and moved on, ER membranes (Figure 3.8j-s).

DISCUSSION

To date, protein localization studies for characterizing plant-adapted rhabdoviruses have been conducted outside the context of viral infections (Goodin et al., 2002; Goodin et al., 2001; Tsai et al., 2005) using fluorescence microscopy or in virus-infected cells for a limited number of proteins (Martins et al., 1998). Furthermore, the relationship between the localization of viral proteins and relocalized host cell and nuclear membranes has not been determined. By expressing autofluorescent protein fusions in SYNV-infected cells, we have revealed novel insight into the protein and membrane dynamics in the SYNV/N. benthamiana pathosystem.

Using TIRFM and confocal microscopy, we did not observe GFP in any cellular loci other than those contiguous with the endomembrane system. Therefore, the source of GFP in virus-infected nuclei should be the ER and lumen of the nuclear envelope. This contention is supported by FRAP analyses of GFP in the nuclear envelope and intranuclear membranes. Since there was no statistical difference in the FRAP kinetics of GFP in any of these loci, we conclude that the intranuclear membranes remain contiguous with the ER and are not confined to covalently closed intranuclear membranes. This finding is significant because it allows for the possibility that the intranuclear membranes are bona fide sites of virion assembly and not simply alterations in host membranes that do not participate in viral biology per se. Contiguity of the intranuclear membranes with the ER is essential for delivery of the glycosylated SYNV-G protein to the INM from the ER and Golgi. It is noteworthy that the G protein did not on its own induce formation of intranuclear membranes nor did coexpression of M and G, which for some negative-strand RNA viruses results in the budding of empty particles from transfected cells (Swenson et al., 2004). Therefore, we suspect that formation of the intranuclear membranes may require additional viral proteins and perhaps, RNA. Furthermore, while overexpression of viral glycoproteins commonly results in adverse cytopathic effects, such effects seen in mock-inoculated cells were absent or markedly reduced in virus-infected cells. Following budding into the perinuclear space, contiguity with the ER might provide rhabdoviruses with a continuous conduit by which to travel cell to cell. Although the current models for cell-to-cell movement, developed primarily from studies with plus-strand RNA viruses, do not favour virus movement through ER tubules, such a mechanism cannot be ruled out for plant-adapted rhabdoviruses. Indeed, MMV has been observed in ER tubules in cells of its insect vector (Herold & Munz, 1965). An alternate means for cell-to-cell movement that also requires contiguity of the ER with virus-induced intranuclear membranes is budding of mature virions from the perinuclear space to release the core particle, which could function as a movement complex. This mechanism would be akin to the bud-in bud-out, envelopment and de-envelopment of Herpes virus
particles which allow this virus to move from sites of replication and assembly in the nucleus to the cytoplasm and subsequently, by additional budding events, exit from the infected cell (Mettenleiter, 2004, 2006).

Both of the movement models above require that viral proteins be delivered to the intranuclear membranes in a manner consistent with the known function of rhabdoviral proteins in assembly (Jayakar et al., 2004). Therefore, we expressed all of the SYNV proteins as RFP fusions in plant cells, except for the 240 kDa L protein. In contrast to expression in mock-inoculated cells, the localization patterns in virus-infected cells were consistent with models for rhabdovirus assembly and morphogenesis proposed by (Green et al., 2006; Green et al., 2000) and (Jayakar et al., 2004). The first step in the budding process is formation of nucleocapsids by delivery of the N protein to nascent genomic-length RNAs via an N/P complex. The majority of P should be excluded from the RNA/N/P complex (Green et al., 2006) to form the mature nucleocapsid, which is in turn delivered to an M/G complex that has formed on membranes (Jayakar et al., 2004). The membrane-anchored nucleocapsid, as suggested by our “slow FRAP” data, is condensed by M to form a core particle which buds through the INM to form a mature virion. Consistent with this model (Figure 3.7) we found that the majority of the P protein did not colocalize with the intranuclear membranes. In fact, this protein appears to be excluded immediately adjacent to these membranes at loci occupied by the N and M proteins. In addition to colocalizing in part with membranes, the N protein was found in a highly mobile (“fast FRAP”) region in the nucleoplasm, consistent in location to the viroplasm, which is the proposed site of rhabdoviral replication (Martins et al., 1998).

That the intranuclear membranes upon which the N, M and G proteins associate, are derived from the nuclear envelope has been established by electron microscopy (Martins et al., 1998). Consistent with these results is the demonstration that the relative fluorescence intensity of the LBR:GFP marker on SYNV-induced intranuclear membranes is almost exactly half (53%) that of the fluorescence on the nuclear envelope. Because the LBR:GFP marker does not contain the lamin-binding domains, the distribution of this fusion on the outer and inner nuclear membranes is expected to be the same under steady-state observations. Therefore, as predicted, the fluorescence per unit area of a single membrane was half that of a double membrane. We do not suspect that the reduction in fluorescence is due to occlusion of LBR:GFP by SYNV-encoded proteins because SYNV-G does not hyper-accumulate on intranuclear membranes relative to the nuclear envelope.

During the course of our localization studies, we discovered heretofore unreported complexes in the cytoplasm of virus-infected cells that incorporated matrix protein fusions to CFP or RFP. Further analyses showed that these complexes were liberated from nuclei of virus-infected cells, which then proceeded to track on ER membranes. We have not yet been able to label these complexes with fluorescent fusions of other SYNV proteins, most notably N or P, which might indicate that nucleocapsid cores were also associated with these complexes. However, given the small amounts of P protein in virus particles relative to M and N (data not shown), it may not be possible to detect these complexes in the same
manner that VSV nucleocapsids have labeled using GFP:P fusions (Das et al., 2006). Extensive analyses failed to reveal such complexes in mock-inoculated leaves in which RFP:M was coexpressed. Further, that these complexes are ER-associated is intriguing as it suggests that G could also be a part of the complex. However, that the observed matrix protein complex is the *bona fide* SYNV movement complex will require extensive characterization by electron microscopy in planned future studies. Intriguingly, one way such complexes could arise, if they are derived from mature virions in the perinuclear space, is via budding through the outer nuclear membrane, which would release cores into the cytoplasm. Therefore, when considered with our FRAP data which show that virus-induced nuclear membranes are contiguous with the ER, it is conceivable that SYNV moves cell to cell via matrix-protein condensed cores that track on ER membranes. Further investigation into the characterization of these complexes is thus warranted in future studies.

Taken together, our live-cell imaging conducted in the context of virus-infected cells revealed the spatial relationship between viral proteins that suggests a contiguous pathway from the putative sites of viral replication to those of morphogenesis. The protein and localization data presented here could not be gleaned from studies conducted in the traditional manner of expression in virus-free cells. Therefore, the ability to express autofluorescent protein fusions in the context of virus-infection represents a significant advance in our ability to study plant:rhabdovirus interactions in live cells.
Figure 3.1. (a, b) TIRFM micrographs showing the distribution of ER tubules in protoplasts derived from leaves of mock-inoculated (a) or SYNV-infected (b) mGFP5-ER *N. benthamiana* plants. Bars, 10 μm. (c, d) Confocal micrographs showing nuclear membranes (green) of mock-inoculated (c) or SYNV-infected (d) leaf epidermal cells. Nuclei were counterstained with the DNA-selective dye 4,6-diamidino-2-phenylindole (DAPI; blue). Bars, 2 μm.
Figure 3.2. (a) Normalized FRAP data following photobleaching of GFP in the nuclear envelope (N.E.) or three different regions of interest (ROIs 1–3) in the same nucleus. Experiments were repeated three times and the mean±SD fluorescence intensity for each time point was plotted. The origin of the y-axis is defined as the point at which a 50 ms pulse from the bleaching laser was activated. (b) Confocal micrographs used to generate the fluorescence data presented in (a). The photobleaching event is shown as the zero time point. Micrographs taken immediately before and after activation of the photobleaching laser, at the times indicated, are shown. The photobleached area, and corresponding area in the pre-bleach image, are indicated by white arrows. Bars, 2 μm.

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Figure 3.3. Localization of a nuclear-envelope marker in SYNV- infected nuclei. Confocal micrographs of an LBR–GFP fusion in mock-inoculated (a–c) and SYNV-infected (d–f) mGFP5-ER transgenic plants. Fluorescence images for the DNA-selective dye DAPI, GFP and the corresponding overlay are shown. Bars, 2 μm. (g) Relative GFP fluorescence intensity on the nuclear envelope (N.E.) compared with that for SYNV-induced intranuclear membranes. Shown are the means ±SD for measurements of fluorescence intensity in 0.5 mm diameter circular ROIs positioned on the nuclear or intranuclear membranes.
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Figure 3.4. Confocal micrographs of RFP fusions of SYNV proteins expressed in SYNV-infected and mock-inoculated mGFP5-ER plants. Fluorescence images for GFP, RFP and the corresponding overlay are shown for each fusion expressed in SYNV-infected cells. Only the overlay is shown for fusions expressed in mock-inoculated leaves. (a–c) Localization of RFP–P in an SYNV-infected nucleus. (d) Localization of RFP–P in the nucleus of a mock-inoculated cell. (e–g) Localization of RFP–N in an SYNV-infected nucleus. (h) Localization of RFP–N in the nucleus of a mock-inoculated cell. (i–k) Localization of RFP–M in an SYNV-infected nucleus. (l) Localization of RFP–M in the nucleus of a mock-inoculated cell. (m–o) Localization of RFP–G in an SYNV-infected nucleus. (p) Localization of RFP–G in the nucleus of a mock-inoculated cell. (q–s) Localization of RFP in an SYNV-infected nucleus. (t) Localization of RFP in the nucleus of a mock-inoculated cell. Regions of colocalization are shown in yellow. Bars, 2 μm.
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Figure 3.5. Confocal micrographs showing the coexpression of CFP (cyan) and RFP (red) fusions in SYNV-infected nuclei. In order to permit unambiguous differentiation between fluorescent protein fusions, expression was conducted in wild-type instead of mGFP5-ER plants. (a–c) Coexpression of CFP–P and RFP–N. (d–f) Coexpression of CFP–P and RFP–M. (g–i) Coexpression of CFP–M and RFP–N. (j–l) Coexpression of CFP–G and RFP–N. Regions of colocalization are shown in white. Bars, 2 μm.
Figure 3.6. (a) Normalized FRAP data following photobleaching of RFP–N localized on intranuclear membranes (mem.) or nucleoplasm (nucleo.) in nuclei of virus-infected cells, or in nuclei of mock-inoculated cells in which RFP–N and CFP–P were coexpressed (N/P mock). Experiments were repeated three times and the mean±SD fluorescence intensity for each time point was plotted. The origin of the y-axis is defined as the point at which a 50 μs pulse from the bleaching laser was activated. (b) Confocal micrographs used to generate the fluorescence data presented in (a). The photobleaching event is shown as the zero time point. Micrographs taken immediately before and after activation of the photobleaching laser, at the times indicated, are shown. The photobleached area, and corresponding area in the pre-bleach image, are indicted by white arrows. Bars, 2 μm.

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Figure 3.7. Model for the spatial relationship between sites of SYNV replication and morphogenesis. Our localization studies, in addition to a large body of published literature, suggest that the rhabdoviral N protein is delivered to the nascent strands of viral RNA via an N–P complex, which, being soluble, would result in rapid fluorescence recovery following photobleaching (‘Fast FRAP’). However, much of the P is removed from the intermediate N–P–RNA complex during maturation of the nucleocapsid, which in turn associates with the M protein, resulting in condensation of the nucleocapsid into core particles and association with G on the INM. Membrane-anchored N protein should have markedly reduced FRAP kinetics (‘Slow FRAP’) compared with the soluble N–P complex. This results in a lack of P accumulation at the loci where N, M and G are colocalized. Following assembly on the INM, core particles bud into the perinuclear space. The fate of mature virions in the perinuclear space is currently unknown. However, nucleorhabdovirus particles have been observed in the ER and cytoplasm, suggesting that virion accumulation in the perinuclear space may not be a terminal event (see Discussion). Adapted from Green et al. (2000).
**Figure 3.8.** Localization of sc4 in SYNV-infected (a–c) and mock-inoculated (d) cells. Punctate accumulations were most prevalent in virus-infected cells (arrows). (e–i) Confocal micrographs showing the time course of liberation of complexes (white arrow) containing M protein from the nucleus (red area, bottom left-hand corner). These micrographs are overlays of fluorescence of RFP–M (red) and GFP (green) in an SYNV-infected cell. Bars, 5 μm. (j–n) Confocal micrographs showing the time course of movement of RFP–M-containing complexes (red) on ER membranes (green). Bars, 2 μm. (o–s) Green channel only of the confocal micrographs shown in (j–n). Bars, 2 μm. M-containing complexes were observed only when CFP–M or RFP–M fusions were expressed in virus-infected cells and were never observed when these fusions were expressed in mock-inoculated cells (data not shown).

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CHAPTER IV
Comparison of the functional domains of Sonchus yellow net virus and Lettuce necrotic yellows virus glycoproteins.

Rhabdoviruses are single-stranded negative-strand RNA viruses in the order Mononegavirales. There are two genera of plant-infecting rhabdoviruses, Nucleorhabdovirus and Cytorhabdovirus, so classified based on their sites of virus replication. As their name implies, the nucleorhabdoviruses like Sonchus yellow net virus (SYNV) replicate in the nucleus of the infected plant cells, and cytorhabdoviruses such as Lettuce necrotic yellows virus (LNYV), replicate in the cytoplasm (Dietzgen et al., 2006; Jackson et al., 2005). Both viruses contain six open reading frames corresponding to the nucleoprotein (N), the phosphoprotein (P), the movement protein (sc4 in SYNV, 4b in LNYV), the matrix protein (M), the glycoprotein (G) and the polymerase protein (L). The single-stranded RNA is associated with the N, P and L proteins to form a viral core. The matrix protein condenses this viral core, and it associates with the viral glycoprotein as it buds through the host membrane (Jackson et al., 2005). The movement protein is thought to facilitate the cell-to-cell movement of the virus (Huang et al., 2005; Melcher, 2000; Min et al., 2010; Scholthof et al., 1994).

It has been determined previously that SYNV G has 6 predicted N-linked glycosylation sites and treatment with tunicamycin, an N-linked glycosylation inhibitor, results in a protein with an apparent molecular weight 10% smaller compared to wildtype (Jones & Jackson, 1990). Tunicamycin also negatively affects the ability of the virus to complete morphogenesis in protoplasts (van Beek et al., 1985). In comparison, LNYV has three predicted N-linked glycosylation sites and no studies on tunicamycin have been conducted (Dietzgen et al., 2006). Other than this, much of what we know about the glycoprotein comes from the animal rhabdovirus model, Vesicular stomatitis virus (VSV), which replicates in the cytoplasm and buds at the plasma membrane. The glycoprotein of VSV is co-translationally inserted into the endoplasmic reticulum, forms a trimer and localizes to the plasma membrane (Brown & Lyles, 2003; Lingappa et al., 1978; Roche et al., 2006; Roche et al., 2007). The viral core condensed by matrix proteins associates with the glycoprotein at the carboxy terminal cytoplasmic side and budding through the plasma membrane occurs (Lyles et al., 1992; Whitt et al., 1989). Once the virion reaches a new cell, VSV G binds to an unknown receptor(s) at the plasma membrane and endocytosis of the virion occurs. Inside the endosomes, the low pH triggers the fusion of the viral envelope to the endosome membrane and the nucleocapsid is released into the cytoplasm and the infection cycle begins anew (Johannsdottir et al., 2009; Le Blanc et al., 2005).

Compared to VSV, studies of fluorescent protein fusions to SYNV G during infection show localization to the inner nuclear membrane (Goodin et al., 2007b). Based on electron micrographs, this is the expected placement of SYNV G during infection, as the mature virion of SYNV accumulates in the perinuclear space after budding through the inner nuclear membrane (Christie et al., 1974; Ismail et al., 1987). SYNV G has also been shown to self-associate in bimolecular fluorescence complementation (BiFC) assays, consistent with a multi-meric form. It has also been
shown to interact with sc4 (the movement protein) in a possible movement complex that still occurs when G is truncated to 400 aa, the size of a truncated form of G that is detected in western analysis (Min et al., 2010). Compared to SYNV G, LNYV G localizes to the endoplasmic reticulum (ER) membranes with some accumulation around the nucleus. The electron microscopy of LNYV reveals aggregations of mature virions in the ER with an expected viroplasm in the cytoplasm (Chambers et al., 1965). There is no localization or interaction data for LNYV G.

In comparison to VSV, there is limited knowledge of how either SYNV G or LNYV G arrives at the final localization during infection. Specifically of interest is the localization of SYNV G into the inner nuclear membrane. We postulated that this might be the role of the predicted nuclear localization signals (NLSs) as these are not present in LNYV G, which replicates in the cytoplasm. To determine if this assumption is correct, we localized fragments of both the SYNV and the LNYV glycoprotein to determine if the functional domains involved in final localization of these proteins can be identified.

METHODS

Plant growth

Wildtype and transgenic plants of Nicotiana benthamiana expressing either green fluorescent protein or red fluorescent protein targeted to the ER were grown in the greenhouse under ambient conditions.

