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Development of Transgenic Lines to Support Plant Cell Biology Research

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I graduated from UK College of Fine Arts in 2004 with a double major in Art History and Arts Administration. Upon graduation, I worked at the UK Art Museum for three years as Curatorial Assistant. While I loved working with art, I felt a deepening desire to return to my childhood dream of becoming a scientist. In the fall of 2007, I chose to return to UK to pursue a Bachelor of Science in Agricultural Biotechnology. I am fortunate to have had the opportunity to work in the Goodin lab, where my art background ties in nicely with Dr. Goodin's focus on obtaining the highest quality micrographs to accompany publications.

The past two summers, I have been awarded a eUreKa! Summer Research & Creativity Grant to support my research in the lab. A portion of the research conducted last summer is currently in press to appear in *The Plant Journal*, a leading plant research journal (of 145 plant science journals, *The Plant Journal* has the sixth highest cumulative impact factor [www.journal-ranking.com]).

I plan to continue my education in a biology-related field after graduation. My dream is to study in the Graduate Program at the Florida Institute of Technology in Melbourne, FL, where I may continue to conduct research. Having access to marine ecosystems, I could apply my studies in biotechnology to the field of marine biology. At FIT, I would have the opportunity to work with some of the leading researchers in the field.

Faculty Mentor: Dr. Michael M. Goodin
Department of Plant Pathology

Ms. Kopperud has worked in my lab since January, 2008. During this time she has assisted in the development of several transgenic plant lines that are essential to the success of a federally-funded research project aimed at identifying plant factors that interact with viral proteins. Construction of protein interaction maps for *Sonchus yellow net virus* and *Potato yellow dwarf virus* (PYDV) are essential for the long-term goal of understanding how host and viral factors function in concert at the molecular level leading to disease. Additionally, Ms. Kopperud is accelerating the characterization of proteins encoded by the genome of PYDV, which needs to be included in the aforementioned interaction maps. The genomic sequence of PYDV has only recently been determined and now it is time to characterize the encoded proteins using fluorescence microscopy techniques to determine their protein localization and interaction. Dedication to her research project has resulted in inclusion of her contributions as second author in a major paper accepted in the *Plant Journal*, one of the highest impact factor journals in plant biology. This and continued experiences are essential to developing this talented scientist who is preparing to go to graduate school next year.

Development of Transgenic Lines to Support Plant Cell Biology Research

Summer Research Project

Abstract

Viruses pose a threat to humans in a variety of ways — from direct infection to imposing devastating effects on the agriculture industry. Prevention of such infection requires knowledge of the mechanisms of viral infection, both in animal- and plant-infecting species. The goal of this project was to identify plant factors that interact with viral proteins in the course of infection leading to disease. *Sonchus yellow net virus* (SYNV) and *Potato yellow dwarf virus* (PYDV) were used to test the hypothesis that the viruses utilize different subsets of host factors in order to infect *Nicotiana benthamiana,* despite both being members of the genus *Nucleorhabdovirus.* Protein:protein interactions were validated with the use of bimolecular fluorescence complementation (BiFC). In addition, these experiments required the use of transgenic *Nicotiana benthamiana* plants that express fluorescent markers targeted to histone 2B as an aid to establishing localization of the viral proteins within the host plant cell. Taken together, BiFC experiments conducted in "blue nuclei" transgenic plants that express CFP-H2B significantly improve image quality and information content of the experiments, providing simultaneous localization and interaction data. The utility of novel techniques and the transgenic plants developed in the Goodin lab provided the necessary tools required to fulfill the objectives of a major NSF-funded research project.

