ABSTRACT

New Viral Vectors for the Expression of Antigens and Antibodies in Plants

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Plants viruses are increasingly being examined as alternative recombinant protein expression systems. Future development of plant virus expression vectors needs to focus on the most important economic hosts, namely cereals and legumes, to develop tools to aid breeding of such hosts and systems for edible vaccine production.

Sunn hemp mosaic virus (SHMV) is a tobamovirus, which infects leguminous plants. This work reports on new SHMV-based viral vectors for high yield of target proteins in legumes. In the SHEC vector series, the coat protein gene of SHMV was substituted by a reporter gene. In the SHAC vector series, the coat protein was substituted by a reporter gene and the coat protein gene from another tobamovirus, tobacco mild green mosaic virus (TMGMV). Co-agroinoculation of SHEC/GFP with an RNA silencing suppressor resulted in high levels of local GFP expression by 3 days post inoculation. Co-agroinoculation with SHAC/GFP led to systemic fluorescence in 12-19 dpi.

Foxtail mosaic virus (FoMV) is a species of the group *Potexvirus*, which infects cereal plants. A new viral vector series named FECT was constructed by eliminating the

triple gene block and coat protein genes, reducing the viral genome by 29%. Interestingly, agroinoculation of the vector alone results in only slight transient expression, whereas co-inoculation with silencing suppressor genes allows for GFP expression of 40% total soluble protein. Full-sized HC and LC components of an anti-langerin IgG, each carried by a separate FECT vector, expressed and folded into immunologically functional antibody upon co-inoculation. This may prove a useful and environmentally safe vector for both transient expression and perhaps transgenic plants.

Mountain cedar (*Juniperus ashei*) pollen causes severe allergies in Texas and the central USA. Jun a 1 is the dominant allergen protein of mountain cedar pollen and would be a good allergen vaccine candidate. Recombinant Jun a 1 was expressed in *Nicotiana benthamiana* using an agroinoculation-compatible tobacco mosaic virus vector and isolated in good quantity from the apoplast by vacuum infiltration (100 μg/g leaf material). The recombinant protein samples were characterized. Pectate lyase activity was detected from plant extracts, suggesting the cause of severe necrotic reaction in plants.

New Viral Vectors for the Expression of Antigens and Antibodies in Plants

by

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TABLE OF CONTENTS

LIST OF FIGURES v
LIST OF TABLES vii
ACKNOWLEDGMENTS viii
CHAPTER ONE 1
Literature Review 1
Plant Virus Expression of Recombinant Proteins 1
DNA Virus Overexpression Vectors 4
RNA Virus Overexpression Vectors 6
Tobacco Mosaic Virus as an Expression Vector 9
PVX-based Expression Systems 13
Other Plant Viruses Used as Expression Vectors 18
New Approaches for Increased Efficacy and Biosafety 25
Virus Induced Gene Silencing (VIGS) 31
Recombinant Antibodies that are Expressed in Plants 33
Post-translational Modification 35
Allergens and Allergy 37
CHAPTER TWO 46
Efficiency Protein Expression in Legumes and Nicotiana from Agroinfection-Compatible Sunn Hemp Mosaic Virus Expression Vectors 46
Abstract 46
Materials and Methods 50
Results 58
Discussion 70
CHAPTER THREE 75
Highly Efficient Suppressor-dependent Protein Expression in Plants with A Foxtail Mosaic Virus Vector 75
Abstract 75
Introduction 76
Methods and Materials 81
Results 91
Discussion 106
CHAPTER FOUR 113
Plant-expressed Recombinant Mountain Cedar Allergen Jun a 1 is Immunogenic and has Pectate Lyase Activity 113
Abstract 113
Introduction 114
Results 124
Discustion 131
APPENDIX 136
Bench Protocol 136
Formaldehyde-Agarose Gel Electrophoresis 136
Quantification of RNA Using Spectrophotometry 137