Predicted domains of SYNV and LNYV glycoproteins

Sequences corresponding to the full length of either the SYNV or LNYV glycoprotein were analyzed for protein domains utilizing a variety of programs available on the Expasy webpage (http://expasy.org). These were PSORT for prediction of protein localization (Nakai & Kanehisa, 1991), SignalP for prediction of signal peptide cleavage sites (Bendtsen et al., 2004), TMpred for prediction of transmembrane domains (Hofmann & Stoffel, 1993) and NetNGlyc for prediction of N-linked glycosylation sites (Blom et al., 2004). Comparison of the similarities between the glycoproteins of all sequenced plant rhabdoviruses was done using the same algorithms, using the amino acid sequences provided in Genbank (see Table 4.2).

Protein expression in N. benthamiana

The cDNA corresponding to the SYNV full-length glycoprotein was used as a template for the polymerase chain reaction (PCR) amplification of truncation mutants. Primers designed to amplify fragments of SYNV G were utilized to create mutants which contained only the signal peptide (Fragment 1), the signal peptide to the first glycosylation site (Fragment 2), the signal peptide to the second glycosylation site (Fragment 3), the signal peptide to the beginning of the transmembrane domain (Fragment 4), the signal peptide to the end of the transmembrane domain (Fragment 5) and finally from the end of the transmembrane domain to the carboxy terminus of SYNV G, not including the stop codon (Fragment 6). Primers for construction are included as Table 4.1. A similar
strategy was utilized to create fragments of the glycoprotein of LNYV, however, the second truncation mutant does not correlate to the second glycosylation site of this protein. Primers for construction are also included as Table 4.1. Each fragment of SYNV G or LNYV G, as well as, full-length G proteins of the two viruses were cloned into pDONR221 and sequenced. From pDONR221, recombination reactions were carried out to move the fragments into pSITE vectors for localization. Vectors used during the course of this study are pSITE-2CA (green fluorescent protein fusions) and pSITE-4CA (red fluorescent protein fusions). Fusions were infiltrated either transiently or in transgenic plants containing an ER targeted protein. The full-length or carboxy terminus of SYNV G containing NLSs were tested with bimolecular fluorescence complementation (BiFC) to determine interactions with importin proteins of *N. benthamiana* as was described previously (Martin *et al.*, 2009). The SYNV G carboxy terminus was cloned into pSITE-nEYFP-C1 or pSITE-cEYFP-C1 and tested with previously constructed clones for importin alpha (Martin *et al.*, 2009). Glutathione-S-transferase (GST) was used as a non-binding control.

**Laser Scanning Confocal Microscopy**

All microscopy was conducted on an Olympus FV1000 laser scanning confocal microscope as described previously (Goodin *et al.*, 2005). BiFC analyses were done as was described previously (Martin *et al.*, 2009).

**RESULTS**

**Comparison of SYNV and LNYV glycoprotein genes in silico**

The open reading frame for SYNV G is 1899 nucleotides in length and 633 amino acids including a stop codon. In a previous study of SYNV G sequences, a signal peptide, six glycosylation sites (Asn-X-Ser/Thr), one transmembrane domain, and a NLS was identified (Goldberg *et al.*, 1991). In this study, the positions of the signal peptide, the glycosylation sites and the transmembrane domain were confirmed as similar to the original study with the exception of the transmembrane domain position originally described as spanning amino acids 570 to 594 (Goldberg *et al.*, 1991). The prediction program used in this study identified the transmembrane domain as spanning amino acids 561 to 577. This study also identified the presence of not one NLS at 591, but two at 590 aa and 616 aa (Figure 4.1).

The open reading frame for LNYV G is 1656 nucleotides in length and 552 amino acids including a stop codon. Originally described as having an N-terminal signal peptide, three glycosylation sites and a C-terminal transmembrane domain (Dietzgen *et al.*, 2006). This was confirmed in this study with the prediction of a 25 aa signal peptide, three glycosylation sites (Asn-X-Ser/Thr) at positions 28, 241 and 272 aa, and a carboxy terminal transmembrane domain spanning amino acids 504 to 524 (Figure 4.1).

Based on predictive algorithms, there is a difference in length of the signal peptides between SYNV G and LNYV G, with LNYV G’s signal peptide 5 amino acids longer. SYNV also contains three additional glycosylation sites when compared to LNYV. LNYV has a longer transmembrane domain when compared to SYNV. One
difference is the presence of two predicted NLSs in SYNV G that are absent from LNYV G.

**Localization of fragments of the SYNV and LNYV Glycoproteins**

Full-length SYNV G localizes to membranes with accumulation around the nucleus and in the ER (a-c, Figure 4.2). Fragment 1 corresponding to the signal peptide localizes to the cell periphery and membrane around the nucleus (d-f, Figure 4.2). Fragment 2, amino acids 1-39, localizes to the ER and shows colocalization with the RFP-ER marker (g-i, Figure 4.2). Fragment 3, amino acids 1-93, localizes to the ER and shows colocalization with the RFP-ER marker (j-l, Figure 4.2). Fragment 4, corresponding to amino acids 1-561 or the start of the transmembrane domain, the localization is surrounding the nucleus, and colocalizes with the RFP-ER marker surrounding the nucleus (m-o, Figure 4.2). Fragment 5, amino acids 1-578, corresponding to the end of the transmembrane domain, localization surrounding the nucleus and colocalizes with the RFP-ER surrounding the nucleus (p-r, Figure 4.2). Fragment 6, corresponding to the carboxy terminus, amino acid positions 578-632, localizes to the nucleus and nucleolus (s-u, Figure 4.2).

Full-length LNYV G localizes to the ER with some accumulation near the nucleus (a-c, Figure 4.3). Fragment 1, corresponding to the signal peptide of LNYV-G, localizes to the nucleus and cell periphery and colocalizes with the RFP-ER marker at the cell periphery and around the nucleus (d-f, Figure 4.3). Fragment 2, amino acids 1-28, localizes to the cell periphery and around the nucleus and colocalizes with the RFP-ER marker (g-i, Figure 4.3). Fragment 3, amino acids 1-93, localizes to the cell periphery and colocalizes with the RFP-ER marker (j-l, Figure 4.3). Fragment 4, amino acids 1-504, localizes to the ER membranes surrounding the nucleus (m-o, Figure 4.3). Fragment 5, amino acids 524-552, localizes to the cell periphery and in the nucleus (p-r, Figure 4.3).

**Bimolecular Fluorescence Complementation of the SYNV glycoprotein with Nicotiana benthamiana importins**

An interaction between SYNV N and *N. benthamiana* importin α 1 and 2 was described in a previous study (Martin *et al.*, 2009). As SYNV N contains a predicted NLS that has been shown to interact with importins to enter the nucleus, it remains to be determined if the predictable NLSs in SYNV G also interact with either known isoform of importin in *N. benthamiana* (Goodin *et al.*, 2001; Martin *et al.*, 2009). To test this theory we conducted BiFC analyses of both the full length G and the carboxy terminus alone with the two isoforms of importin α. Full-length G interacts with itself (a-c, Figure 4.4). Full-length G does not interact with either importin α 1 (d-f, Figure 4.4) or importin α 2 (g-i, Figure 4.4). Fragment 6 corresponding to the carboxy terminus, amino acids 578-632, does not interact with either importin α 1 (j-l, Figure 4.4) or importin α 2 (m-o, Figure 4.4). Full-length G or any of the fragments do not interact with GST, however, full-length G is shown as an example (p-r, Figure 4.4).
SYNV G and LNYV G are similar in their conserved domains including the presence of a signal peptide, glycosylation sites and a transmembrane domain (Dietzgen et al., 2006; Goldberg et al., 1991). SYNV G has NLSs in the carboxy terminus that are not present in LNYV G. The localization of various fragments of both glycoproteins from either LNYV or SYNV G reveal that the signal peptide directs GFP to the ER consistent with the idea that the glycoprotein begins translation in the cytoplasm, but is directed into the ER similar to VSV (Lingappa et al., 1978). The two slightly larger fragments corresponding to the first and second glycosylation sites in SYNV G also localize to the ER. This is similar for LNYV G. The fragment corresponding to the entire glycoprotein truncated at the beginning of the transmembrane domain localizes to a site similar to the full-length protein in uninfected N. benthamiana cells. This may indicate that in the absence of infection, the transmembrane domain and the carboxy terminus are not required for localization in the membranes surrounding the nucleus for SYNV and to various sites in the ER for LNYV. It is also not uncommon for full-length LNYV G and LNYV G fragment 4 (truncation of transmembrane domain and carboxy terminus) to accumulate near the nucleus. This may be because the virus is also known to be present in the ER surrounding the nucleus (Chambers et al., 1965).

Fragments lacking the carboxy terminus of SYNV G (4 and 5) were infiltrated into virus infected cells for comparison of these truncated pieces with the full-length protein. However, there was no expression detected in these tissues. As it is extremely difficult to infiltrate into tissues infected with SYNV, it is unclear whether continued attempts may reveal the significance of the carboxy terminus on localization during infection. However, this knowledge will bring only limited insight, as it is impossible to determine if the truncation would hinder viral fitness due to the lack of a reversible genetics system for this virus. Similar localizations of LNYV G pieces into infected cells were not attempted.

The glycoprotein of SYNV presents an interesting study in terms of nuclear import of a membrane bound protein, the final localization of this protein in virus-infected cells is in the inner nuclear membrane (Goodin et al., 2007b). The carboxy terminus of SYNV G expressed alone localizes to the nucleus and the nucleolus in uninfected cells, indicating that there is some means of nuclear localization of this piece. Although the fragment is small, only 54 amino acids, this GFP fusion is localized only to the nucleus and is not on the cell periphery (s.u; Figure 4.2). If a signal peptide is added to this construct (Signal peptide-GFP-SYNV G cterm), the localization pattern resembles that of the wildtype protein (data not shown). This may indicate that the final localization of SYNV G is due to signals within the carboxy terminus only when first directed to the ER. Moreover, SYNV G contains predicted NLSs but does not interact with importin α 1 or 2, either in the full-length glycoprotein or in the carboxy terminus sequences expressed separately. This could mean that the form of importin responsible for this import into the inner nuclear membrane has not yet been identified. In insect cells, a form of importin α (importin α-16) was identified which specifically targeted proteins to the inner nuclear membrane, and it may be that a similar protein in plants is responsible for final
localization of SYNV G (Saksena et al., 2006). Another possibility is that viral infection is also required for the interaction of the glycoprotein with either known importin.

Interestingly, NLSs are also absent from the glycoprotein sequences of the other plant rhabdoviruses currently available in Genbank, with the exception of Maize mosaic virus (MMV). This virus also contains one predicted NLS, but it is before the transmembrane domain unlike SYNV whose NLSs are both present in the cytoplasmic carboxy terminus (Table 4.2). However, as there are no other plant rhabdoviruses with predictable NLSs after the transmembrane domain, the significance of this remains unknown. Added to that, the predictable NLSs do not interact with known importin alphas present in N. benthamiana. Although previous studies with the yeast protein helix-extension-helix-2 (Heh2) showed that mutation of the NLSs caused this protein to be excluded from the inner nuclear membrane (wildtype localization), the specific mutation of the NLSs of SYNV G has yet to be done (King et al., 2006; Lusk et al., 2007). In contrast, the LNYV G carboxy terminus is localized to both the nucleus and the cell periphery without the presence of NLSs (p-r, Figure 4.3). The small size of the LNYV G carboxy terminus (28 amino acids) may allow for free diffusion of this fragment into the nucleus similar to what is observed for GFP alone.

The carboxy terminus of rhabdoviruses are also predicted to interact with the matrix proteins during morphogenesis to enable the viral cores to bud through the membrane of choice, for nucleorhabdoviruses, the inner nuclear membrane, and for cytorhabdoviruses the ER membrane. This prediction is based on the interactions of the animal rhabdovirus VSV (Lyles et al., 1992; Whitt et al., 1989). However, in SYNV an interaction between M and G has not been demonstrated (Min et al., 2010). An interaction of the c-terminus alone also does not interact with M (data not shown). However, this differs in the Potato yellow dwarf virus (PYDV) glycoprotein where interaction between M and G was detected in BiFC assays (Bandyopadhyay et al., 2010). However, it should be noted that the interaction detected between M and G of PYDV was with the N terminus of PYDV G and not the carboxy terminus as was shown for VSV.

The previous work of SYNV has determined that G associates with itself and with the movement protein. This is added to the new information that the carboxy terminus and transmembrane domain are not needed for aggregation around the nucleus. This is similar to the localization of the truncated version of LNYV G. In uninfected cells, this fragment shares the same localization as full-length. This may indicate that the targeting signals for the final localization reside between the second glycosylation site and the transmembrane domain. However, the carboxy terminus is also capable of aggregating around the nucleus when first directed to the ER. Further mutation analysis is required to determine if the domains for final localization are in a similar location between LNYV and SYNV, and if multiple domains may be necessary for the final localization of the glycoprotein of these viruses.
Table 4.1. Primers for the amplification of fragments of the Glycoproteins of SYNV and LNYV

**SYNV Glycoprotein Fragments**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-21 aa</td>
</tr>
<tr>
<td>2</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-39 aa</td>
</tr>
<tr>
<td>3</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-93 aa</td>
</tr>
<tr>
<td>4</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-561 aa</td>
</tr>
<tr>
<td>5</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-578 aa</td>
</tr>
<tr>
<td>6</td>
<td>AAAGAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>578-632 aa</td>
</tr>
</tbody>
</table>

**Full Length**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-632 aa</td>
</tr>
</tbody>
</table>

**LNYV Glycoprotein Fragments**

<table>
<thead>
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<th>Fragment</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-25 aa</td>
</tr>
<tr>
<td>2</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-28 aa</td>
</tr>
<tr>
<td>3</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-93 aa</td>
</tr>
<tr>
<td>4</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-504 aa</td>
</tr>
<tr>
<td>5</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>524-551 aa</td>
</tr>
</tbody>
</table>

**Full Length**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAAAACAGGGCTTATgttctacatatgaacc</td>
<td>AGAAAGCTGGGTAaagacctagatgaacccactgtctaaacaa</td>
<td>1-551 aa</td>
</tr>
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</table>
Table 4.2. Comparison of the Glycoproteins for all sequenced Plant-adapted Rhabdoviruses in Genbank

<table>
<thead>
<tr>
<th>Virus</th>
<th>Length (aa)</th>
<th>Signal Peptide</th>
<th>Glycosylation sites</th>
<th>TM</th>
<th>C-terminus</th>
<th>NLS</th>
<th>Genbank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize mosaic virus</td>
<td>591</td>
<td>1-21 aa</td>
<td>76, 239, 275, 511</td>
<td>551-573</td>
<td>RKKP at 431 aa</td>
<td>NONE</td>
<td>YP_052854</td>
</tr>
<tr>
<td>Taro vein chlorosis virus</td>
<td>587</td>
<td>1-16 aa</td>
<td>72, 84, 235, 271, 448, 505</td>
<td>552-574</td>
<td>13 aa</td>
<td>NONE</td>
<td>AAV92086</td>
</tr>
<tr>
<td>Maize Iranian mosaic virus</td>
<td>594</td>
<td>1-16 aa</td>
<td>76, 88, 239, 275, 452, 509</td>
<td>550-569</td>
<td>25 aa</td>
<td>NONE</td>
<td>ABA60888</td>
</tr>
<tr>
<td>Potato yellow dwarf virus</td>
<td>607</td>
<td>1-18 aa</td>
<td>6, 108, 156, 169, 464</td>
<td>576-591</td>
<td>16 aa</td>
<td>NONE</td>
<td>ADE45273</td>
</tr>
<tr>
<td>Rice yellow stunt virus</td>
<td>669</td>
<td>1-24 aa</td>
<td>114, 117, 149, 302, 333, 473, 480, 513, 548, 555</td>
<td>616-638</td>
<td>31 aa</td>
<td>NONE</td>
<td>NP_620500</td>
</tr>
<tr>
<td>Maize fine streak virus</td>
<td>596</td>
<td>1-20 aa</td>
<td>64, 131, 132, 139, 204, 325, 438, 494</td>
<td>529-551</td>
<td>45 aa</td>
<td>NONE</td>
<td>AAT66749</td>
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<tr>
<td>Sonchus yellow net virus</td>
<td>632</td>
<td>1-20 aa</td>
<td>39, 93, 385, 501, 512, 541</td>
<td>561-578</td>
<td>54 aa</td>
<td>KKKR at 590 aa</td>
<td>AAA47898</td>
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<td>Northern cereal mosaic virus</td>
<td>483</td>
<td>1-21 aa</td>
<td>28, 224, 318, 330, 381, 420</td>
<td>447-464</td>
<td>19 aa</td>
<td>NONE</td>
<td>VGLG_NCMV</td>
</tr>
<tr>
<td>Lettuce necrotic yellows virus</td>
<td>552</td>
<td>1-25 aa</td>
<td>28, 241, 272</td>
<td>504-524</td>
<td>28 aa</td>
<td>NONE</td>
<td>CAC18651</td>
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<tr>
<td>Lettuce yellow mottle virus</td>
<td>548</td>
<td>1-25 aa</td>
<td>28, 437, 472, 527</td>
<td>497-516</td>
<td>32 aa</td>
<td>NONE</td>
<td>ABV56128</td>
</tr>
</tbody>
</table>
Figure 4.1. Comparison of the domains of the glycoproteins of PYDV, SYNV, and LNYV. PYDV G contains a signal peptide from amino acid positions 1-18, 5 predicted glycosylation sites at amino acid positions 6, 108, 156, 169, and 464, and a transmembrane domain spanning amino acid positions 576-591. SYNV G contains a signal peptide from amino acid positions 1-20, six predicted glycosylation sites at amino acid positions 39, 93, 385,501, 512, and 541, a transmembrane domain spanning amino acids 561-578, and two predicted nuclear localization signals at positions 590 and 616. LNYV G contains a signal peptide at from amino acid positions 1-25, three predicted glycosylation sites at 28, 241 and 272, and a transmembrane domain spanning amino acid positions 504-524.
Figure 4.2. Confocal micrographs showing the coexpression of SYNV glycoprotein fragments (GFP) and endoplasmic reticulum (RFP) in *N. benthamiana* leaf epidermal cells. Diagram on left indicates the domains of SYNV G corresponding to those previously described in Figure 4.1. Lines drawn to the top of group of images corresponds to that fragment of the glycoprotein fused to GFP. (a-c) Coexpression of GFP-Full length SYNV G with RFP-ER. (d-f) Coexpression of GFP-SYNV G Fragment 1 with RFP-ER. (g-i) Coexpression of GFP-SYNV G Fragment 2 with RFP-ER. (j-l) Coexpression of GFP-SYNV G Fragment 3 with RFP-ER. (m-o) Coexpression of SYNV G Fragment 4 with RFP-ER. (p-r) Coexpression of SYNV G Fragment 5 with RFP-ER. (s-u) Coexpression of SYNV G Fragment 6 with RFP-ER. Regions of Colocalization are shown in yellow. Scale bar =20 μm.
Figure 4.3. Confocal micrographs showing the coexpression of LNYV glycoprotein fragments (GFP) and endoplasmic reticulum (RFP) in *N. benthamiana* leaf epidermal cells. Diagram on left indicates the domains of LNYV G corresponding to those previously described in Figure 4.1. Lines drawn to the bottom of group of images corresponds to that fragment of the glycoprotein fused to GFP. (a-c) Coexpression of GFP-Full length LNYV G with RFP-ER. (d-f) Coexpression of GFP-LNYV G Fragment 1 with RFP-ER. (g-i) Coexpression of GFP-LNYV G Fragment 2 with RFP-ER. (j-l) Coexpression of GFP-LNYV G Fragment 3 with RFP-ER. (m-o) Coexpression of LNYV G Fragment 4 with RFP-ER. (p-r) Coexpression of LNYV G Fragment 5 with RFP-ER. Regions of Colocalization are shown in yellow. Scale bar =20 μm.
Figure 4.4. Confocal micrographs of differential interactions of SYNV G full-length and SYNV G Fragment 6 (carboxy terminus) with importin alpha 1 or 2 in *N. benthamiana* leaf epidermal cells. (a-c) Interaction of SYNV G full-length with itself. (d-f) Lack of interaction of SYNV G full-length with *N. benthamiana* importin α 1. (g-i) Lack of interaction of SYNV G full-length with *N. benthamiana* importin α 2. (j-l) Lack of interaction of SYNV G carboxy terminus with *N. benthamiana* importin alpha 1. (m-o) Lack of interaction of SYNV G carboxy terminus with *N. benthamiana* importin α 2. (p-r) Lack of interaction of SYNV G full-length with GST as a negative binding control. Scale bar =20 μm.
CHAPTER V