I. Introduction and Significance

Viruses threaten human health and welfare in a variety of ways. In addition to diseases caused by pathogens that are human-infecting, such as HIV, hepatitis, and influenza, diseases caused by plant viruses are equally detrimental. Infection of crop plants affects food quality and yield and, in

turn, may have devastating economic, social, political, or historical consequences (Strange and Scott, 2005). The development of novel anti-viral strategies for application in agriculture requires insight into how viruses interact with their host cells to cause disease. In order to replicate or to be transported into host cells, viruses must manipulate the host's cellular functions to their advantage while avoiding the defense responses it implements. The proteins encoded by the genomes of host cells associate physically with viral proteins. In many cases, these host proteins are recruited away from their normal sites of accumulation to subcellular loci where viral processes such as replication occur. Taken together, viral infection in host cells results from the combination of altered gene expression, protein:protein interactions, and changes in protein localization. In order to integrate these processes into systems biology toward the development of novel anti-viral strategies, a comprehensive view of host:virus protein interaction and localization is required.

Specifically, although the plant-infecting viruses to be studied, *Sonchus yellow net virus* (SYNV) and *Potato yellow dwarf virus* (PYDV), are both members of the genus *Nucleorhabdovirus,* they elicit dramatically different effects from the host, both at the phenotypic and cellular levels (Fig 1). Infection of the host by SYNV (Fig 1A-D) induces an accumulation of green fluorescent protein (GFP) (targeted to the endoplasmic reticulum (ER) lumen of the host) within the nucleus. PYDV infection, on the other hand (Fig 1E-H), stimulates GFP accumulation around the periphery of the nucleus (Jackson et al., 2005). Therefore, in the current research, SYNV and PYDV were used to test the hypothesis that the viruses utilize different host factors in order to infect *Nicotiana benthamiana.* Validation of protein interaction for SYNV and PYDV are essential for the long-term goal of understanding how host and viral factors integrate at the molecular level.

The research reported herein will contribute to a significant project funded by the National Science Foundation, entitled *A Host Protein Interaction and Localization Map for a Plant Rhabdovirus*. The goal of that project is to identify plant factors that interact with viral proteins in the course of infection leading to disease. These experiments require the use of transgenic *Nicotiana benthamiana* plants that express fluorescent markers at specific cellular loci, such as nuclei, as a means of establishing localization of the viral proteins within the host plant cell. Micrographs depicting these interactions have been captured using state-of-the-art confocal microscopy. Given that both SYNV and PYDV replicate in plant nuclei, the host proteins predicted to interact with viral proteins are those that mediate nuclear transport. One such protein is importin-a, a host protein

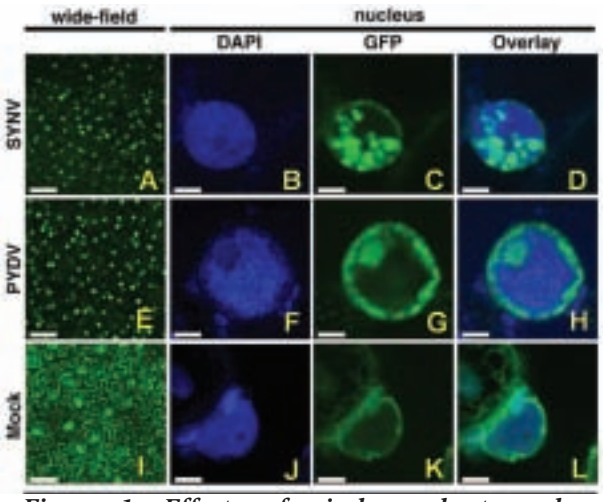

Figure 1. Effects of viral on host nuclear membrane morphology. **Nicotiana benthamiana** ***cells contain DNA-specific dye DAPI, shown here in blue, and transgenically express GFP tagged to the ER lumen. A. E. and I. Wide-field views of SYNV-, PYDV-, and mock-inoculated cells, respectively. B. Nucleaus stained with DAPI (shown in blue) in SYNV-infected cell. C. Effects of viral infection as alteration of the endomembrane system. D. Overlay of images B. and C. F. G. and H. As per B. C. and D., except in PYDV-infected cell. J. K. and L. As per B. C. and D., except in mock-inoculated cell. (From Goodin*** **et al.***, 2005, MPMI 18:703-709)***

that functions to aid in the nuclear import of proteins containing nuclear localization signals, such as the one found in SYNV-N (Ghosh et al., 2008).