RNA Extraction from Plant Tissue 138
RNA Extraction from Plant Tissue with Tri Reagent 139
Alkaline Lysis Miniprep: Small Scale Isolation of Plasmid DNA 141
DNA Plasmid Maxiprep Protocol 143
Preparing E. coli Electrocompentent Cells 148
Bacterial Transformation by Electroporation 151
Bacterial Transformation by Heat-shock 152
Preparation of electroporation competent Agrobacterium *cells* 153
Transformation of Agrobacterium *Cells by Electroporation* 154
Agroinfiltration 155
Histochemical GUS Assay 157
Spectrophotometric GUS Assay. 158
Protein Extraction by Grinding 159
Protein Extraction by Vacuum Infiltration 159
SDS Polyacrylamide Gel Electrophoresis 160
Transfer of Protein from Gel to PVDF Membrane 162
Ponceau Staining (Sensitive Staining of Proteins) 163
Western Blot for the Detection of Jun a 1 163
Alkaline Phosphatase ELISA Protocol 164
Purification of Jun a 1 165
Purification of rJun a 1 167
Enzyme-Linked Immunoassays ELISAs 168
Freezing Cells 169
Beta-hexosaminidase Release Assay 170
Thawing Cells 171
REFERENCES 172

LIST OF FIGURES

Figure 1. Genome organization of tobacco mosaic virus 10

Figure 2. Genome organization of potato virus X 14

Figure 3. Genome organization of cowpea mosaic virus 20

Figure 4. Genome organization of *Potyvirus* 22

Figure 5. Schematic diagram of binary plasmid in SHMV study 52

Figure 6. Infection with wild type SHMV via agroinoculation 59

Figure 7. Electron Microscopy of SHMV virion. 60

Figure 8. Effect of gene silencing suppressor on the agroinfection efficiency of SHEC 62

Figure 9. Time course analysis of GFP expression from SHEC74. 63

Figure 10. Fluorescence microscopy of plants infected with SHEC/SHAC:GFP 66

Figure 11. Histochemical GUS assay for plant leaves agroinfected with SHEC:GUS 67

Figure 12. β-Glucuronidase expression level of SHEC:GUS and 35S:GUS 68

Figure 13. Agroinfection induced systemical expression of SHAC vector 70

Figure 14. Schematic diagram of binary plasmids used in FoMV study 86

Figure 15. Effect of gene silencing suppressor on the agroinfection efficiency of FECT 93

Figure 16. Comparing the expression efficiency of FECT22 and FECT40 95

Figure 17. Time course analysis of GFP expression from FECT40/GFP 96

Figure 18. High efficient expression of FECT40 viral vector 98

Figure 19. Quantification analysis of GFP expression 99

Figure 20. Dilution experiment of agroinfection 100

Figure 21. Fluorescence microscopy of monocots agroinfected with FECT40/GFP 102

Figure 22. Effect of gene silencing suppressor p19 in cis and trans constructs 103

Figure 23. Western blot analysis of full-length recombinant antibody 105

Figure 24. Diagram of binary plasmid pJL36/Jun a 1 and mutant variants 124

Figure 25. Protein analysis of rJun a 1 expression and purification 125

Figure 26. Symptoms of Jun a 1 Expressing Plants 127

Figure 27. Human IgE ELISA assay of native and recombinant Jun a 1. 128

Figure 28: Biological activity of wild type and recombinant Jun a 1 130

LIST OF TABLES

Table 1. Primers used for plasmid construction in SHMV study. 54

Table 2. Primers used for plasmid construction in FoMV study. 84

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CHAPTER ONE

Literature Review

Plant Virus Expression of Recombinant Proteins

As eukaryotes, plants expression systems are able to process human and medically-relevant proteins with post-translational modifications and folding such as glycosylation and signal peptide cleavage. This "molecular pharming" strategy is effective, scalable and inexpensive. Recombinant proteins expressed in plants are also free of bacterial toxins and free of animal pathogens. Thus, these make them suitable for pharmaceutical uses.

There are two types of plant expression systems: transgenic and transient. Transgenic plant expression involves integrating the foreign DNA into the chromosomes of host plants. Most commonly, the Ti ("tumor induction") plasmid of the plant pathogenic bacterium, *Agrobacterium tumefaciens*, is used (Zupan *et al*., 2000). The Ti plasmid contains a sequence, T-DNA, which is transposed from this plasmid to the plant chromosome via proteins produced by other genes located on the Ti plasmid. T-DNA can be constructed with a foreign gene and one strong promoter (e.g., the cauliflower mosaic virus (CaMV) 35S promoter) to drive the expression of the gene of interest (Hull *et al*., 2002). However, stable transformation of plants using the *Agrobacterium* method can take three to nine months in tissue culture to produce transgenic plants available for testing the function of the expressed protein.