Lettuce necrotic yellows virus protein localization and interaction map and comparison to two nucleorhabdoviruses, Sonchus yellow net virus and Potato yellow dwarf virus.

Rhabdoviruses that infect plants are assigned to two taxonomic genera, *Nucleorhabdovirus* and *Cytorhabdovirus*. Nucleorhabdoviruses replicate and assemble in the nucleus, whereas, this occurs in the cytoplasm for the cytorhabdoviruses. *Sonchus yellow net virus* (SYNV) and *Potato yellow dwarf virus* (PYDV) are members of the *Nucleorhabdovirus* genus with SYNV the best characterized and PYDV the type species of this genus. *Lettuce necrotic yellows virus* (LNYV) is the type species of the *Cytorhabdovirus* genus. In 1954, LNYV was first recognized as a destructive pathogen of *Lactuca sativa* (lettuce) causing a chlorotic and flattened appearance in the mature leaves with varying degrees of necrosis (Stubbs & Grogan, 1963). The virus is sap transmissible from lettuce or sowthistle to several indicator species including *Nicotiana glutinosa* and petunia but not to lettuce. In Australia and New Zealand, LNYV is present in a circulative, persistent manner in the aphid vector, *Hyperomyzus lactucae* (Dietzgen et al., 2006; Stubbs & Grogan, 1963). Phylogenically, LNYV is the most closely related to other cytorhabdoviruses: *Strawberry crinkle virus* (SCV) and *Lettuce yellow mottle virus* (LYMoV) (Dietzgen et al., 2006). Unfortunately, sequence information for comparison is limited, for many cytorhabdoviruses, only electron micrographs are available and the full sequence is available only for *Northern cereal mosaic virus* (NCMV) and LYMoV (Heim et al., 2008; Tanno et al., 2000).

Rhabdoviruses are single-stranded, negative-sense RNA viruses with genomes that encode at least five genes. These genes include a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and a polymerase (L). The RNA, N, P and L complex composing the viral core is the minimally infectious unit that is condensed by matrix proteins and surrounded by a host membrane embedded with viral glycoproteins (Jackson et al., 2005). Compared to the animal rhabdoviruses such as *Vesicular stomatitis virus* (VSV), plant rhabdoviruses also contain one additional gene that is considered to be a putative movement protein (Jackson et al., 2005). Although the name of this protein differs depending on which virus is being studied, it will be referred to here as Mv when comparing multiple viruses and 4b when referring to solely LNYV.

Previous studies of the protein-protein interactions of two nucleorhabdoviruses, SYNV and PYDV have already been completed (Bandyopadhyay et al., 2010; Min et al., 2010) and reveal conservation among some of the protein-protein interactions; however, this work has not been done in relation to the cytorhabdoviruses. This study will focus on LNYV, primarily on localization, colocalization and completion of an interaction map for the viral proteins. Comparison of the interaction map for LNYV proteins to the previously published maps for SYNV and PYDV reveals that although these viruses all share a similar genome organization and viral structure, few interactions are shared. This reveals a possible role for host proteins in the viral replication and morphogenesis to bridge the gaps between viral protein interactions.
METHODS

Plant growth

Wildtype and transgenic *Nicotiana benthamiana* plants expressing fluorescent markers targeted to the endoplasmic reticulum (ER) and nucleus were maintained in a greenhouse under ambient conditions (Martin *et al.*, 2009).

Protein expression in *N. benthamiana*

Clones corresponding to all open reading frames of LNYV except L were sequenced in the vector pDONR221. The LNYV sequences used for amplification are from the fully sequenced accession 318, an Australian isolate found originally in garlic, genetic material provided by R. Dietzgen (Dietzgen *et al.*, 2007; Dietzgen *et al.*, 2006). The pDONR clones were then recombined into the final binary vectors for expression of autofluorescent protein fusions in plant cells for localization and bimolecular fluorescence complementation (BiFC) as previously described (Chakrabarty *et al.*, 2007; Goodin *et al.*, 2007b; Martin *et al.*, 2009). Vectors utilized in this study were pSITE-2CA (GFP fusions), pSITE-4CA (mRFP fusions) for localization experiments and pSITE-nEYFP-C1 and pSITE-cEYFP-C1 for BiFC experiments. Recombinant vectors containing the gene of interest were transformed into *Agrobacterium tumefaciens* strain LBA4404. Agroinfiltration for expression of protein fusions in plant cells was conducted as described previously (Goodin *et al.*, 2005). BiFC analyses were conducted in transgenic *N. benthamiana* with a cyan fluorescent histone 2B protein (CFP H2B) for simultaneous localization of the nucleus. Each expression construct was examined in a minimum of three leaves from three independent plants and at least three high-quality images were acquired for each construct. BiFC assays were conducted as described for the production of a protein interaction map for PYDV and SYNV (Bandyopadhyay *et al.*, 2010; Min *et al.*, 2010). The comparison of the LNYV BiFC interaction map was done with images from SYNV and PYDV similar to those previously published (Bandyopadhyay *et al.*, 2010; Min *et al.*, 2010).

Laser Scanning Confocal Microscopy

All microscopy was conducted on an Olympus FV1000 laser scanning confocal microscope as described previously (Goodin *et al.*, 2005). BiFC analyses were done as was described previously for SYNV and PYDV (Bandyopadhyay *et al.*, 2010; Min *et al.*, 2010). All proteins were tested as carboxy terminal fusions to the amino (nec) or carboxy (cec) terminal portions of yellow fluorescent protein. Glutathione-S-transferase (GST) served as a negative binding control.

RESULTS

Localization of LNYV proteins relative to the nucleus or endoplasmic reticulum

LNYV is the type species of the cytorhabdoviruses, however, no studies of localization of the viral proteins have yet been published. To this end, we localized each of the viral proteins fused to both red fluorescent protein (RFP) and green
fluorescent protein (GFP) in transgenic *N. benthamiana* plants expressing fluorescent markers for the nucleus and the endoplasmic reticulum (Martin et al., 2009; Ruiz et al., 1998). In RFP-H2B (nuclear marker) plants, GFP fusions to LNYV proteins N, P and G localized to the cell periphery and the nuclear membranes (a-c, d-e, m-o; Figure 5.1). LNYV 4b and M-GFP fusions colocalized with the RFP-H2B marker and outside of the nucleus on the cell periphery (g-i, j-l; Figure 5.1). In addition, LNYV 4b and M also showed the presence of small bodies in the nucleus and the cell periphery that are not present in LNYV N, P or G. GFP alone localized to the nucleus and cell periphery (p-r, Figure 5.1). In studies with the LNYV RFP fusions in the GFP-ER plants (Ruiz et al., 1998). LNYV N, P and G colocalized with the ER membranes (a-c, d-f, m-o; Figure 5.2). LNYV G-RFP caused massive aggregations of the ER with areas of G-RFP that exclude the ER membranes. The LNYV M and 4b-RFP fusions colocalized with the ER membranes and to the nucleus as were shown with the GFP fusions and the RFP-H2B marker (j-l, g-i; Figure 5.2). Small RFP bodies with LNYV M and 4b are not present as observed with GFP fusions.

**Colocalizations of LNYV proteins**

Previous studies of LNYV using electron microscopy showed viral particles in the ER of infected cells, never in the nucleus, though particles were seen in the ER closely adjoining the nucleus (Chambers et al., 1965). To determine if the localizations of LNYV proteins are consistent with the expected sites of cytorhabdovirus replication, each protein was colocalized in all pairwise combinations fused to either GFP or RFP. In colocalizations of the N and P proteins of LNYV reveal that GFP-N and RFP-P localize to a location distinct from the localization of both N and P alone and that this is not in the nucleus (a-c, Figure 5.3) GFP-N and RFP-4b colocalize to the cell periphery with some accumulation of the two around the nucleus (d-f, Figure 5.3). GFP-P/RFP-M colocalize to the cell periphery, RFP-M localizes exclusively to the nucleus (g-i, Figure 5.3) GFP-P/RFP-4b colocalize to the cell periphery and the membrane around the nucleus (j-l, Figure 5.3). GFP-M and RFP-N colocalizes to the cell periphery with some brighter accumulations of punctate loci present (m-o, Figure 5.3). GFP-4b/RFP-M colocalize to the nucleus and cell periphery (p-r, Figure 5.3). RFP-G/GFP-N appears to be associated with the ER (a-c, Figure 5.4). GFP-P/RFP-G localizes to the cell periphery and with the most dramatic sites of colocalization at the sites of G accumulation (d-f, Figure 5.4). GFP-4b/RFP-G localizes to membranes of the cell, colocalization is most apparent at sites of G accumulation (g-i, Figure 5.4); this is similar to GFP-M/RFP-G localization (j-l, Figure 5.4). All reciprocal fusions were also conducted to eliminate any affect by the fluor and these localizations are identical.

**Bimolecular florescence complementation of LNYV proteins**

Bimolecular fluorescence complementation (BiFC) was done with all pairwise interactions of LNYV proteins to determine the binary interactions and localizations except L due to its large size and difficulty in cloning. BiFC offers the advantage of localization, interaction and comparison to other rhabdoviruses previously tested (Bandyopadhyay et al., 2010; Citovsky et al., 2006; Martin et al., 2009; Min et al., 2010). LNYV N, P, M, 4b and G were tested in pairwise
combinations with each other and with GST as a negative control. The M/P interaction was detected outside the nucleus in aggregations (a-c, Figure 5.5). The M/M interaction was detected inside the nucleus and on the cell periphery (d-f, Figure 5.5). The N/P interaction was detected outside the nucleus also in aggregations similar to M/P (g-i, Figure 5.5). The P/P interaction was outside of the nucleus at the cell periphery (j-l, Figure 5.5). In all other pairwise combinations of LNYV proteins, there were no interactions detected and were not included in Figure 5.5. One GST control was included as an example of the negative interactions between GST and LNYV proteins (m-o, Figure 5.5).

**Comparison of LNYV protein interactions to those of other plant rhabdoviruses**

Previous comparisons of plant rhabdovirus interactions have not been done to determine if there are similarities between them. Three viruses were compared for their similarities in BiFC interaction assays, LNYV, PYDV and SYNV. LNYV is a cytorhabdovirus and PYDV and SYNV are nucleorhabdoviruses that have previously been shown to induce differential nuclear morphology during viral morphogenesis (Goodin et al., 2005).

A positive interaction was detected for all cognate N/P proteins for LNYV, PYDV and SYNV. LNYV N/P is localized outside of the nucleus (a-c, Figure 5.6). PYDV N/P is localized inside the nucleus (d-f, Figure 5.6), and so is SYNV N/P (g-i, Figure 5.6). All three viruses also share a M/M interaction. LNYV M/M is localized in the nucleus and on the cell periphery (j-l, Figure 5.6). PYDV M/M and SYNV M/M are both localized inside the nucleus (m-o, PYDV; p-r, SYNV; Figure 5.6).

SYNV and LNYV share a P/P interaction on the cell periphery (a-c, LNYV; d-f, SYNV, Figure 5.7). PYDV and SYNV share three interactions. The first is N/N, both viruses show this interaction inside the nucleus (g-i, SYNV; j-l, PYDV, Figure 5.7). The second is Mv/Mv, which localizes to on the cell periphery (m-o, SYNV; p-r, PYDV, Figure 5.7). The third interaction shared is G/G, which is localized outside the nucleus in both viruses (s-u, SYNV; v-x, PYDV, Figure 5.7).

LNYV has one unique interaction not detected with the other viruses, that of M/P which is localized outside the nucleus (a-c, Figure 5.8). PYDV has three unique interactions, G/M localized outside the nucleus on the cell periphery and on the nuclear membrane (d-f, Figure 5.8), Mv/M localized in the nucleus (g-i, Figure 5.8), and lastly, N/M localized in the nucleus (j-l, Figure 5.8). SYNV also has one unique interaction not detected with the other viruses, G/Mv, which localized to the cell periphery and around the nuclear membrane (m-o, Figure 5.8).

**DISCUSSION**

Prior to the advent of sequencing, rhabdoviruses in plants were classified into two genera, *Nucleorhabdovirus* and *Cytorhabdovirus*, based on serology, electron micrographs of the cell and particle morphology. As sequencing of these viruses is completed, the distinction of the two genera has been maintained (Bandyopadhyay et al., 2010; Dietzgen et al., 2006; Ghosh et al., 2008; Redinbaugh et al., 2002). The sequence for LNYV became publically available in 2006 and is the
second cytorhabdovirus to be completely sequenced after NCMV (Dietzgen et al., 2006; Tanno et al., 2000). LNYV is most closely related to SCV based on the phylogeny constructed on the L gene sequences, (Dietzgen et al. 2007), however, the entire sequence of SCV is not available for comparison although it has been reported as completed (Schoen et al., 2004). The closest relatives with complete sequences are other cytorhabdoviruses, NCMV and LYMol, and no protein localizations are known (Heim et al., 2008; Tanno et al., 2000). This paper is the first report of the localization of proteins from a cytorhabdovirus to further characterize their similarities and differences to the nucleorhabdoviruses, SYNV and PYDV, which are better characterized.

All LNYV proteins localize to outside of the nucleus, and 4b and M also localize inside the nucleus. All proteins also colocalize with the ER marker in transgenic plants. This is consistent with the localization of the virion in electron micrographs done previously (Chambers et al., 1965). Cytorhabdoviruses, unlike nucleorhabdoviruses, do not associate with the nucleus and nuclear membranes. The model for cytorhabdovirus replication begins with the entry of a virion into the cell, uncoating to release the viral core, synthesis of viral mRNAs which leads to the synthesis of viral proteins, the formation of a viroplasm in the cytoplasm (presumably near or the ER membranes) that leads to budding of the mature virion through the ER membranes (Jackson et al., 2005). The localizations of each of the LNYV proteins to the ER agree with this model of replication. The partial nuclear localization of 4b and M is unexpected and the role of these proteins in the nucleus is unknown. In VSV, which also replicates in the cytoplasm, the M protein localizes to the nuclear rim to block export of host mRNAs in the infected cells through interactions with nuclear export proteins (Faria et al., 2005). This has been proposed as a means of limiting competition of resources for the viral proteins (Faria et al., 2005; von Kobbe et al., 2000). This may be a reason for the localization of LNYV M in the nucleus of plant cells, however, no tests to determine the binding of M to nuclear export factors have been done to determine this. However, 4b is also present in the nucleus, and this localization of 4b and M may also be related to viral movement, possibly in the recruitment of host transcription factors as seen in SYNV (Min et al. 2010). In the nucleorhabdoviruses, SYNV and PYDV, the movement protein localizes to the cell periphery, but both M proteins localize to the nucleus and are considered to be part of a movement complex (Bandyopadhyay et al., 2010; Goodin et al., 2007b; Min et al., 2010).