Objective 1: Identifying the best plant lines for use in experiments

The level of fluorescence varies with the expression of the plant's transgene. Thus, all plants used for experimentation and microscopy were screened for the presence and degree of fluorescence. In order to obtain the highest-quality micrographs for publication, only those plants that exhibited an intense and consistent level of fluorescence were retained for seed amplification or use in experiments. Diligent records of each plant tested were kept, to ensure that only the best plants were available for use by lab members.

Objective 2: Amplifying seed stocks of homozygous plant lines

As a result of genetic assortment, the acquisition of a plant line that exclusively produces progeny positive for the expression of the desired transgene may take several generations. Prior to "true breeding" status, a fraction (approximately 1/4) of the progeny are negative for transgene expression and must be discarded. True breeding is achieved through the collection of seeds from those plants with the highest level of fluorescence and repeating the cultivation process until the yield of progeny positive for expression is 100%. This result indicates that no segregation is occurring and the plant line is

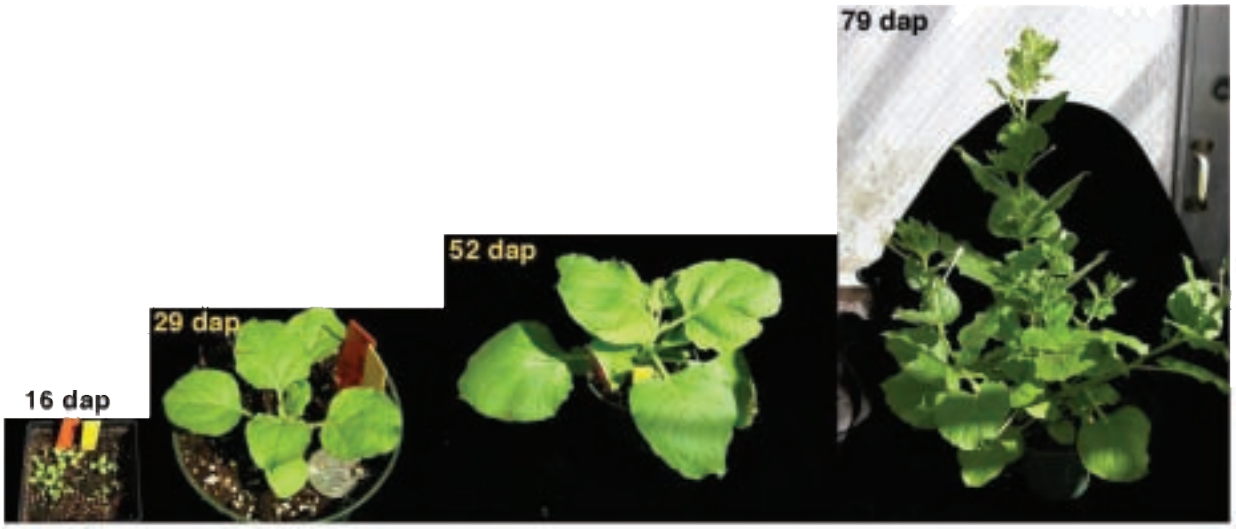

Figure 2. Photographic documentation of transgenic* Nicotiana bethamiana *plants at various stages of growth: 16 days after planting; 29 dap; 52 dap; and 79 dap. Optimal age for inoculation for use in experiments is 29 days after planting, when leaves are approximately 1" long (bottom center).

homozygous for the expression of the transgene. Depending on the line, four to six generations of consecutive cultivation are required to reach true breeding.

Objective 3: Maintaining plant stocks to support experiments

In conjunction with the plant screening process, It is also necessary to maintain a continuous supply of the various transgenic plants lines in the optimal size and age ranges for experiments. In order to meet the demand of the lab, approximately sixty plants per week were planted and the seedlings were transplanted at 21 days after germination. It was determined that the optimal age of plants for use in experiments is 29 days after germination (Fig 2).