Transient expression uses plant viruses as vectors for the foreign gene expression (Dawson *et al*, 1986). Both transgenic and transient plant expression systems are useful in creating recombinant proteins, but transient expression has several advantages over transgenic plants (Kearney *et al*., 1994). Transient expression systems are more cost-effective, and also give considerably higher yields of proteins than transgenic systems (Twyman *et al*., 2003). For infectious viruses, recombinant viral vectors are able to move into whole plants by single inoculation. The virus spreads throughout the plant allowing the target gene expression in all cells (Lico *et al*., 2008). Infected cells yield large amounts of virus, so recombinant viral vectors have the potential for high-level protein expression. Viral infections are rapid, so large amounts of recombinant proteins can be produced in a few weeks (Kearney *et al*., 1994).

Many different plant viruses have been developed for recombinant protein expression, including DNA virus such as geminiviruses and caulimoviruses. However, RNA viruses, such as tobacco mosaic virus (TMV) and potato virus X (PVX), are the most effective expression systems (Lico *et al*., 2008), and allow rapid replication of genes and thus high expression levels. Large amounts of green fluorescent protein (GFP) are produced throughout the entire plant and can be easily visualized with a handheld ultraviolet light. GFP is produced systemically with viral vectors and can be seen in the leaves, stems, and even roots of the plant (Casper and Holt, 1996). Some plant viruses have a wide host range allowing the same viral vectors to be used in many different species, beyond the plant virologist's model plant, *Nicotiana benthamiana*. It should be mentioned that *N. Benthamiana* is extraordinarily capable of supporting viral replication because it lacks one of the RNA dependent RNA polymerases critical for gene silencing

of viruses (Ding *et al.*, 2004). Thus, it has become the main host used by virology researchers as well as by the plant-made pharmceutical industry.

The viral vector can be introduced into isolated plant cells or to whole plants by leaf rubbing, *Agrobacterium* or direct DNA transfer. With traditional virus vectors, the viral RNA or DNA was introduced to protoplasts, or to whole leaves by rubbing. In the case of RNA viruses, transcription of the cDNA of viral genome produces replicable viral RNA, which is amplified in the cytosol for high-level expression. In case of DNA virus, the rubbing method was replaced by *Agrobacterium*-mediated transient transformation with T-DNA containing viral genome can introduce the intact viral genome into cytosol. The *Agrobacterium*-mediated delivery of viral genomes is known as "agroinfection" or "agroinoculation" and provides highly efficient gene transfer. This method was then transferred to RNA viruses. Agroinfection of RNA viral vectors have emerged as the most efficient approache to achieving high-level expression of recombinant proteins (Lindbo, 2007; Marillonnet *et al*., 2005; Komarova *et al*., 2006).

There are other uses for viral vectors not covered in this work. Some plant viruses have been developed to present short peptides on the virion surfaces. Epitope-display technology have been developed as a potential source of vaccines in plant viruses based on cowpea mosaic virus, alfalfa mosaic virus, potato virus X, and tomato bushy stunt virus (Lomonossoff and Hamilton, 1999; Pogue *et al.*, 2002; Yusibov and Rabindran, 2004; Twyman *et al.*, 2005). Other applications of viral vectors include their use in virus-induced gene silencing (VIGS) (Robertson, 2004) and investigating of viral functions in plants (Yusibov *et al*., 1999).