The colocalizations of the LNYV proteins also agree with the localizations of the individual proteins with the exception of N-P. When N and P are co-expressed they localize to aggregate formations outside of the nucleus. This is similar to SYNV, PYDV and Maize fine streak virus (MFSV) where N-P localize to subnuclear loci distinctly different from the localization of either protein alone (Bandyopadhyay et al., 2010; Goodin et al., 2001; Tsai et al., 2005). A N-P interaction is also conserved in the animal rhabdovirus, VSV (Takacs & Banerjee, 1995; Takacs et al., 1993), and may represent a conserved interaction and localization pattern in all rhabdoviruses. The LNYV colocalizations were done in uninfected N. benthamiana cells and may change if the virus is introduced. Changes in the localization of the proteins of SYNV were noticed when done in virus-infected cells (Goodin et al., 2007b). Using SYNV
as an example, the viroplasm may be more identifiable using colocalizations in LNYV if done in the context of infection. Such experiments would have to be done in another plant species, *N. glutinosa*, which unlike *N. benthamiana* is a systemic host for LNYV.

When testing the protein interactions of LNYV, there were only four positive interactions, N-P, P-P, M-P, and M-M. Compared to SYNV and PYDV, the only two plant rhabdoviruses whose interaction maps have been completed, this is the fewest number of interactions detected (Figure 5.9). The N-P interaction is similar to the N-P colocalization observed in Figure 5.3 with aggregations outside of the nucleus. The P-P interaction is similar to that observed from SYNV and PYDV as it resembles the single infiltration of P. The M-M interaction is conserved in SYNV, PYDV and LNYV. It has also been described in the animal rhabdoviruses, VSV and *Lagos bat virus* (Ge *et al.*, 2010; Graham *et al.*, 2008). Unlike SYNV, PYDV, and VSV, LNYV has no detectable G-G interaction. In VSV, the G-G self-interaction is characterized and the protein is part of a homotrimer (Roche *et al.*, 2006; Roche *et al.*, 2008). It is interesting that this not seen in LNYV, however, the YFP halves in the BifC assay may not be able to come together due to steric constraints. The YFP halves were attached to the N-termini of the protein, but unfortunately, adding the YFP halves to the carboxy termini of G did not result in interaction (data not shown) and does not rule out the possibility that interaction occurs. Conservation of only the N-P and M-M interactions is present, and other interactions between these viruses do not appear to be conserved.

We tested the interactions of LNYV proteins in a non-host plant, *N. benthamiana*, due to the ease of infiltration, however, when the same interactions were tested in a host plant, lettuce, no discernable differences were observed other than a decrease in the amount of aggregations present in the P-P interaction (data not shown). This suggests that the interactions present in *N. benthamiana* represent the interactions in lettuce as well. Also, since it was initially done in a non-host plant, these interactions were not tested in the context of virus. It may be possible that the presence of the whole virus is required for some interactions to be detected. This was the case for the VSV M-N interaction, which does not occur unless the cell is infected. The authors hypothesized that interaction during infection was detected because either the presence of multiple viral proteins is needed or there is host protein recruitment (Flood & Lyles, 1999; Lyles & McKenzie, 1998). VSV also incorporates a number of host proteins into the virion during assembly (Moerdijk-Schauwecker *et al.*, 2009) and these may be responsible for bridging the gaps that are seen even between the proteins in a VSV interaction map (Moerdijk-Schauwecker *et al.*, 2011). When host factors are added to the SYNV interaction map, the gaps are bridged between viral proteins (Min *et al.*, 2010). It is expected that this will also be true for LNYV.

This is the first report of protein localization and interaction in a cytorhabdovirus. The protein localizations and interactions are very different from the previously described nucleorhabdoviruses, PYDV, SYNV and MFSV. The localization of LNYV more closely resembles that of the animal rhabdovirus model VSV, however, there are several differences in this comparison as well. Although both viruses replicate in the cytoplasm, VSV buds from the plasma membrane, but
LNYV buds into the ER. This study clearly demonstrates that although the rhabdoviruses may have analogous genes, gene order and virus structure, there are clear differences in the protein interactions that occur.
Figure 5.1. Confocal micrographs of the localization of LNYV proteins in relation to a red nuclear marker, histone 2B (RFP-H2B), in transgenic *N. benthamiana*. From left to right, the first column is GFP-gene fusion, the second the RFP-H2B, and the last column is the Overlay between the two. (a-c) Coexpression of GFP-LNYV N with RFP-H2B. (d-f) Coexpression of GFP-LNYV P with RFP-H2B. (g-i) Coexpression of GFP-LNYV 4b with RFP-H2B. (j-l) Coexpression of GFP-LNYV M with RFP-H2B. (m-o) Coexpression of GFP-LNYV G with RFP-H2B. (p-r) Coexpression of GFP with RFP-H2B. Regions of Colocalization are shown in yellow. Scale bar =20 μm.
Figure 5.2. Confocal micrographs showing the localization of LNYV protein fusions in relation to a green fluorescent protein endoplasmic reticulum marker (GFP-ER) transgenic *N. benthamiana*. From left to right, the first column is RFP-gene fusion, the second the GFP-ER, and the last column is the Overlay between the two. (a-c) Coexpression of RFP-LNYV N with GFP-ER. (d-f) Coexpression of RFP–LNYV P with GFP-ER. (g-i) Coexpression of RFP–LNYV 4b with GFP-ER. (j-l) Coexpression of RFP–LNYV M with GFP-ER. (m-o) Coexpression of RFP–LNYV G with GFP-ER. (p-r) Coexpression of RFP with GFP-ER. Regions of Colocalization are shown in yellow. Scale bar =20 μm.
Figure 5.3. Confocal micrographs showing the coexpression of GFP (green) and RFP (red) LNYV protein fusions in uninfected *N. benthamiana*. (a-c) Coexpression of GFP-LNYV N and RFP-LNYV-P. (d-f) Coexpression of GFP-LNYV N and RFP-LNYV 4b. (g-i) Coexpression of GFP-LNYV P and RFP-LNYV M. (j-l) Coexpression of GFP-LNYV 4b and RFP-LNYV P. (m-o) Coexpression of GFP-LNYV M and RFP-LNYV N. (p-r) Coexpression of GFP-LNYV M and RFP-LNYV 4b. Scale bar =20 μm.
Figure 5.4. Confocal micrographs showing the coexpression of GFP (green) and RFP (red) LNYV protein fusions in uninfected *N. benthamiana*.  (a-c) Coexpression of GFP-LNYV N and RFP-LNYV G.  (d-f) Coexpression of GFP-LNYV P and RFP-LNYV G.  (g-i) Coexpression of GFP-LNYV 4b and RFP-LNYV G.  (j-l) Coexpression of GFP-LNYV M and RFP-LNYV G. Scale bar = 20 μm.
Figure 5.5. Confocal micrographs showing LNYV protein interactions determined by bimolecular fluorescence complementation (BiFC). Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing cyan fluorescent protein fused to the nuclear marker histone 2B (CFP-H2B). Shown are the localization of CFP-H2B (nucleus, column 1), interaction assay (BiFC, column 2), and merge of the two preceding panels (overlay, column 3). Proteins listed first in the pair of interactors were expressed as fusions to the amino-terminal half of yellow fluorescent protein (YFP). Those listed second were expressed as fusions to the carboxy-terminal half of YFP. However, protein fusions to each half of YFP were tested in all pairwise interactions, of which a subset is shown here. All pairwise interactions for LNYV proteins, excluding L, were tested. BiFC-positive interactions were observed for (a-c) M/P, (d-f) M/M, (g-i) N/P, (j-l) P/P. The following pairwise combinations were BiFC negative: N/N, N/M, N/4b, N/G, P/4b, P/G, M/4b, M/G, 4b/4b, 4b/G, and G/G. Only one representative of the results obtained using glutathione-S-transferase (GST) as a nonbinding control is provided here (m-o). However, no LNYV proteins shown here interacted with GST. Scale bar = 20 µm.
Figure 5.6. Confocal micrographs showing protein interactions shared between LNYV, SYNV and PYDV determined by bimolecular fluorescence complementation (BiFC). Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing cyan fluorescent protein fused to the nuclear marker histone 2B (CFP-H2B). Shown are the localization of CFP-H2B (nucleus, column 1), interaction assay (BiFC, column 2), and merge of the two preceding panels (overlay, column 3). (a-c) N/P interaction of LNYV, (d-f) N/P interaction of PYDV, (g-i) N/P interaction of SYNV. (j-l) M/M interaction of LNYV, (m-o) M/M interaction of PYDV, (p-r) M/M interaction of SYNV.
Figure 5.7. Confocal micrographs showing protein interactions only detected in two viruses. LNYV, SYNV and PYDV were compared and interactions shown are those seen in two viruses. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing cyan fluorescent protein fused to the nuclear marker histone 2B (CFP-H2B). Shown are the localization of CFP-H2B (nucleus, column 1), interaction assay (BiFC, column 2), and merge of the two preceding panels (overlay, column 3). (a-c) P/P interaction of LNYV, (d-f) P/P interaction of SYNV, (g-i) N/N interaction of SYNV. (j-l) N/N interaction of PYDV, (m-o) Mv/Mv interaction of SYNV, (p-r) Mv/Mv interaction of PYDV, (s-u) G/G interaction of SYNV, (v-x) G/G interaction of PYDV.
Figure 5.8. Confocal micrographs showing protein interactions only detected in one virus, LNYV, SYNV or PYDV. Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing cyan fluorescent protein fused to the nuclear marker histone 2B (CFP-H2B). Shown are the localization of CFP-H2B (nucleus, column 1), interaction assay (BiFC, column 2), and merge of the two preceding panels (overlay, column 3). (a-c) M/P interaction of LNYV, (d-f) G/M interaction of PYDV, (g-i) Mv/M interaction of PYDV, (j-l) N/M interaction of PYDV, (m-o) G/Mv interaction of SYNV.
Figure 5.9. Schematic diagram of the three viral interaction maps for SYNV, LNYV and PYDV. Self-interactions are indicated by curved arrow. Lines indicate heterologous interactions.
CHAPTER VI*

A host-factor interaction and localization map for a plant-adapted rhabdovirus implicates cytoplasm-tethered transcription activators in cell-to-cell movement.

In order to infect systemically, plant viruses recruit host factors that facilitate cell-to-cell and long distance movement (Chen & Citovsky, 2003; Epel, 2009; Harries et al., 2009; Lewis & Lazarowicz, 2010; Tzfira & Citovsky, 2008; Wang et al., 2009). Despite the wealth of information for the movement of many genetically diverse plant viruses, there is a paucity of such information for the plant-infecting rhabdoviruses.

The nucleorhabdoviruses are those plant-adapted rhabdoviruses that replicate in nuclei of infected plant cells (Jackson et al., 2005; Tordo et al., 2005). These viruses share many of the structural features of animal rhabdoviruses, such as Vesicular stomatitis virus (VSV; Jackson et al., 2005). As such, their minus-sense single-stranded RNA genome is not infectious. Instead, the minimal infectious unit of these viruses is a nucleocapsid composed of the genomic RNA encapsidated over its entire length by a nucleocapsid (N) protein and associated with this complex are the phospho (P) and polymerase (L) proteins. During morphogenesis, the nucleocapsid is condensed by the matrix (M) protein to form viral ‘cores’ that bud through the inner nuclear membrane into the perinuclear space, acquiring a host-derived lipid envelope and viral-encoded glycoprotein (G) in the process (van Beek et al., 1985). Currently, molecular details of how plant-adapted rhabdoviruses move from sites of replication and morphogenesis into adjacent cells are lacking. Two possible models for cell-to-cell movement of these viruses are considered here. First, mature virions may bud from the perinuclear space through the outer nuclear membrane akin a model proposed for release of herpes virus particles (Farnsworth et al., 2007; Mettenleiter et al., 2009; Sagou et al., 2010). Alternatively, the nucleocapsid may be exported from the nucleus, as occurs with influenza and plant DNA viruses (Boulo et al., 2007; O’Neill et al., 1998; Sanderfoot et al., 1996). Whichever model is correct, it has long been suspected that the sc4 protein of Sonchus yellow net virus (SYNV) facilitates cell-to-cell movement (Goodin et al., 2001; Huang et al., 2005; Melcher, 2000; Scholthof et al., 1994). Also implicated in the formation of SYNV movement complexes is the M protein, which has been shown to form mobile complexes associated with the endoplasmic reticulum in virus-infected cells (Goodin et al., 2007b). Therefore, using SYNV proteins as baits, screens of a high-resolution Nicotiana benthamiana yeast-two hybrid library and live-cell imaging were performed. We provide evidence that the cell-to-cell movement of plant-adapted rhabdoviruses requires cytoplasm-tethered transcription activators that facilitate formation and transport of an ER- and microtubule-associated complex.

This chapter was originally published as: Min, B.E., Martin, K., Wang, R., Tafelmeyer, P., Bridges, M. and Goodin, M. (2010) A host-factor interaction and localization map for a plant-adapted rhabdovirus implicates cytoplasm-tethered transcription activators in cell-to-cell movement. Mol Plant Microbe Interact 23, 1420-1432. Copyright permission was granted by the publisher for inclusion in this dissertation.
METHODS

Plant materials and plasmid
Wild-type and transgenic *N. benthamiana* plants expressing fluorescent marker proteins targeted to the nucleus, ER or actin filaments, were maintained in the greenhouse under ambient conditions (Martin et al., 2009). SYNV was maintained in a similar manner in *N. benthamiana*. The marker construct for highlighting microtubules (GFP-MBD) was described by (Marc et al., 1998).

Isolation of total RNA, RT-PCR
Total RNA was extracted from plant tissues using the RNeasy Plant minikit (Qiagen) according to the manufacturer’s instructions. Except where noted, first strand cDNA synthesis and PCRs were carried out using Superscript reverse transcriptase III (Invitrogen) and Phusion high fidelity DNA polymerase (Finnzymes), respectively. To allow full-length host proteins to introduce into Gateway compatible vectors, PCR reaction was conducted by gene specific primers flanked by *attB* sequence.

High-resolution yeast two-hybrid screens
The coding sequences for the sc4, M and N proteins of SYNV were PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA-bait-C) and into pB66 as a C-terminal fusion to the Gal4 DNA-binding domain (N-Gal4-bait-C). The constructs were checked by sequencing the entire insert and used as a bait to screen a random-primed *N. benthamiana* cDNA library constructed into pP6. pB27, pB66 and pP6 derive from the original pBTM116 (Vojtek & Hollenberg, 1995), pAS2ΔΔ (Fromont-Racine et al., 1997) and pGADGH (Bartel et al., 1993) plasmids, respectively. To maximize proteome representation, the relative percentage contributions of mRNA for library construction from the following sources were: fully expanded leaves (50%), roots of mature plants (10 %), whole 2-week old seedlings (10 %), leaves treated with salicylic acid (10 %), and leaves infected with *Sonchus yellow net virus* (SYNV; 10 %) or an RNA silencing mutant of *Tomato bushy stunt virus* (10%). The random-primed cDNA library contains an average insert size of 800 nt and a complexity of 50 million independent fragments in *E. coli* and 10 million independent fragments in yeast. To ensure exhaustive and reproducible Y2H results, the library was screened to saturation using an optimized cell-to-cell mating procedure using a Y187 (mata) and L40DGal4 (mata) yeast strains as previously described (Fromont-Racine et al., 1997) for the LexA constructs and Y187 (mata) and CG1945 (mata) yeast strains for the Gal4 constructs. On average 108 million interactions per screen were tested, corresponding to approximately 10-fold coverage of the library.

For the N protein, 79.3 million and 63.6 millions interactions were tested in screens conducted with pB27 and pB66, respectively. For the M protein, 76.6 million and 365 million interactions were tested in screens conducted with pB27 and pB66, respectively. For the sc4 protein, 85.6 million and 82.8 million interactions were tested in screens conducted with pB27 and pB66, respectively. The prey fragments
of the positive clones were amplified by PCR and sequenced at their 5’ and 3’ junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005). Interactors for further study were selected based on confidence scores (A or B), our ability to recover full-length clones, and that the recombinant proteins could be expressed efficiently as fusions to autofluorescent proteins.

**DNA sequence analysis**

Assembly of nucleotide sequences into full-length sequences encoding each interactor was performed using the DNASTAR v.7 software package. Homology searches by various BLAST tools were conducted on the National Center for Biotechnology Information (NCBI) server. Open reading frames (ORFs) were identified using ORF finder search tool (Tatusov & Tatusov, 2007). The deduced amino acid sequences of proteins encoded by host genes were analyzed using a variety of algorithms provided by the Expasy proteomics server (Gasteiger et al., 2003), including Compute PI/MW (Bjellqvist et al., 1993), PSORT for prediction of protein localization (Nakai & Kanehisa, 1991), SignalP for prediction of signal peptide cleavage sites (Bendtsen et al., 2004), NetNGlyc for prediction of N-glycosylation sites (Blom et al., 2004), and NetNES for prediction of nuclear export signals (La Cour et al., 2004).