The various transgenic lines in greatest demand this summer were those with fluorescent subcellular markers targeted to the endoplasmic reticulum (ER), nucleoli, and nucleus, expressing different colors of fluorescent proteins. Each line of plants was cultivated to express one color in a specific locus and was designated by the number on a seed stock list. Transgenic plants that contained a cyan (blue) fluorescent protein fused to histone 2B (CFP-H2B) were used for bimolecular fluorescence complementation (BiFC) experiments to study protein:protein interaction *in vivo,* providing enhanced image quality over assays conducted without the benefit of a subcellular marker. Plants with red markers on the endoplasmic reticulum (RFP-ER) were used to observe changes in the host cell membrane as a result of viral infection. Finally, those with a red histone marker (RFP-H2B) were used by the Goodin lab for nuclear localization signal (NLS) mapping experiments.

II. Materials and Methods

A. Development of transgenic plant lines

Each line of plants was cultivated as described above to express one color in a specific locus. The various transgenic lines in greatest demand were those with fluorescent markers targeted to nuclei.

In addition to the CFP-H2B plant lines that benefit BiFC experiments, the Goodin lab has developed a series of transgenic *N. benthamiana* lines that express red fluorescent markers targeted to the nucleus or the endoplasmic reticulum (ER). These plants were utilized in experiments conducted in the context of probing plant:virus interactions to gain insight into the membrane and protein dynamics that take place in virus-infected cells (Chakrabarty et al., 2007; Goodin et al., 2007).

B. Agroinfiltration of transgenic *N. benthamiana*

Agrobacterium tumefaciens transformed with expression vector is streaked onto LB plates with the appropriate antibiotics and incubated at 28°C. Following incubation, cells are harvested and resuspended in agroinfiltration buffer, and the cell suspension is adjusted to an $O.D._{600}$ of 0.6-1.0. Acetosyringone is added and the cells are further incubated at room temperature for 2-3 hours.

To inoculate *N. benthamiana* plants expressing CFP-H2B with the *Agrobacterium* culture, a 1 mL syringe barrel is filled with the cell suspension and appressed to the tip to the abaxial surface of the leaf. The leaf is infiltrated by gently depressing the plunger while maintaining a seal between the syringe tip and leaf. Following incubation with illumination, agroinfiltrated tissues are suitable for microscopy or biochemical analyses.

C. Bimolecular Fluorescence Complementation (BiFC)

Bimolecular Fluorescence Complementation (BiFC) is a powerful new technique that permits simultaneous determination of protein:protein interaction and localization (Ohad et al., 2007). In this technique,

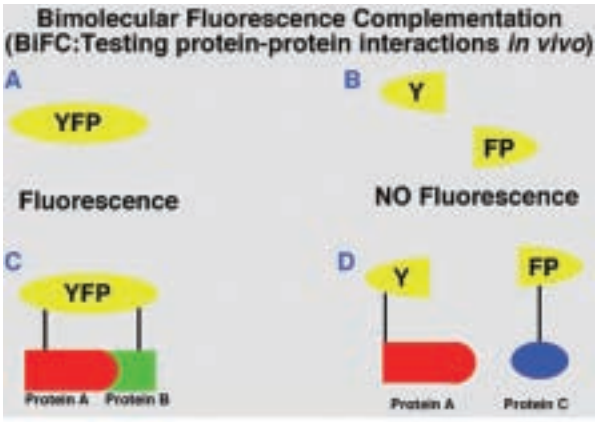

Figure 3. Bimolecular Fluorescence Complementation (BiFC) A. Complete yellow fluorescent protein (YFP); produces fluorescence. B. Unfused halves of YFP; produce no fluorescence. C. Split halves of YFP fused to Proteins A and B; interaction between test proteins results in reconstitution of YFP halves and fluoresces. D. YFP halves fused to Proteins A and C; lack of interaction between test proteins prevents reconstitution of YFP halves and does not fluoresce.

the yellow fluorescent protein (YFP; Fig 3A) is expressed in two approximately equal halves, neither of which fluoresces (Fig 3B). Each half is, in turn, fused to different protein(s) whose ability to interact is being determined. A positive interaction between proteins results in reconstitution of the YFP halves and, subsequently, fluorescence (Fig 3C). A lack of interaction between test proteins prevents association of the YFP halves and, thus, no fluorescence (Fig 3D).