DNA Virus Overexpression Vectors

The first plant viral vectors were based on DNA viruses because their small and simple viral DNA genomes were easier to manipulate. Two groups of DNA viruses, caulimoviruses and genimiviruses, were developed as vectors. Cauliflower mosaic virus (CaMV) is the type species of the *Caulimovirus group* and was the first plant virus overexpression vector for recombinant proteins production (Brisson *et al*., 1984). Since CaMV has a pre-formed icosahedral virion capsid, the maximum capacity for CaMV DNA packaging is 8.3 kb. After removing the only two non-essential genes, the maximum insert size is less than 1kb (Daubert *et al*., 1983). A number of small peptides were expressed with CaMV vector, such as the 240 bp bacterial *dhfr* gene (Brisson *et al*., 1984) and the 500bps human interferon gene (De Zoeten *et al*., 1989). However, the 35S RNA promoter and terminator sequences of CaMV are now used extensively in plant biotechnology, even though the caulimovirus vectors themselves are rarely utilized.

The DNA virus family, *Geminiviridae,* has been a good source of expression vectors because of their ability of producing high-yield recombinant protein as functional replication virus (Stanley, 1993). The coat protein is not required for replication, so the coat protein gene was replaced by a foreign gene, with the strong promoter of coat protein being used to drive gene expression in this way (Timmermans *et al*., 1994). The bacterial gene, neomycin phosphotransferase, was expressed with a tomato golden mosaic virus (genus *Begomovirus,* family *Geminivirus*) vector in systemically infected leaves of *Nicotiana tabacum* (Hayes *et al*., 1988). Mastreviruses, also in the *Geminivirus* family, can achieve much higher viral titers than that of begomoviruses, and achieved the high-level transient expression of foreign protein (neomycin phosphotransferase,

chloramphenicol acetyltransferase, and beta-galactosidase) in protoplasts of wheat (*Triticum monococcum*) (Matzeit *et al.*, 1991).

Recently, a promising geminivirus-based expression vector was created from tomato yellow leaf curl virus (Peretz *et al*., 2007). The viral dsDNA genome can move cell-to-cell and systemically in host plants and replicate by the host without the help of any viral polypeptides. The gene insertion capacity of 5 kb is longer than the viral monopartite genome and is much greater than the 1.5 kb capacity of the best viral vectors. The partially deleted coat protein gene is responsible for the attenuation of viral disease symptoms. The target protein yields were up to 6% of the total soluble leaf proteins in *Nicotiana benthamiana*, which is far less than the 40-80% yields reported by others for the TMV vector (Lindbo, 2007; Marillonnet *et al*., 2005), but is still higher than the 1% maximum yield for nonviral vectors using the 35S promoter (Twyman *et al*., 2003). This vector-virus was able to replicate and spread in a very broad range of monocots and dicots and woody plants, but it was, unfortunately limited to the phloem cells in many hosts. Thus, its use as an expression vector or a VIGS vector beyond the few hosts in which is moves beyond the phloem (*N. benthamiana*, wheat, dill) is somewhat limited. However, because it can be inoculated as DNA rather than by agroinoculation, and because agroinoculation is ineffective in grasses such as wheat, its potential in wheat and some other crops is exciting.

DNA viruses were largely developed in early experiments because cDNA copies of RNA genomes could not be generated. However, foreign sequences inserted into DNA viruses were often shown to decrease the stability of the viral vector. As well, DNA viruses usually have limited insertion capacity for foreign sequences because the

pre-formed virion capsid can accommodate restricted genetic material. Deletion of non-essential genes can overcome this limitation, but it also interfere the replication and infection of viruses. Other than the recently introduced TYLCV vector mentioned above, the use of DNA viruses as expression vectors has been mostly abandoned now because cDNA forms of RNA viruses can be produced routinely. Many RNA viruses build their capsids with filamentous morphology, so there is less of a packaging limitation in vector genome construction.

RNA Virus Overexpression Vectors

Most plant virus expression vectors are based on RNA viruses because they can accept larger foreign genes than DNA viruses and can be used to express proteins in high yield. The possibility of using RNA viruses as protein expression vectors began when it was discovered that viral RNA can be converted into complementary DNA (cDNA) and its cDNA fragment could be inserted into plasmid vectors and cloned into *E. coli*. The plasmid construct can be replicated and manipulated in bacterial hosts and then *in vitro* transcribed back into infectious viral RNA. Over time, techniques were developed to allow viral cDNA to be directly infectious.