**Protein expression in plant cells**

**Expression of SYNV proteins for Bimolecular fluorescence complementation (BiFC) assays for virus protein-protein interactions (Figure 6.1) was conducted by Kathleen Martin. The cloning and BiFC assays for G* (Figure 6.8) was conducted by Kathleen Martin.**

Protein expression in plant cells for protein localization or bimolecular fluorescence complementation was conducted essentially as described (Bandyopadhyay et al., 2010). Briefly, sequence-validated full-length clones in vector pDONR221 (Invitrogen) and pDONRzeo (Invitrogen) of all relevant ORFs were recombined into appropriate binary vectors for the expression of autofluorescent protein fusions in plant cells for localization and BiFC assays using a variety of pSITE or pSITEII vectors (Chakrabarty et al., 2007; Goodin et al., 2007; Martin et al., 2009). Vectors employed in this study were pSITE-2CA (GFP fusions) and pSITEII-6C1 (TagRFP fusions) for localization experiments, and the pSITE-BiFC-nEYFP and pSITE-BiFC-cEYFP vectors for BiFC assays. BiFC assays were conducted as described for production of a protein interaction map for *Potato yellow dwarf virus* (PYDV; Bandyopadhyay et al., 2010). Recombinant vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404. Agroinfiltration for expression of protein fusions in plant cells was conducted essentially as described previously (Goodin et al., 2005). Each expression construct was examined in sections taken from a minimum of three leaves from each of three separate plants (nine leaves total). Several hundred cells were examined for each experiment and at least three high-resolution micrographs were acquired for each construct.
For expression of recombinant proteins containing an amino-terminal FLAG tag, we converted pSAT6-FLAG (gift from Tzvi Tzfira) to its pSITE equivalent as described (Martin et al., 2009). Cloning and expression from this vector, pSITE-FLAG, were as described above.

**Laser scanning confocal microscopy**

*Kathleen Martin performed the microscopy for Figures 6.1 and 6.8.*

All microscopy was performed with an Olympus FV1000 laser-scanning confocal microscope as described previously (Goodin et al., 2005). BiFC assays were conducted as described for production of a protein interaction map for PYDV, as described (Bandyopadhyay et al., 2010). Briefly, all proteins were tested as fusions to the amino (nec) and carboxy (cec) terminal portions of YFP. Glutathione-S-transferase (GST) or maltose-binding protein (MBP) served as non-binding controls.

**Drug treatments for depolymerization of tubulin and actin**

Stock solutions of latrunculin B (Lat B; Sigma) and oryzalin (Fluka) were made in concentrations of 10mM and 20mM in DMSO, respectively. 100μM oryzalin or 10μM LatB were co-infiltrated with tagRFP fusions to host proteins. Distruption of actin and tubulin by drugs was confirmed by confocal microscopy at two days post infiltration.

**Construction of pNIA for nuclear import assays in yeast cells**

Recombinant pNIA (Bandyopadhyay et al., 2010) plasmids for expression of host proteins, Mi7, Sc4i17, Sc4i21 and Ni67, as well as genes for maltose-binding protein (MBP) and histone 2B (H2B), were transformed into *Saccharomyces cerevisiae* strain L40 (Zaltsman et al., 2007). The transformed yeast cells were grown for 4 days at 30°C on minimal media lacking tryptophan (Trp-). Yeast colonies were then re-streaked onto minimal media lacking both tryptophan and histidine (His-) containing 50 mM 3-amino-1,2,4-triazole (3AT). Growth of yeast cultures on Trp-/His- media was indicative of a functional nuclear localization signal in proteins expressed from pNIA (Zaltsman et al., 2007).

**Immunodetection of proteins**

*This section was conducted by Kathleen Martin.*

Tissue to be prepared for immunodetection was sampled from *N. benthamiana* leaves with a number 8 cork-borer (1 cm i.d.). Protein extracts were prepared in a 1.5 ml eppendorf tube, by grinding three leaf discs in 200 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer [0.5 M Tris–HCl (pH 6.8), 10% SDS, 7.5% glycerol, 5% β-mercaptoethanol and 0.05% Bromophenol Blue] and heated in a boiling water bath (100 °C) for 5 min. Proteins were separated by discontinuous SDS-PAGE using 12% gels. Following electrophoresis, gels were developed using PageBlue dye to stain total proteins (Fermentas Life Sciences) or subjected to western immunoblot analysis after transfer of the proteins to nitrocellulose membranes. Antibodies for SYNV-G raised in mouse (Goldberg et al., 1991) or M2 anti-flag antibody (Sigma) were used for detection. Immunoblots were developed using a colorimetric assay employing the
appropriate secondary antibody conjugated to alkaline phosphatase with Nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyphosphate p-toluidine (BCIP).

RESULTS

Construction of an interaction matrix for SYNV proteins

Bimolecular fluorescence complementation (BiFC) was used to determine the binary interactions and localization patterns of SYNV protein complexes. We chose to use BiFC given that it provided simultaneous interaction and localization data in planta (Bandyopadhyay et al., 2010; Citovsky et al., 2006; Martin et al., 2009). The SYNV-N, -P, -sc4, -M and -G proteins were tested in all pair-wise interactions and against glutathione-S-transferase (GST), which served as a non-binding control (Figure 6.1). The L protein was not included in these studies due to the inability to express this 240 kDa protein. We did not observe binding between any of the SYNV proteins and GST. Therefore, we provide only one example of this control experiment (Figure 6.1A1-A3). The sc4/sc4 interaction was detected exclusively on the periphery of cells (Figure 6.1B1-B3). In contrast, the sc4/G interaction was detected on both on the cell periphery and nuclear membranes (Figure 6.1C1-C3). The G/G interaction was detected primarily on perinuclear membranes (Figure 6.1D1-D3). The M protein self-interaction was detected in sub-nuclear loci (Figure 6.1E1-E3), whereas the N/N interaction was dispersed throughout the nucleus, but excluded from the nucleolus (Figure 6.1F1-F3). As reported previously (Goodin et al., 2001; Martin et al., 2009), the N/P interaction was subnuclear (Figure 6.1G1-G3), while that of P/P was nuclear and cytoplasmic (Figure 6.1H1-H3). All other pair-wise combinations of SYNV proteins were negative in BiFC assays.

Characteristics of host factors that interact with SYNV proteins

We used high-resolution screens of an N. benthamiana yeast two-hybrid library to identify proteins that interact with the N, M and sc4 proteins. The high-resolution of this library refers to the number of independent cDNA clones that compose the library. For this study, a library containing 10 million independent clones was constructed. This contrasts to a previously reported N. benthamiana yeast two-hybrid library composed of 1.2 million clones (Jimenez et al., 2006). The greater the number of independent clones the better the possibility of finding rare or weak-interacting host factors.

Following protocols reported previously (Formstecher et al., 2005), as well as bioinformatic characterization, the interactors were assigned confidence scores of ‘A’ (very high confidence), ‘B’ (high confidence), ‘C’ (good confidence) and ‘D’ (moderate confidence, likely to include false positives). Low confidence interactors were removed from the dataset. Additionally, interactors that did not have significant matches in protein sequence databases, were also excluded. A total of 31 unique interactors were given priority for further study: eight N protein interactors; ten sc4 interactors and sixteen M protein interactors (data not shown). Five interactors, including sc4i17, were common to the M and sc4 screens. Four of the
very highest confidence interactors, sc4i17, sc4i21, Mi7 and Ni67, were selected for this study.

Using a variety of web-based proteomics tools we characterized the selected SYN1 host factors selected for this study (Table 6.1). The deduced amino acid sequence of the M interactor, Mi7, encodes a small 21 kDa basic protein with a pI of 9.7. Mi7 contains a DUF640 domain conserved in such transcription factors including the Arabidopsis Light sensitive hypocotyl 10 protein (TAIR reference AT1G78815; Genbank accession NM_106529). Mi7 was predicted to contain both a PYKKKKK nuclear localization signal (NLS) starting at amino acid 155, and a VLEFLRYLD nuclear export signal (NES) starting at amino acid residue 66. The steady-state localization pattern for Mi7, shown below, was nuclear.

The sc4i17 protein is predicted to be an 80 kDa protein with a pI of 6.0. This protein is 98% identical to the N. tabacum BY-2 kinesin-like protein 10 (Matsui et al., 2001). Consistent with the predicted motor domain at amino acids 186-508 in the deduced protein sequence, sc4i17 localized to punctate loci on microtubules in N. benthamiana leaf epidermal cells.

The sc4 interactor, sc4i21, is a 53 kDa, pI 5.8, protein that contains a conserved “no apical meristem” (NAM) domain found in some transcription factors, such as the phloem-associated Arabidopsis vascular one zinc-finger protein 1 (AtV0Z1; Mitsuda et al., 2004), with which it shares 65% identity (E-value 3e-165). Although sc4i21 contains a predicted NLS (314-KPRR-317) and NES (382-LYRLEKLVD-391) its steady state localization was on microtubules. Interestingly, Ni67, a 53 kDa, pI 5.5, protein, shares 84% sequence identity with sc4i21, with identically positioned NLS and NES sequences. However, the steady-state localization of Ni67 was located in the ER, as shown below.

**Microtubule association of sc4i17 and sc4i21**

To validate the protein prediction data, we expressed full-length ORFs for sc4i17 and sc4i21 as tagRFP (tRFP) fusions in fluorescent maker lines of N. benthamiana (Goodin et al., 2007a; Martin et al., 2009). Sc4i21 localized to filaments that only partially colocalized with the ER (Figure 6.2A1-A3). To establish the nature of the sc4i21 filaments we expressed tRFP-tagged sc4i21 in transgenic N. benthamiana plants expressing GFP:talin to label actin filaments (Figure 6.2B1-B3). Treatment of this tissue with 10 mM Latrunculin B resulted in complete disruption of actin filaments, whereas there was little effect on localization of sc4i21 (Figure 6.2B4-B6). Coexpression of tRFP-tagged sc4i21 (Figure 6.2C1-C3) or sc4i17 (Figure 6.2C4-C6) with a GFP-microtubule binding domain fusion (GFP:MBD; Marc et al., 1998)) suggested that both tRFP fusions colocalize with microtubules. To provide further support for microtubule-association, we treated leaves expressing tRFP-sc4i21 or tRFP-sc4i17 with 100 mM Oryzalin. Under drug treatment, sc4i21 filaments were completely disrupted (Figure 6.2D1-D2), while sc4i17, which was most easily viewed at the cell periphery, was detected only at punctate loci on cell walls following oryzalin treatment (Figure 6.2D3-D4).
Homologous and heterologous interactions between host factors and SYNV proteins.

Given that transcription factors often function as dimers or in higher-order oligomers (Amoutzias et al., 2008), we examined the ability of Mi7, Ni67, sc4i17 and sc4i21 to associate with each other as well as with SYNV proteins. In the sections below, we provide micrographs for the results of BiFC experiments conducted with host factors and SYNV proteins. It was found that their interactions with different partners could dramatically alter the localization of some host factors. The results of these assays are summarized in Table 6.2. Note that Ni67 was detected within nuclei, on ER membranes or at the cell periphery depending on its interacting partner. Similarly, sc4i17 was found in the nucleus or on microtubules when interacting with M or sc4, respectively.

Localization and interaction of Ni67

We expressed tRFP-tagged Ni67 in transgenic N. benthamiana plants expressing GFP-ER. Under steady-state observations, Ni67 accumulated in punctate loci on ER (Figure 6.3A1-A3). Examination of these puncta revealed Ni67 complexes surrounded by ER-derived rings (Figure 6.3B1-B3). In contrast to its steady-state localization pattern, BiFC established that Ni67 interacts with the N protein in nuclei (Figure 6.3C1-C3).

Localization and interaction of Mi7

Mi7 localized exclusively to nuclei when expressed as tRFP fusions in GFP-ER transgenic plants (Figure 6.4A-C). BiFC validated the yeast two-hybrid results given that M interacted with Mi7 in nuclei (Fig. 4D-F). Additionally, we show that Mi7 interacted with itself (Figure 6.4G-I), but not with GST (Figure 6.4J-L).

Binary complexes between sc4, M and host factors sc4i17 and sc4i21

The host factor sc4i17 was detected in two-hybrid screens using either sc4 or M as bait. Therefore, we determined whether interaction with different viral proteins could change the localization pattern of sc4i17. Interaction between M and sc4i17 was detected as mobile complexes within nuclei (Figure 6.5A-C). The sc4/sc4i21 interaction was detected on the periphery of cells (Figure 6.5D-F). In contrast to the M/sc4i17 interaction, the sc4/sc4i17 was detected on filamentous complexes in the cytoplasm and around nuclei (Figure 6.5G-H).

Binary complexes between host factors

In addition to interactions between SYNV proteins and host factors, we tested the ability of the host factors to form binary complexes. Sc4i17/sc4i17 complexes formed on filamentous structures that were dispersed throughout cells (Figure 6.6A1-A3). Similar to its steady-state localization pattern using tRFP fusions, Ni67/Ni67 complexes accumulated in a reticulate pattern in the cytoplasm (Figure 6.6B1-B3). The sc4i21/Ni67 complexes were detected almost exclusively on punctate loci on cell periphery (Figure 6.6C1-C3). The sc4i21/sc4i21 interaction was detected on cell periphery (Figure 6.6D1-D3) as well as on cortical filamentous complexes (Figure 6.6D4).
Mi7, sc4i21 and Ni67 contain functional NLSs

The predicted NESs and NLSs in Sc4i17 and Ni67, and the intranuclear Ni67/N interaction, suggested that despite their cytoplasmic accumulation at steady-state, these proteins are capable of nuclear import. Therefore, Mi7, sc4i17, sc4i21, and Ni67 were screened in a yeast-based nuclear import assay (NIA, Figure 6.7). In this assay, only proteins containing a functional NLS will facilitate the nuclear import of a transcriptional activator required for expression of a reporter gene in yeast cells (Bandyopadhyay et al., 2010; Zaltsman et al., 2007). Entirely consistent with computational predictions, Mi7, sc4i21 and Ni67 were all NIA positive as was the control protein *N. benthamiana* histone 2B. The sc4i17 protein and the control using maltose-binding protein (MBP) were NIA-negative.

Multiple forms of SYNV-G accumulate in plant cells

Interpretation of the data showing the interaction of *sc4* and *G* in BiFC assays presented a topological problem in that the amino terminus of *G* should be ER-luminal and thus inaccessible to *sc4*, which lacks a signal peptide. However, it is known that several negative-strand RNA viruses produce truncated and soluble forms of their *G* proteins (Graeve et al., 1986; Teng et al., 2001; Volchkov et al., 1998). We postulated that SYNV may do the same and that it is in fact a truncated form of *G* that interacts with *sc4*. Western immunoblotting showed indeed that two stable forms of *G* accumulated in leaf tissues both in the context of viral infections and agroinfiltration-based expression of a recombinant *G* protein (Figure 6.8A and 8B). The mature form of *G* migrated at 70 kDa on denaturing polyacrylamide gels, while the truncated form, hereafter called *G**, has an estimated molecular weight of 51 kDa.

To determine the ability of *G* to interact with *sc4*, we utilized a 51 kDa amino terminal portion of *G* that lacked its transmembrane domain and carboxy terminus in BiFC experiments. *G* was detected in binary complexes located primarily in perinuclear ER (Figure 6.8 C1-C3). *G* retained the ability to interact with full-length *G* and binary complexes of these proteins were found on nuclear and endomembranes (Figure 6.8 D1-D3). *G*/sc4 complexes were found on the nuclear envelope and on punctate loci along the cell periphery (Figure 6.8 E1-E4). *G* did not form complexes with GST (Figure 6.8 F1-F3). Although the precise carboxy terminus of the native *G* has not been mapped, these results were reproducible with both 51 and 53 kDa amino-terminal portions of *G* (data not shown).

A host factor protein interaction map for SYNV

The protein localization and interaction data presented above were integrated into a comprehensive map (Figure 6.9). All nine proteins used in this study were capable of self-interactions. Two host factors, sc4i21 and Ni67, formed heterologous interactions with each other that resulted in a change in their steady-state localization patterns. One host factor, sc4i17, interacted with both sc4 and M. In contrast to *Potato yellow dwarf virus* (PYDV) (Bandyopadhyay et al., 2010), SYNV proteins formed few heterologous associations (N/P and sc4/G).
DISCUSSION

Protein interaction maps, particularly when supported by protein localization data are critical for understanding molecular mechanisms that underlie viral infection and transmission processes (Bandyopadhyay et al., 2010; Guo et al., 2008; Shapira et al., 2009; Uetz et al., 2006). Moreover, they permit a rapid means to infer different mechanisms by which related viruses might conduct particular processes. For example, a comparison of protein interaction data for henipaviruses suggests at least two different strategies for cell-to-cell movement may be employed by Rice yellow stunt virus (RYSV), PYDV and SYNV, the movement proteins for which are referred to as P3, Y and sc4, respectively. In the case of RYSV, the P3 protein is thought to bind directly to nucleocapsids. In contrast, no comparable Y/N or sc4/N interactions have been detected ((Bandyopadhyay et al., 2010), this study). For PYDV, Y interacts with the matrix and glyco proteins, which may permit the formation of a movement complex similar to that proposed here for SYNV. For both PYDV and SYNV, interactions with either M or G proteins results in the relocalization of the movement proteins from the cell periphery to the nucleus, the site of viral replication and morphogenesis. Overall PYDV and SYNV protein interaction maps are similar with all proteins, except for the PYDV phosphoprotein, capable of forming at least binary associations (Bandyopadhyay et al., 2010).