III. Results and Discussion

A. Validation of BiFC vectors

In order to validate protein interactions in plant cells, a graduate student, Kathleen Martin, developed an improved series of expression vectors for conducting BiFC experiments. These "pSITE-BiFC" vectors were validated in the contexts of both soluble and membrane-associated viral protein complexes (Fig 4A-H). Expression of the two YFP halves from the pSAT-BiFC vectors resulted in insignificant background fluorescence when the two non-fused halves of YFP were coexpressed (Fig 4A and E) or when non-fused halves were coexpressed with protein fusions (Fig 4B, C, F and G). Thus, actual interactions could be scored easily, such as in the case of the soluble PYDV-N/P (Fig 4D) complex or the membrane self-association of SYNV-G (Fig 4H).

Despite the exceptional utility of the pSITE-BiFC vectors, image-quality and data/information content of micrographs could be further increased by conducting BiFC experiments in transgenic *N. benthamiana* plants that expressed CFP fused to histone 2B (CFP-H2B; Fig 4I-Q). Consistent with previous reports (Deng et al., 2007; Goodin et al., 2001), the SYNV-N protein complex localized to the nucleus exclusively (Fig 4I-K), whereas coexpression of -N and -P resulted in relocalization of both proteins to a subnuclear locale (Fig 4L-N). The SYNV-P protein complex showed accumulation in both the nucleus and cytoplasm (Fig 4O-Q).

B. Differential interaction of "cargo" with importin-α

Given that the aim of the NSF-funded project is to identify plant factors that interact with viral proteins, the new BiFC vectors were further validated by testing the interaction of rhabdovirus -N proteins with importin-α. Although the association of SYNV-N and -P proteins has been studied in detail, the mechanism by which these proteins are transported into the nucleus is less well-defined. 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 to importin-α *in vitro* (Deng et al., 2007). Moreover, although the cognate proteins from *Potato yellow dwarf virus* (PYDV) lack predictable NLSs, both are localized exclusively to the nucleus (Ghosh et al., 2007).

However, the nuclear transport of proteins lacking canonical NLSs have been shown to be mediated via an importin-α dependent pathway (Wolff et al., 2002). Additionally, while higher eukaryotes typically encode multiple alleles of importin-α with at least two known to exist in *N. benthamiana* (NbImpα1 and NbImpα2), the cargo specificities of these various isoforms are poorly understood (Kanneganti et al., 2007; Palma et al., 2006). Therefore, in order to advance our understanding of plant importin-α proteins, BiFC was used to study the ability of these proteins to bind proteins with non-canonical NLSs, and to determine if isoforms of importin-α differ in their cargo specificity (Fig 4). These experiments showed that, consistent with *in vitro* binding data, SYNV-N interacted with both NbImpα1 and NbImpα2 (Fig 5).

IV. Conclusions

- "Blue nuclei" transgenic plants that express CFP-H2B can be used to significantly improve image quality and information content of BiFC experiments.
- "Red nuclei" transgenic plants that express RFP-H2B greatly increase the accuracy of scoring nuclear localized proteins, essential for NLS-mapping and related projects.
- "Red-ER" plants permit facile visualization of membrane alteration induced by plant-infecting viruses.
- Validation of the utility of transgenic plants and novel expression vectors developed in the Goodin lab provide the necessary tools required to fulfill the objectives of a major NSF-funded research project.

V. Acknowledgements

This project was conducted in the Goodin Laboratory, Department of Plant Pathology, College of Agriculture. I sincerely wish to thank Drs. Michael Goodin and Anindya Bandyophadyay for their guidance, and the members of the Goodin lab for their support. This research was funded in part by a eUreKa! Summer Research & Creativity Grant and by National Science Foundation awards to Michael Goodin.