In order to gain an in-depth understanding of viral infection processes viral protein interaction maps must be expanded to include host proteins (Shapira et al., 2009; Uetz et al., 2006). Although it is generally established that in order to move from initially infected cells into adjacent cells plant viruses employ the ER (Bamunusinghe et al., 2009) and at least one type of protein filament, be it actin (Prokhnevsky et al., 2005) or (Epel, 2009; Sambade & Heinlein, 2009), no additional host factors have been implicated in the movement of plant-adapted rhabdoviruses.

Under the assumption that binding to the N protein is equivalent binding to the nucleocapsid, we propose a model for SYNV movement that requires the N, M, sc4 and G, or G*, proteins of SYNV and the host factors sc4i17, sc4i21, Mi7 and Ni67 (Figure 6.10). We believe this assumption, also made by (Huang et al., 2005), to be valid, given that the N protein readily forms viroplasm-like complexes even in the absence of other viral proteins (Deng et al., 2007; Martins et al., 1998), and that N proteins can self-assemble into ribonucleoprotein complexes in the absence of viral RNA (Green et al., 2000).

By virtue of their much larger molecular weights, it is clear that Ni67 and sc4i21 are distinct from the 10 kDa MBF1 transcriptional coactivator that was shown to interact with p30 of TMV (Matsushita et al., 2002). Interestingly, both sc4i21 and Ni67 contain a NAM domain, as does the smaller 32 kDa ATAF2 that binds to the helicase domain of the TMV replicase and that is involved in suppression of systemic host defense (Wang et al., 2009). Thus, to our knowledge, neither the host factors reported here, nor their homologues in other plants, have been shown to participate in the biology of other plant viruses.

We propose that nucleocapsids that are not condensed and budded into the perinuclear space associate with Ni67 and Mi7, both of which contain functional
nuclear import and predicted export signals. The use of two factors for export is attractive as it could provide a regulatory mechanism for exporting only nucleocapsids, or partially condensed cores, and not simply oligomers of the N and M proteins. We find it intriguing that VOZ1, the closest named homologue of both sc4i21 and Ni67, is expressed primarily in the phloem (Mitsuda et al., 2004). It stands to reason that a nucleocapsid destined for cell-to-cell movement would associate with phloem-associated proteins, particularly if it was a non-cell autonomous protein (NCAP) with intrinsic ability to move between cells (Oparka, 2004). Once exported, Ni67 may anchor nucleocapsids onto ER membranes bringing with it the associated M and Mi7 proteins, both of which associate with mobile ER-associated complexes ((Goodin et al., 2007b) and data not shown). The ER-associated complex could then be tethered to microtubules via a complex containing the motor-kinesin, sc4i17, as well as sc4i21, and G*. Once formed, the complete ER-associated movement complex could track on microtubules towards plasmodesmata in a manner similar to that of NCAPs. Given the punctate localization of the Ni67/sc4i21 on cell periphery, which could be plasmodesmata, we propose that upon reaching this structure, an Ni67/sc4i21 interaction could provide a mechanism to release the nucleocapsid into the adjacent cell. Some such release of the nucleocapsid from the movement complex is required in order to allow the nucleocapsid to be ferried to the nucleus, probably via an importin-mediated process (Deng et al., 2007), to initiate the next infection cycle (Jackson et al., 2005). Unfortunately, the requirement for these host factors in cell-to-cell movement could not be supported by RNA-silencing experiments (Park et al., 2009), due to the inability of virus-induced gene silencing (Liu et al., 2002a) to significantly reduce the steady-state levels of host factor mRNAs, which accumulated to very low levels in both mock-inoculated and SYNV-infected plants (data not shown). A greater a degree of silencing, probably in the context of transgenic plants, will be required to elucidate the function of the proteins reported here in viral movement. However, our ability to demonstrate protein-protein interactions via two independent yeast or plant-based assays provides strong support for which host factors should be pursued in future studies.

Our model for transport of rhabdoviral nucleocapsids is distinctly different from that of (Huang et al., 2005), who proposed that P3-mediated movement of RYSV required direct binding of P3 to nucleocapsids. However, these authors only investigated binding between P3 and N, whereas we have considered all pairwise interactions for SYNV proteins, except for L. Additionally, our model requires integration of data that demonstrates binding of sc4 to G (this study) and the report that sc4 co-purifies with membrane-associated nucleocapsids but not with purified nucleocapsids treated with organic solvents to remove membranes (Scholthof et al., 1994). It is possible that different rhabdoviruses, particularly those with dicot versus monocot hosts, could utilize different mechanisms for cell-to-cell transport. In this regard, this study strongly implicates cytoplasm-tethered transcription factors in the cell-to-cell movement of plant-adapted rhabdoviruses, a finding that heretofore has not been reported.

Transcription factors that are tethered in the cytoplasm are typically membrane-associated (Chen et al., 2008; Kim et al., 2010), and are typically
members in the bZIP (basic leucine zipper) and NAC (NAM/ATAF1-2/CUC2) transcription factor families, which include AtVOZ1, Ni67 and sc4i21. At present, known cytoplasm-tethered transcription factors utilize transmembrane anchors to mediate their retention in the ER, and are released from these membranes upon proteolytic cleavage. Ni67 is ER-associated, but lacks a predicted transmembrane domain or signal peptide, while sc4i21 is microtubule-associated. This suggests that there may be multiple mechanisms to retain transcription factors outside of the nucleus. This contention is supported by the reports from animal and plant systems that implicate microtubules in transcriptional activation by NFκB and RNA processing (Hamada et al., 2009; Jackman et al., 2009). Consistent with the localization of Ni67, AtVOZ fused to tagRFP, accumulated in ER-associated complexes in N. benthamiana leaves (Min and Goodin, unpublished data). It is curious that the related proteins Ni67 and sc4i21 have such different localization patterns, the reasons for which will be investigated in future studies.

Taken together, the reported two-hybrid screens provided high confidence interactions that were all positive in independent binding assays with their corresponding bait proteins. More importantly, all the associated localization studies returned biologically relevant information that fit logically into a model for cell-to-cell movement of nucleorhabdoviruses. Thus, there should be high confidence and strong support for preparing comprehensive protein interaction and localization maps for genetically diverse viruses, in a manner similar to the generation of transcriptional profiles (Ascencio-Ibanez et al., 2008; Dardick, 2007; Senthil et al., 2005; Whitham et al., 2003). Collectively, these data will be critical for systems biology approaches to understanding plant-virus interactions (Mendez-Rios & Uetz, 2010).
Table 6.1. Sonchus yellow net virus (SYNV) host factors characterized in this study.

<table>
<thead>
<tr>
<th>INTERACTER</th>
<th>MW/pI</th>
<th>DOMAIN</th>
<th>HOMOLOGUE</th>
<th>NLS</th>
<th>NES</th>
<th>LOCALIZATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mi7</td>
<td>21/9.7</td>
<td>DUF640/TF</td>
<td>AtLSH10</td>
<td>+</td>
<td>+</td>
<td>nucleus</td>
</tr>
<tr>
<td>sc4117</td>
<td>80/6.0</td>
<td>motor</td>
<td>MT-kinesin</td>
<td>-</td>
<td>-</td>
<td>microtubule</td>
</tr>
<tr>
<td>sc4121</td>
<td>53/5.8</td>
<td>NAM/TF</td>
<td>AtVOZ1</td>
<td>+</td>
<td>+</td>
<td>microtubule</td>
</tr>
<tr>
<td>Ni67</td>
<td>53/5.5</td>
<td>NAM/TF</td>
<td>AtVOZ1</td>
<td>+</td>
<td>+</td>
<td>ER</td>
</tr>
</tbody>
</table>

Features of these proteins were determined by predictive algorithms. Molecular weight (MW) in kilodaltons and isoelectric point (pI) of each protein were predicted from the deduced amino acid sequence for each gene. Domain searches identified conserved domains in transcription factors (TF) or motor proteins. Protein with the highest named homologue in BLAST searches included the Arabidopsis light-sensitive hypocotyl 10 (AtLSH10), a Nicotiana benthamiana BY-2 microtubule-associated motor kinesin (MT-kinesin), and the Arabidopsis vascular one-zinc-finger 1 (AtVOZ1) proteins. The presence or absence of nuclear localization signal (NLS) and nuclear export signal (NES) sequences are also shown, as is the predominant site of localization of these proteins in steady-state observations.
Table 6.2. Subcellular loci at which binary complexes of host factors and the nucleocapsid (N), matrix (M), and sc4 proteins were found.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>M</th>
<th>sc4</th>
<th>Ni67</th>
<th>Mi7</th>
<th>sc4i17</th>
<th>sc4i21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni67</td>
<td>Nu</td>
<td>-</td>
<td>-</td>
<td>ER</td>
<td>-</td>
<td>-</td>
<td>Per</td>
</tr>
<tr>
<td>Mi7</td>
<td>-</td>
<td>Nu</td>
<td>-</td>
<td>-</td>
<td>Nu</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sc4i17</td>
<td>-</td>
<td>Nu</td>
<td>MT</td>
<td>-</td>
<td>-</td>
<td>MT</td>
<td>MT</td>
</tr>
<tr>
<td>sc4i21</td>
<td>-</td>
<td>-</td>
<td>Per</td>
<td>Per</td>
<td>-</td>
<td>MT</td>
<td>Per/MT</td>
</tr>
</tbody>
</table>

a Nu = nucleus, ER = endoplasmic reticulum, Per = periphery, and MT = microtubule; – indicates that no interaction was detected.
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Figure 6.1. Single-section confocal micrographs showing SYNV protein interactions determined by bimolecular fluorescence complementation (BiFC). Interaction assays were conducted in leaf epidermal cells of transgenic *N. benthamiana* expressing cyan fluorescent protein fused to the nuclear marker histone 2B (CFP-H2B). Shown are the localization of CFP-H2B (nucleus, column 1), interaction assay (BiFC, column 2), and merge of the two preceding panels (overlay, column 3). Proteins listed first in the pair of interactors were expressed as fusions to the amino-terminal half of yellow fluorescent protein (YFP). Those listed second were expressed as fusions to the carboxy-terminal half of YFP. However, protein fusions to each half of YFP were tested in all pairwise interactions, of which a subset is shown here. A1 to A3, Only one representative of the results obtained using glutathione-S-transferase (GST) as a nonbinding control is provided here. However, no SYNV proteins shown here interacted with GST. All pairwise interactions for SYNV proteins, excluding L, were tested. BiFC-positive interactions were observed for B1 to B3, sc4/sc4; C1 to C3, G/sc4; D1 to D3, G/G; E1 to E3, M/M; F1 to F3, N/N; G1 to G3, N/P; and H1 to H3, P/P. The following pairwise combinations were BiFC negative: N/M, N/sc4, N/G, P/M, P/sc4, P/G, M/sc4, and M/G.
Figure 6.2. A, Single-plane confocal micrographs showing the relationship between the localization patterns of A1, green fluorescent protein–endoplasmic reticulum (GFP-ER) and A2, sc4i21. A3, Merger of A1 and A2. B, Z-stacks of confocal micrographs to show the relationship between B1, GFP-labeled actin filaments in leaf epidermal cells of transgenic *N. benthamiana* plants expressing GFP-Talin and B2, sc4i21 expressed by agroinfiltration. B3, Overlay of B1 and B2. B4, Under conditions that completely disrupt actin filaments (10 μM LatB), B5, localization of sc4i21 was unaffected. B6, Overlay of B4 and B5. C1 and C4, GFP-labeled microtubules colocalize with C2, sc4i21 and C5, sc4i17. C3 and C6, Overlay of these images. D, Confocal micrographs to further establish the microtubule association of sc4 interactors. D1, Under control conditions, RFP:sc4i21 appeared as filaments. D2, Following treatment with 100 μM oryzalin, RFP:sc4i21 was detected as disorganized aggregates. D3, In single-plane confocal micrographs taken at mid-cell, RFP:sc4i17 was easily detected continuously around the cell periphery. D4, Following treatment with oryzalin, RFP:sc4i17 is detected primarily in discrete puncta on the cell periphery.

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Figure 6.3. Single-plane confocal micrographs showing the relationship between the localization patterns of A1, green fluorescent protein–endoplasmic reticulum (GFP-ER) and A2, Ni67 and A3, the merger of these two panels (overlay). B, Region (green box) selected in A1 shows that B1, ER rings encircle B2, Ni67 complexes. B3, Merger of B1 and B2. Despite its steady state localization in the cytoplasm, use of a nuclear marker line of C1, *N. benthamiana* shows that the C2, interaction of Ni67 and *Sonchus yellow net virus* N protein was nuclear. C3, Merger of C1 and C2. Protein localization was conducted in leaf epidermal cells of transgenic *N. benthamiana* cells expressing GFP targeted to the ER (GFP-ER) or cyan fluorescent protein targeted to the nucleus (CFP:H2B).

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Figure 6.4. Single-plane confocal micrographs showing the relationship between the localization patterns of A, Mi7 in B, green fluorescent protein–endoplasmic reticulum plants (GFP-ER). C, Merger of A and B. D, G, and J, Bimolecular fluorescence complementation assays conducted in leaf epidermal cells of transgenic *N. benthamiana* plants expressing a nuclear marker protein show that E, the Mi7/M interaction was F, nuclear. H, The Mi7/Mi7 interaction was also I, nuclear. K and L, Control experiments showed that Mi7 did not interact with the nonbinding control glutathione-S-transferase.

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Figure 6.5. Single-plane confocal micrographs of bimolecular fluorescence complementation assays conducted in leaf epidermal cells of transgenic *N. benthamiana* plants expressing a nuclear marker protein (CFP:H2B). A to C, The M/sc4i17 interaction resulted in the formation of B, mobile complexes that are C, nuclear localized. D through F, The sc4/sc4i21 interaction was detected most strongly E, on the cell periphery. G, The sc4/sc4i17 was detected on filamentous complexes that radiate from the H, nucleus (inset).

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Figure 6.6. Single plane confocal micrographs of bimolecular fluorescence complementation (BiFC) assays conducted in leaf epidermal cells of transgenic *N. benthamiana* plants expressing a nuclear marker protein. Panels show the location of nuclei (CFP-H2B) and protein interaction assay (BiFC) and mergers of these two images (overlay). A1 to A3, Sc4i17/sc4i17 form filamentous complexes. B1 to B3, Ni67/Ni67 complexes were dispersed throughout the cytoplasm. C1 to C3, Sc4i21/Ni67 complexes accumulated in punctate loci on the cell periphery. Sc4i21/sc4i21 complexes accumulated in punctate loci on the cell periphery in D2 and D3, mid-cell micrographs and D4, on filaments when viewed at the top or bottom of cells. None of these host factors interacted with the nonbinding control glutathione-S-transferase (data not shown).
Figure 6.7. Yeast-based assay for identification of proteins containing a functional nuclear localization signal (NLS). Positive- (H2B) and negative control (MBP) proteins or *N. benthamiana* proteins (Mi7, sc4i17, sc4i21, and Ni67) were expressed from pNIA-DEST in yeast strain L40. Top panel: yeast dropout media supplemented with histidine. Lower panel: yeast dropout media lacking histidine. Only those proteins containing a predicted NLS (Mi7, sc4i21, and Ni67) were able to facilitate yeast growth on media lacking histidine.

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Figure 6.8. Two forms of the SYNV glycoprotein accumulate in A, virus-infected cells (SYNV) or B, leaf-epidermal cells of *N. benthamiana*, in which a recombinant G protein was expressed by agroinfiltration (FLAG·G). The full-length G protein (G) is approximately 70 kDa whereas the truncated form, G*, is approximately 50 kDa. A truncated G protein lacking its transmembrane, and carboxy-terminus interacted with C1 to C3, itself; D1 to D3, full-length G; and E1 to E3, sc4 but not F1 to F3, glutathione-S-transferase. The boxed area in E3 is enlarged in E4 to show that the G*–sc4 interaction was detected primarily on punctate loci on the cell periphery. Bimolecular fluorescence complementation (BiFC) conducted in leaf epidermal cells of transgenic *N. benthamiana* plants expressing a fluorescent nuclear marker (CFP:H2B) was used to determine protein interactions.
Figure 6.9. Host factor and viral protein interaction map for SYNV. Proteins forming heterologous interactions are connected by a straight line. Self-interactions are indicated by curved arrows. The superscript indicates steady-state localization: MT = microtubule, ER = endoplasmic reticulum, m = membranes, n = nuclear, and p = cell periphery.

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Figure 6.10. Model of the SYNV cell-to-cell movement complex. Sc4i21 and sc4i17 are a microtubule-anchored transcription activator and motor-kinesin, respectively. Ni67 is an endoplasmic reticulum (ER)-associated transcription activator that may shuttle into the nucleus where it interacts with the SYNV-N protein and, presumably SYNV, nucleocapsids. Mi7 is also a transcription factor that interacts with the M protein, and which is also likely a nuclear shuttle protein. Current data suggest that Mi7 and Ni67 could function cooperatively to export nucleocapsids from the nucleus to establish a movement complex with sc4 and G or G* in the cytoplasm.

Nucleocapsids that are not exported bud through the inner nuclear membrane to form mature virions that accumulate in the perinuclear space. Based on electron microscopy data, it is likely that most nucleocapsids undergo morphogenesis (bold arrow) given that the movement complex has not yet been observed or isolated.
CHAPTER VII
Concluding Remarks

Rhabdoviruses that infect plants are assigned to two taxonomic groups, the genera *Nucleorhabdovirus* and *Cytorhabdovirus*. Nucleorhabdoviruses replicate and assemble in the nucleus, whereas this occurs in the cytoplasm for the cytorhabdoviruses. *Sonchus yellow net virus* (SYNV) and *Potato yellow dwarf virus* (PYDV) are nucleorhabdoviruses with SYNV the best characterized and PYDV the type species. *Lettuce necrotic yellows virus* (LNYV) is the type species of the cytorhabdoviruses. These three plant rhabdoviruses, LNYV, PYDV and SYNV, were studied in a common host, *Nicotiana benthamiana*, the most widely used experimental host in plant virology (Goodin et al., 2008). Direct comparisons of viral protein localization and interactions with viral and host proteins provided insights how each of these viruses are capable of infecting the host, replicating, undergoing morphogenesis and moving cell-to-cell within the same system.

Rhabdoviruses are single-stranded, negative-sense RNA viruses with genomes that encode at least five genes. These genes include a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and a polymerase (L). Compared to animal rhabdoviruses such as *Vesicular stomatitis virus* (VSV), plant rhabdoviruses also contain one additional gene that is considered to be a putative movement protein (Jackson et al., 2005). Very little is known about the structure of plant rhabdoviruses. Through electron microscopy, plant rhabdoviruses appear as bacilliform particles. They are composed of negative sense RNA surrounded by N, P and L proteins. The RNA, N, P and L complex is called the viral core because it is the minimum infectious unit and is encased in a coating of the matrix proteins surrounded by a host membrane embedded with viral glycoproteins.

To test this model of viral structure, SYNV proteins were expressed as fusions to cyan fluorescent protein (CFP) and red fluorescent protein (RFP) in *N. benthamiana*. This was done in both infected and non-infected plants to determine the effect of virus on localization (Goodin et al., 2007). These experiments generated a general model where N (already bound to the negative-sense RNA) associates with P in the nucleus for replication, the P protein is then removed and N associates with M, then M may associate with G at the inner nuclear membrane for morphogenesis. This study agrees with previously published models (Jackson et al., 2005) as a model for the assembly of rhabdoviruses.

To extend this research, a system was developed to determine protein interactions and localization simultaneously in plant cells. The hypothesis was that LNYV, SYNV and PYDV, which all share a similar genome organization and viral structure, would share common interactions between each of their viral proteins. With this knowledge, inferences could be made about how these viruses replicate, assemble and move from cell-to-cell. A method was required that would allow for the determination of not only possible interactions, but also the site of interaction in the same host, *N. benthamiana*. This was achieved by the construction of a set of *Agrobacterium*-mediated transformation vectors that would enable an assay called Bimolecular Fluorescence Complementation (BiFC) in plants (Hu et al., 2002). This
assay is based on yellow fluorescent protein (YFP); however, it can be done with any autofluorescent protein (AFP). When the AFP is expressed in two halves, no fluorescence is detected. However, if one expresses fusions of the two halves of the AFP to two proteins that interact, the AFP fluorescence is reconstituted. These vectors and those designed with previously untested AFPs in plants are described in more detail in Chapter II.

Pair-wise combinations of viral proteins excluding L, encoded by LNYV, SYNV and PYDV, were tested for cognate protein interactions. The L protein is difficult to express due to its large size (240kD) and was not included in these assays. In contrast to my original hypothesis, only two interactions are common between all three viruses, the N-P interaction and M self-interaction (see Figure 5.9). When comparing these interaction patterns to the patterns predicted from the co-localization assays, it was unexpected that only PYDV fits this model. SYNV has an N-P interaction, but no interaction between either P and M or N and M. LNYV has an N-P interaction, but also a P-M interaction unlike either SYNV or PYDV. This suggests two possibilities: the interactions missing may only occur during infection (these assays were done in uninfected plants) or the second, that there may be host factors involved. The presence of host factors either within the mature virion or associated with viral cores may explain why there are so few interactions detected between the viral proteins of SYNV and LNYV when compared to PYDV (see Figure 5.9 for viral interaction map comparisons).

The interaction of N and P proteins was compared to that of other rhabdoviruses published in the literature and the re-localization of the N-P complex (when seen in BiFC and compared to expressing these proteins as fluorescent protein fusions) is similar to Maize fine streak virus (MFSV) (Tsai et al., 2005). This suggested that the N-P interaction and re-localization effect might be conserved among plant rhabdoviruses. Viral protein interactions in the animal rhabdovirus, VSV, include an N-P interaction as well (Moerdyk-Schauwecker et al., 2011). Further tests on the expression and interactions of N and P proteins from other plant and animal rhabdoviruses are required to determine if this effect is conserved in all members of the Rhabdoviridae or specific to only certain members of the family. It is expected that as N, P and L form the viral polymerase complex and that this may be one interaction conserved in all rhabdoviruses. However, other interactions between these three viruses, SYNV, PYDV and LNYV, do not appear to be conserved, which suggests that these viruses, although genetically related, are very different from each other structurally.

The continuation of this work will expand these interaction maps to include host factors, which will create a whole host-virus “interactome” map. This is the first step in a systems biology approach that will incorporate genomics, proteomics and cell biology to gain a more complete picture of how these viruses are able to infect, replicate, assemble and move from cell-to-cell. A host-virus interaction map was created using SYNV-N, M and sc4 as baits in a high-resolution N. benthamiana yeast two-hybrid screen to identify host factors. A total of 31 host factors of interest were identified, four of which were considered to be relevant in terms of movement of the virus from cell-to-cell during infection. These were chosen because they contained domains necessary for movement in and out of the nucleus (nuclear
localization signals [NLSs] and nuclear export signals [NESs]) or movement throughout the cell (cytoskeletal proteins). When these host factors were tested in further BiFC analyses, they interacted with viral proteins in a manner consistent with the model that these host factors are involved in viral movement.

The four host factors identified were called Mi7, sc4i21 and Ni67 that have NLSs and NESs, and sc4i17 which contains a motor protein domain (see Table 6.1). SYNV-N and P interact in the nucleus (shown in the viral interaction map comparisons) to form part of the viral core also containing the L protein and the negative-sense genomic RNA. This core interacts with SYNV-M, possibly for condensation or perhaps for movement alone. This core interacts with Ni67 and Mi7 that have NLSs and NESs necessary not only to enter the nucleus, but also to exit the nucleus in association with viral proteins through the nuclear pores. This larger complex also interacts with sc4i21, sc4i17, SYNV-sc4 and SYNV-G outside of the nucleus for movement along the microtubules. The G protein thought to be part of the movement complex is a smaller truncated G that does not contain a transmembrane domain.

This model of viral movement answers some interesting questions about SYNV, one question being why are gaps in the SYNV interaction map present compared to the map of PYDV? Amazingly, the four host factors described in a model of viral movement can fill these gaps (see Figure 6.9). It seems a viral core can avoid budding out of the nuclear envelope in a two-step process to exit the nucleus with associations with host factors containing NES sequences for exit via nuclear pores. This suggests that the mature virions accumulating in the perinucleolar space are separate from the viral cores that exit the nucleus through the nuclear pores. One further question remains in regards to these two pathways: what are the controlling factors that determine whether the viral core will leave the nucleus through the nuclear pores or bud into the perinucleolar space to accumulate? Can this be answered with associations with host factors? If the availability of host proteins is a limiting factor, then those cores associated with host factors can move out of the nucleus and along the microtubules and those not associated simply bud into the perinucleolar space? If the associations with host factors determine the movement out of the nucleus, silencing these host factors will result in accumulation of the mature virus in the perinucleolar space. A systematic approach to silence each of the identified host factors in N. benthamiana through VIGS vectors or siRNA vectors described in Chapter I and infecting the silenced plants with SYNV will determine if the associations with these host factors are necessary for infection to be successful.

Once out of the nucleus, through SYNV protein associations with host proteins, it was determined that the microtubules are the means of moving from the nucleus to the cell periphery. Once at the cell periphery, the viral core still needs to move to the adjacent cells. It is proposed that this occurs through the plasmodesmatal channels. Preliminary results have shown SYNV-sc4 and a plasmodesmata-localized protein from Arabidopsis thaliana, AtPDLP1a (Thomas et al., 2008), colocalize to the plasmodesmata. Further analysis is needed to determine if a N. benthamiana homolog can be found and if the same co-localization and possible interaction can occur. The viral core would need to exit out of the
plasmodesmata to infect the next cell, and once there, the infection cycle would continue until the virus is able to infect cells adjacent to the phloem cells of the plant. Once in the phloem, the viral cores may move throughout the plant for systemic infection. Interestingly, two of the host factors (sc4121 and Ni67) associated with the viral core in movement have homology to AtVoz1, an *Arabidopsis* phloem-associated transcription factor (Mitsuda *et al.*, 2004), and one begins to wonder if these same host factors may be necessary for systemic movement as well as cell-to-cell movement. If this is the case, the silencing of these host factors would not only impact cell-to-cell movement, but systemic movement as well.

For PYDV, the interaction map does not have any gaps between each of its cognate proteins (see Figure 5.9). This does not preclude the importance in host factors for infection, as VSV has only one gap between M and N in its interaction map (Moerdyk-Schauwecker *et al.*, 2011), however the purified virion contains up to 64 host proteins (Moerdyk-Schauwecker *et al.*, 2009). If a similar movement complex for PYDV (compared to SYNV) occurs, then virus-host factor associations with microtubules and the plasmodesmata are predicted to occur. Similar to SYNV: the movement protein of PYDV does not localize to microtubules, but to the cell periphery, thus host factors may enable movement of viral cores along the microtubules. Although PYDV proteins do not contain NLSs that can be predicted through *in silico* algorithms, they localize to the nucleus with the exception of the movement protein, which localizes to the cell periphery (Bandyopadhyay *et al.*, 2010). The identification of the specific host factors associated with PYDV proteins should be determined. Tests to determine if PYDV interacts with any of the same host factors as SYNV, as these have already been determined, is the first step. BiFC analysis with the SYNV host factors with PYDV proteins may determine if any conservation of host factor associations exists between these two viruses. Then PYDV proteins should also been screened against the high resolution *N. benthamiana* yeast two-hybrid library to find other PYDV specific host factors. Screens with PYDV proteins would need to be compared to BiFC assays as was done for SYNV to determine the site of interaction *in planta*. An initial screen with PYDV-N has been completed, but further analyses have yet to be done. Similar domains may be present in the host factors associated with PYDV infection including possible NLSs and NESs for movement of the core out of the nucleus and possibly also a motor protein for movement along the microtubules.

It remains to be seen if the interaction map for LNYV can also be filled with host factors from its native host, lettuce. LNYV does not infect *N. benthamiana*, and although initial BiFC tests have shown that the virus protein-protein interactions are the same in both *N. benthamiana* and lettuce, the host factor associations may differ between the two hosts. This suggests a possible reason why LNYV cannot infect *N. benthamiana*. If so, how similar are LNYV host factors in comparison to those from SYNV or PYDV? LNYV is a cytorhabdovirus, and the requirement to enter and leave the nucleus is absent, but as LNYV matures in the endoplasmic reticulum (ER), this necessitates the viral core either leaving the ER for cell-to-cell movement or movement through the ER to the plasmodesmata. Having left the ER, the viral cores of LNYV could move throughout the cell in association with
cytoskeletal proteins. Interestingly, unlike SYNV-sc4 and PYDV-Y, LNYV-4b seems to associate with the microtubules alone without the requirement of additional factors. However, it is unknown whether 4b associates with the viral cores directly, or with host factors that act as a bridge to the viral core as seen with SYNV. Similar to SYNV, the interaction map for LNYV has no direct viral protein interactions between the viral core and the movement protein (see Figure 5.9). Identification of host factors associated with LNYV infection could be achieved using yeast two-hybrid screens of LNYV protein baits with a library built from lettuce similar to SYNV proteins with N. benthamiana (Min et al., 2010).

To complement the interaction maps, two viral proteins were characterized, SYNV-G and L. The domains of these proteins were mapped using in silico methods and compared to the published sequences of plant rhabdoviruses. Common to all sequenced plant rhabdovirus glycoproteins are: signal peptides, varying numbers of glycosylation sites, a single transmembrane domain and carboxy termini of various lengths. When the glycoproteins, SYNV-G and LNYV-G, were compared in truncation analyses, the localization results suggest that the carboxy terminus is not required for the final localization of both proteins, and the signal peptide is functional and directs a fusion to the endoplasmic reticulum. Interestingly, the carboxy terminus of SYNV-G is unique among plant rhabdoviruses and contains functional nuclear localization signals and is localized to the nucleus when expressed as a GFP fusion. When first directed into the ER, the carboxy terminus also directs GFP to the same location as the full-length glycoprotein fusion. Signals for the final localization seem to be in multiple regions of the glycoprotein.

Unfortunately, the effect of these mutations on localization during infection has yet to be determined. Expression of the truncation mutants of the SYNV glycoprotein in the context of infection need to be repeated, however, this is an indirect assay. The localization of the truncation mutants would the most conclusive if tested in the context of an infectious clone. In this case, the glycoprotein truncations would be in the viral genome and determination of localization and viral infectivity would be more relevant. The role of each domain could then be analyzed in the context of how it impacts the infection cycle of the virus directly, instead of indirectly through fluorescence microscopy. However, as these viruses require the presence of not only the genomic RNA, but N, P and L proteins during the initial stages, expressing the L protein remains the largest hurdle towards the creation of an infectious clone.

To determine the best way to express the L protein, in silico domain analysis was conducted. For the L protein, there are multiple regions of similarity between all sequenced plant-adapted rhabdoviruses. These conserved blocks have also been identified in the animal infecting rhabdovirus, VSV (Poch et al., 1990) and other members of the Mononegavirales. For the fragments SYNV-L expressed, it is difficult to detect the fragments and the last fragment is undetectable (see Figure A.2). Previous attempts to detect a full-length SYNV-L have also failed. It is possible that the L protein may need to be expressed in the context of other viral proteins for stability. A full-length L and each of the L fragments should be expressed together with other viral proteins to determine if this is the case. Initial attempts with SYNV P were undertaken, however, this needs to be repeated with N as well, to determine
if the presence of other viral proteins has any effect. It is possible that in
combinations of viral proteins, expression of SYNV L could be detected.

Taken together, the domain analysis suggests that for the glycoprotein and
the polymerase, individual proteins may have areas of conservation necessary for
the virus to replicate, undergo morphogenesis and move cell-to-cell. Further in
silico analysis and comparison of the other four viral proteins with other published
rhabdoviruses is needed to determine if conservation among the domains of other
proteins occurs. This is striking when comparing the individual protein domain
analyses with the whole virus interaction maps. The protein functions seem to be
conserved, domains are conserved, but the protein interactions necessary for viral
functions differ.

Although the data presented in this dissertation provides more detailed
information of viral replication, morphogenesis and cell-to-cell movement in SYNV,
only basic comparisons to two other plant rhabdoviruses, PYDV and LNYV, have
been completed. These comparisons demonstrate that the interaction maps from
different viruses differ; however, the domains in two proteins are conserved. The
interaction maps may differ in these three viruses because of important host factor
associations, which in the case of SYNV, fill the gaps to create a map more
comparable to that of PYDV. These host factor associations are responsible for the
cell-to-cell and possibly for systemic movement of SYNV. At this point, the host
factor associations of LNYV and PYDV remain unknown.
APPENDIX I*
Characterization of the Sonchus yellow net virus polymerase protein domains.

Members of the Rhabdoviridae are single-stranded negative-sense RNA viruses that share a common elongated, rod-like, or bacilliform shape that separate them from other members of the order Mononegavirales. In addition to the plant-infesting rhabdoviruses, other genera in this family include the lyssaviruses (Rabies virus), the vesiculoviruses (Vesicular stomatitis virus) and the emphemoroviruses (Bovine ephemeral fever virus). The plant-infesting rhabdoviruses are further classified into two genera, Nucleorhabdovirus and Cytorhabdovirus. As their names suggest, the nucleorhabdoviruses replicate in the nucleus and the cytorhabdoviruses replicate in the cytoplasm (Jackson et al., 2005).

The nucleorhabdovirus, Sonchus yellow net virus (SYNV), contains six open reading frames corresponding to the nucleoprotein (N), the phosphoprotein (P), the movement protein (sc4), the matrix protein (M) and the polymerase protein (L). The single stranded RNA is associated with the N, P and L proteins to form a viral core. This viral core interacts with the matrix protein, which condenses it to associate with the glycoprotein as it buds through a membrane to complete morphogenesis (Jackson et al., 2005). The movement protein is thought to facilitate the cell-to-cell movement of the virus (Huang et al., 2005; Melcher, 2000; Min et al., 2010; Scholthof et al., 1994).