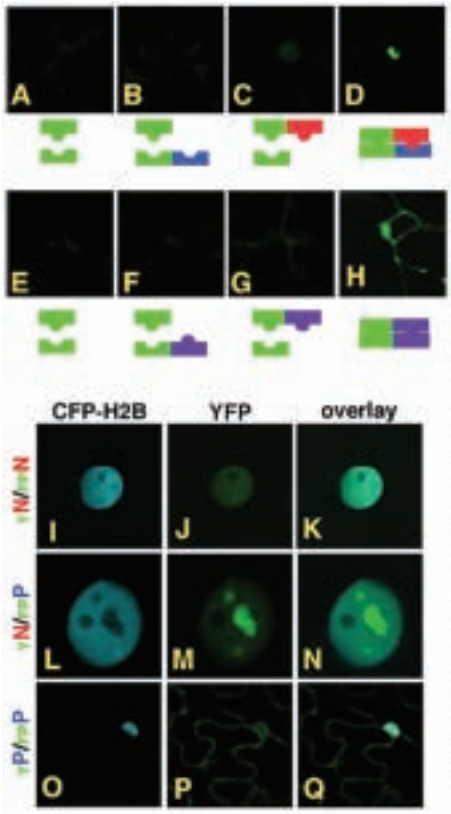

Figure 4. Validation of the pSITEII-BiFC vectors for detecting inter-actions between soluble (A-D) or integral membrane protein complexes (E-H). Identical detector settings were used to acquire all images. Expression was conducted in leaf epidermal cells of *N. benthamiana* using agro-infiltration. Micrographs taken 48 h post-infiltration show results of coexpressed **A** and **E**. nYFP + cYFP; **B**. nYFP + cYFP:PYDV-P (blue); **C**. nYFP:PYDV-N (red) + cYFP; **D**. nYFP:PYDV-N + cYFP:PYDV-P. **F**. nYFP + cYFP:SYNV-G (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 BiFC experiments. **I**, **L**, 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:SYNV-P. **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 bar A-H and O-Q = 10 μm; I-K = 5 μm; L-M = 3 μm (Martin *et al.*)

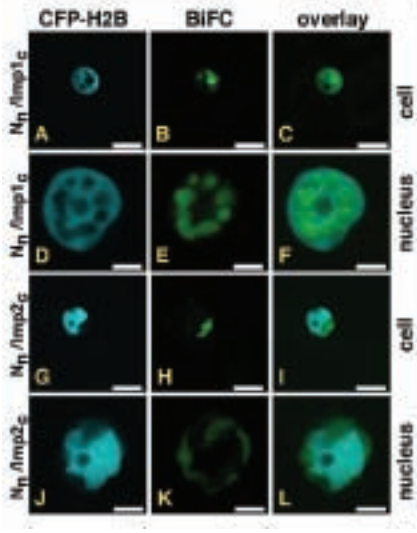

Figure 5. Single-section confocal micrographs of BiFC showing differential interactions of NbImpα1 and -2 with SYNV-N. Importin-α proteins were expressed as fusions to the carboxy-terminal half of YFP (Imp$_C$). SYNV-N was expressed as a fusion to the amino-terminal half of YFP (N_N). **A-F**. Interaction of SYNV-N with NbImpα1. **A-C**. Whole cell (cell) views showing fluorescence from CFP-H2B, BiFC interaction of N_N and NbImpα1 and resultant overlay of images, respectively. **D-F**. Confocal sections of nuclei in cells (nucleus) expressing the same fusions shown in A-C. **G-I**. Whole cell (cell) views showing fluorescence from CFP-H2B, BiFC interaction of N_N and NbImpα2 and resultant overlay of images, respectively. **J-L**. Confocal sections of nuclei in cells (nucleus) expressing the same fusions shown in G-I. Scale bar = 10 μm, except for D-F and J-L = 5 μm (Martin *et al.*)

The publication that includes data from this research conducted in the Goodin lab last summer:

Martin, K., Kopperud, K., Chakrabarty, R., Banerjee, R., Brooks, R., and Goodin, M.M. (2009) Transient expression in *Nicotiana benthamiana* fluorescent marker lines provides enhanced definition of protein localization, movement and interactions *in planta*. *Plant J.* in press.

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