Specifically the polymerase protein (L or Large protein) comprises 6, 401 bp out of the total 13, 720 bp genome. It is 2116 amino acids and predicted to be 241 kDa. L also contains two predicted nuclear localization sites at amino acid positions 1648 and 2055. Compared with other members of the Rhabdoviridae, SYNV L also associates with the N and P proteins to facilitate the replication of the viral genome and transcription of the mRNA that are both capped and poly-adenylated (Wagner & Jackson, 1997; Wagner et al., 1996). There is a single initiation site for the polymerase at the 3’ terminus of the viral genome, and with polar transcription, there are higher levels of mRNA present for the genes at the 3’ end compared to those at the 5’ end (Wagner & Jackson, 1997; Wagner et al., 1996). This is also consistent to what is found with other members of the family including Vesicular stomatitis virus (VSV). SYNV L has also been previously compared with other members of the same order, Mononegavirales, and several conserved amino acid sequence domains are present (Choi et al., 1992; Poch et al., 1990). The functions of all these domains have not yet been identified, however the RNA-dependent RNA polymerase domain is predicted to be block III and mRNA capping are determined to be blocks V and VI in VSV and Sendai virus (SV) (Galloway et al., 2008; Li et al., 2008; Murphy & Grdzelishvili, 2009; Murphy et al., 2010).

Previous attempts to localize the L protein in SYNV have not met with success. The expected localization is in the nucleus due to the site of replication and the presence of nuclear localization signals (NLSs). In addition to determining the similarities of

SYNV L to the well-characterized L protein of VSV, an approach to localize fragments of the protein may lead to information about the localization of the whole protein.

METHODS

Plant growth
This section has not been previously published.

Wildtype plants of *Nicotiana benthamiana* were grown in the greenhouse under ambient conditions.

Predicted domains of SYNV polymerase
This section has not been previously published.

Sequences corresponding to the full length of the SYNV polymerase were analyzed for protein domains. This was done with PSORT for determination of protein localization (Nakai & Kanehisa, 1991). The comparison of the previously sequenced polymerases of plant rhabdoviruses to previously published members of the *Mononegavirales* was done utilizing the CLUSTAL W algorithm (Choi et al., 1992; Poch et al., 1990; Thompson et al., 1994). Amino acid sequences used for comparison include: Lettuce necrotic yellows virus (LNYV; NC_007642), Northern cereal mosaic virus (NCMV; NC_007642), Rabies virus (RABV; NC_001542), Vesicular stomatitis Indiana virus (VSIV; EF197793.1), Maize mosaic virus (MMV; NC_005975), Taro vein chlorosis virus (TaVCV; NC_006942), Maize Iranian mosaic virus (MIMV; DQ186554), Rice yellow stunt virus (RYSV; NC_003746), SYNV (M87829), and Maize fine streak virus (MFSV; NC_005974).

Multiple sequence alignments
This section has been previously published, but was conducted by Kathleen Martin.

Except for PYDV, all L protein sequences used in the sequence alignment study were obtained from data deposited in the NCBI database. The deduced amino acid sequences of the L genes were aligned using the CLUSTAL W algorithm (Thompson et al., 1994), included in the MegAlign program of the DNASTAR software package. The alignments were analyzed by MEGA4.0.2 (Tamura et al., 2007). The phylogenetic tree derived from these datasets was generated using the neighbor-joining method (Saitou & Nei, 1987) with a bootstrap test with 1000 replicates (Felsenstein, 1985) to determine the percentage of replicate trees in which the taxa clustered together. The evolutionary relationship of these polymerase proteins was computed using the Dayhoff matrix-based method (Schwartz & Dayhoff, 1979). In contrast to other algorithms for determining phylogenetic relationships, the Dayhoff method is more effective when using small datasets of closely related proteins, which is the assumption made here given that only rhabdoviral sequences were considered. L gene sequences utilized in phylogenetic analyses are the same as those used for domain comparison described above.
Protein expression in *N. benthamiana*
*This section has not been previously published.*

Total RNA was extracted from plant tissues using the RNeasy Plant minikit (Qiagen) according to the manufacturer’s instructions. First strand cDNA synthesis and PCRs were carried out using Superscript reverse transcriptase III (Invitrogen) and Phusion high fidelity DNA polymerase (Finnzymes), respectively. PCR amplification from cDNA of SYNV L fragments corresponding to between 1-2 kb of sequence was done and fragments were cloned into pDONR221 and sequenced. Primers for amplification are included in Table A.1. Recombination of fragments was done into pSITE-2CA (green fluorescent protein) and positive clones were transformed into Agrobacterium tumefaciens strain LBA 4404. Infiltration into wildtype *N. benthamiana* was done as described previously (Goodin *et al.*, 2005).

**Laser Scanning Confocal Microscopy**
*This section has not been previously published.*

All microscopy was conducted on an Olympus FV1000 laser scanning confocal microscope as described previously (Goodin *et al.*, 2005).

**RESULTS**

**Prediction of Domains of SYNV L**

SYNV L has two predicted nuclear localization signals at positions 1648 and 2055 aa (Table A2). By comparison of the SYNV L polymerase to the previously published sequences of L proteins in the *Mononegavirales* with attention to the closest characterized relative, Vesicular stomatitis virus, it was determined that SYNV L contains six conserved blocks: block one is 235 - 419 aa, block two is 528 - 631 aa, block three is 631 - 851 aa, block four is 941-1100 aa, block five is 1139-1369 aa and block six is 1685-1755 aa (Figure A.1).

**Localization of Fragments of SYNV L**

Due to the inability to express the full-length polymerase, the construction of fragments of SYNV L was done to determine the expression and localization of specific domains. Primers were designed to ensure overlap of each of the fragments, so as not to break up any of the six conserved blocks predicted in the amino acid sequence. Fragment one corresponding to 1 -627aa localizes to the cell periphery and nucleus. Fragment two corresponding to 526 - 862 aa localizes to the cell periphery. Fragment three, 635 – 1139 aa, localizes to the cell periphery. Fragment four, 891 – 1573 aa, localizes to the cell periphery and fragment five, 1500 – 2116 aa, was not detectable (Figure A.2).

**Taxonomic assignment of SYNV based on L protein sequence comparisons**

We show here, using the primary structure of L proteins that PYDV is most closely related to other leafhopper-transmitted viruses RYSV and MFSV, other nucleorhabdoviruses. Interestingly, the planthopper-transmitted MIMV and MMV clustered with TaVCV, for which planthopper transmission is suspected but not
firmed established (Revill et al., 2005). SYNV, transmitted by the aphid, *Aphis correopsidis*, formed a separate clade to the aforementioned viruses. However, as a group, all of the nucleorhabdoviruses and MIMV clustered together and were well separated from the cytorhabdoviruses and non-plant-associated rhabdoviruses (Figure A.3).

**DISCUSSION**

Previous studies have determined that SYNV L has conserved domains compared to other members of the *Mononegavirales* (Choi et al. 1992). Expanding on this comparison, the conserved domains of L were compared to the six conserved blocks of VSV (Poch et al., 1990) and the positions of the blocks in SYNV were determined (Figure A.1). SYNV was further compared to the other available plant adapted rhabdoviruses and the positions of the conserved domains are located in similar regions of the proteins (*Lettuce yellow mottle virus* was not available at the time of this comparison)(Table A.2). The functions of all of the conserved blocks are not known, however, the third block contains the conserved RNA-dependent-RNA-polymerase domain and the fifth block is responsible for mRNA capping in VSV (Galloway et al., 2008; Li et al., 2008; Murphy & Grdzelishvili, 2009; Murphy et al., 2010). These blocks are conserved among polymerase proteins of viruses in the order *Mononegavirales*. Other members of the same order include the *Bornaviridae*, *Filoviridae* and *Paramyxoviridae*. Initially the studies on the conserved domains included Newcastle disease virus (NDV), Sendai virus (SV), Measles virus (MV), Rabies virus, and Vesicular stomatitis virus (VSV). The first three in this alignment are Paramyxoviruses and the last two are Rhabdoviruses (Poch et al., 1990). The conservation of these domains suggests that they are required for function in viral genome replication, mRNA 5’ capping, cap methylation and 3’ polyadenylation in the viruses of this order (Lyles & Rupprecht, 2007).

The catalytic domains required for those functions described above have been attributed solely to the L protein but the N and P proteins are also considered to be part of the polymerase. They are responsible for the shift in polymerase function from transcription of viral mRNAs to replication of the genome (Lyles & Rupprecht, 2007). The L-P interaction domain is in the N terminus of L in both MV and SV (Cevik et al. 2004; Holmes and Moyer, 2002). The MV L-P interaction domain is from 1-408 aa and the SV L-P interaction domain is from 1-305 aa (Cevik et al., 2004a; Cevik et al., 2004b; Holmes & Moyer, 2002). To determine if this is also the case for SYNV L and P, bimolecular fluorescence complementation of SYNV L fragment one with P was conducted, however no interaction was detected (data not shown). There was also no self-interaction observed with the first L fragment, even though the oligomerization domain of SV L is between 1-174 aa of the protein (Cevik et al., 2004a; Cevik et al., 2004b) (data not shown). This suggests several possible alternatives: the L fragment expressed from 1-627aa may not fold correctly and is not able to associate with itself or with P; other regions of the protein may facilitate this binding by determination of the correct protein localization for binding; and finally, the nucleorhabdoviruses may utilize different regions of the L protein for both oligomerization and P interactions than do the Paramyxoviruses even if they
share other conserved domains. Interactions between P and other fragments of the L protein have yet to be tested.

The last fragment of SYNV L was not detectable; this is the region that contains the predicted NLSs (Figure A.1, Table A.2). Originally an attempt was made to clone this fragment in two pieces, however, sequencing revealed these clones were not correct. A correct clone of the last fragment was not detectable, but an attempt to again split this clone may reveal further fragments that can be localized.

In conclusion, we note that three clades within the genus *Nucleorhabdovirus* were identified that could be distinguished by the particular vector for each virus. Thus, it appears that insect vectors may have a major influence on the evolutionary trajectories of plant-adapted rhabdoviruses. This agrees with previous phylogenetic analyses of the N protein of these viruses (Ghosh *et al*., 2008), however in that study, vectors for the virus were not considered. The relationship between viruses and their vectors is also seen in the animal infecting flaviviruses (Gaunt *et al*., 2001; Gould *et al*., 2003) where virus groups can be distinguished by both insect vector and host. In plants, it has been suggested that the insect vectors of geminiviruses drive viral evolution, this based on the emergence of a new biotype of whitefly which caused the rampant spread of disease to hosts that these viruses previously did not infect (Brown *et al*., 1995; Chare & Holmes, 2004; Power, 2000). Our continued comparative studies with SYNV and PYDV will contribute to a better understanding of the common and unique molecular requirements for infection of a common host by aphid- and leafhopper-vectored viruses.
Table A.1  Primers for amplification of the fragments of the polymerase of SYNV

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>amino acids</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>AAAAAAGCAGGCTTAatggaagggatggaactggg</td>
<td>AGAAAGCTGGGTAatattaaggtggtcataatcgaataa</td>
<td>1-627 aa</td>
</tr>
<tr>
<td>2</td>
<td>AAAAAAGCAGGCTTAacaaagagaagagga tttgagaattg</td>
<td>AGAAAGCTGGGTAcgtagtctttttgtgctgattga</td>
<td>526-862 aa</td>
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<tr>
<td>3</td>
<td>AAAAAAGCAGGCTTAacagttgtaacatagatgccgt</td>
<td>AGAAAGCTGGGTAatagcgtgaccatcctgtgtgtt</td>
<td>635-1139 aa</td>
</tr>
<tr>
<td>4</td>
<td>AAAAAAGCAGGCTTAacaaagagctatgctactagtccccga</td>
<td>AGAAAGCTGGGTAgttctgcatagagataaa ccagctgtg</td>
<td>891-1573 aa</td>
</tr>
<tr>
<td>5</td>
<td>AAAAAAGCAGGCTTAacaaagacattatagaggtgacta</td>
<td>AGAAAGCTGGGTAatcttcaagagatagatgtcata</td>
<td>1500-2116 aa</td>
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Table A.2. Comparing the polymerase protein conserved domains of sequenced plant-adapted rhabdovirus.

<table>
<thead>
<tr>
<th>Virus</th>
<th>total size</th>
<th>I(220-408aa)</th>
<th>II(492-599aa)</th>
<th>III(600-822aa)</th>
<th>IV(888-1052aa)</th>
<th>V(1090-1317aa)</th>
<th>VI(1597-1682aa)</th>
<th>NLS</th>
</tr>
</thead>
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<tr>
<td>Maize Iranian mosaic virus</td>
<td>2260aa</td>
<td>220-408aa</td>
<td>492-599aa</td>
<td>600-822aa</td>
<td>888-1052aa</td>
<td>1090-1317aa</td>
<td>1597-1682aa</td>
<td>NONE</td>
</tr>
<tr>
<td>Maize fine streak virus</td>
<td>1944aa</td>
<td>195-384aa</td>
<td>475-592aa</td>
<td>593-814aa</td>
<td>882-1048aa</td>
<td>1086-1311aa</td>
<td>1596-1683aa</td>
<td>NONE</td>
</tr>
<tr>
<td>Potato yellow dwarf virus</td>
<td>1931aa</td>
<td>201-387aa</td>
<td>482-589aa</td>
<td>590-807aa</td>
<td>878-1044aa</td>
<td>1082-1310aa</td>
<td>1592-1678aa</td>
<td>NONE</td>
</tr>
<tr>
<td>Maize mosaic virus</td>
<td>1922aa</td>
<td>218-406aa</td>
<td>489-596aa</td>
<td>597-819aa</td>
<td>885-1049aa</td>
<td>1087-1314aa</td>
<td>1594-1679aa</td>
<td>KKNPRQSVLDEIRRQ</td>
</tr>
<tr>
<td>Rice yellow stunt virus</td>
<td>1967aa</td>
<td>229-416aa</td>
<td>498-605aa</td>
<td>606-829aa</td>
<td>895-1061aa</td>
<td>1099-1325aa</td>
<td>1618-1708aa</td>
<td>HRKK at 1240</td>
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<tr>
<td>Taro vein chlorosis virus</td>
<td>1928aa</td>
<td>221-407aa</td>
<td>490-597aa</td>
<td>598-820aa</td>
<td>886-1050aa</td>
<td>1088-1315aa</td>
<td>1594-1679aa</td>
<td>KKP LEDHGIVARS</td>
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<td>Sonchus yellow net virus</td>
<td>2116aa</td>
<td>235-419aa</td>
<td>528-631aa</td>
<td>632-851aa</td>
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<td>1685-1755aa</td>
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<td>Northern cereal mosaic virus</td>
<td>2058aa</td>
<td>182-370aa</td>
<td>450-560aa</td>
<td>561-784aa</td>
<td>853-1019aa</td>
<td>1058-1285aa</td>
<td>1630-1753aa</td>
<td>RKF K at 351</td>
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<tr>
<td>Lettuce necrotic yellows virus</td>
<td>2068aa</td>
<td>185-373aa</td>
<td>451-565aa</td>
<td>566-788aa</td>
<td>859-1024aa</td>
<td>1064-1301aa</td>
<td>1633-1739aa</td>
<td>KKKK at 1535,</td>
</tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>KFP DEH GIKR</td>
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<td></td>
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<td></td>
<td>KKT SYTVKNQA</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>KIR at 540</td>
</tr>
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Figure A.1. Diagram of full length SYNV L protein. SYNV L is 2116 aa in length, contains six conserved blocks in white labeled one through six. Block one is 235 - 419 aa, block two is 528 - 631 aa, block three is 631 - 851 aa, block four is 941-1100 aa, block five is 1139-1369 aa and block six is 1685-1755. In addition to the six conserved blocks, there are two predicted nuclear localization signals at 1648 nt and 2055 nt. Numbered line diagrams underneath the protein diagram indicate the sizes of the amplified fragments. Fragment one is 1 - 627aa, fragment two is 526 - 862 aa, fragment three is 635 – 1139, fragment four is 891 – 1573 aa and fragment five is 1500 – 2116 aa.

Kathleen Martin is responsible for previously published figures labeled in RED.
Figure A.2. Localization of fragments of SNYV L fused to green fluorescent protein. Images labeled as the fragment number corresponding to the amino acid lengths and positions shown in Figure A.1.

Kathleen Martin is responsible for previously published figures labeled in RED.
Figure A.3. Phylogeny of plant rhabdoviruses inferred from L protein sequences. Representative rhabdoviruses infecting a variety of hosts were used including viruses that do not infect plants (non-p) as well as plant-adated viruses in the Nucleorhabdovirus (Nucleo) and Cytorhabdovirus (Cyto) genera. Bootstrap values greater than 50% are shown at nodes in the tree. Vectors for the plant-adapted viruses are shown as subscripts, which are aphid (a), leafhopper (l), and planthopper (p). Virus names and Genbank accession numbers are listed in the Materials and Methods. MMV, Maize mosaic virus, TaVCV, Taro vein chlorosis virus, MIMV, Maize Iranian mosaic virus, PYDV, Potato yellow dwarf virus, RYSV, Rice yellow stunt virus, SYN, Sonchus yellow net virus, MFSV, Maize fine streak virus, NCMV, Northern cereal mosaic virus, LNYV, Lettuce necrotic yellows virus, RV, Rabies virus, VSV, Vesicular stomatitis virus – Indiana serotype.
REFERENCES


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