The first RNA virus converted into a cDNA version was Qβ phage (Taniguchi *et al*, 1978) and the plasmid itself was found to be infectious, presumably by random transcription initiation. The same technique was used to create infectious poliovirus cDNA (Racaniello and Baltimore, 1981) and viroid cDNA (Cress *et al*, 1983). The first plant full-length cDNA clone from the genome of brome mosaic virus (BMV) was built in 1984 (Ahlquist *et al*., 1984). Ahlquist used phage promoters to drive *in vitro* transcription of infectious viral RNA that could be rubbed onto a whole plant to create an

infection. The coat protein gene was replaced with the chloramphenicol acetyltransferase (CAT) reporter gene and high-level CAT activity was accomplished. Following the establishment of infectious BMV produced by *in vitro* transcription, many other RNA viruses have been constructed as cDNA versions. Some of these viruses have been extensively developed as vectors for commercial foreign gene expression. The two most popular plant viral vectors were constructed by this principle - tobacco mosaic virus (TMV) and potato virus X (PVX), which are discussed below.

The next step was to create directly infectious cDNA vectors. For plant viruses, the option exists for manually inoculating plasmid DNA containing viral cDNA preceded by the 35S promoter directly onto whole leaves. Abrasive is included to gently disrupt the cells and allow infection. Examples of such constructs include pea early browning virus (*Tobravirus* family) (PEBV, MacFarlane *et al*., 1992); tomato mosaic virus (*Tobamovirus* family) (ToMV, Weber *et al*., 1992); plum pox virus (*Potyvirus* family) (PPV, Maiss *et al*., 1992); cowpea mosaic virus (*Comovirus* family) (CPMV, Dessens *et al*., 1993); alfalfa mosaic virus (*Alfamovirus* family) (AlMV, Neeleman *et al*., 1993); cucumber mosaic virus (*Cucumovirus* family) (CMV) and tobacco mosaic virus (*Tobamovirus* family) (TMV, Dagless *et al*., 1997). Unfortunately, the infectivity of these vectors was generally very low.

To increase infectivity, it was thought that viral genomes could be directly inserted into the plant host genome. Yamaya *et al* (1988) was the first to accomplish this, constructing a full-length cDNA copy of the TMV genome, and creating transgenic plants using the *Agrobacterium* system. They found the transformed plants showed symptoms of TMV infection. The next step involved a technique known as

agroinfiltration, in which whole plants can also be locally transformed by *Agrobacterium* without tissue culture. Turpen *et al.* inserted a 35S-driven cDNA of the TMV genomes into the Ti-plasmid of *Agrobacterium*, and infiltrated the *Agrobacterium* into whole leaves using a syringe ('agroinfiltration") to greatly increase infectivity of the TMV cDNA, since every cell in the infiltrated area now contained the TMV transgene in its chromosomes (Turpen *et al*., 1993). This new approach for plant RNA viruses held great promise for its speed, high expression levels of foreign proteins, and ease of use. Based on the agroinfiltration strategy, Icon Genetics, a German plant biotechnology company, has developed a powerful expression platform of agroinfiltration of TMV vector (Marillonnet *et al*., 2004, 2005), which are discussed below.

To manipulate and organize the heterogous genes in the RNA genome of viral vector, several strategies were developed to produce large scale recombinant protein: epitope presentation vectors, deletion vectors, gene-addition vectors. The first strategy was to replace a non-essential viral gene with the foreign gene. The second approach is involving to insert the foreign gene as an addition to the viral genome without the deletion of any viral genes. However, this approach has size limitations on foreign gene, and genes might be deleted by homologous recombination (Scholthof *et al*., 1996). The third basic type of viral expression system, termed epitope presentation systems, has been developed for the production of immunogenic peptides and proteins in plants (Usha *et al*., 1993). With the inclusion of the foreign ORF fused to the ORF of another gene in the viral genome, the deletion of viral sequence was avoided. In epitope presentation systems, the viral vector is designed so that short antigenic peptides fused to the coat protein (CP) are displayed on the surface of assembled viral particles. Such modified

virions are attractive as potential novel vaccines, because the modified particles can be purified and the presentation of the antigenic peptide on the surface of viral particle can significantly increase its immunogenicity (Lomonossoff and Johnson, 1996). Polypeptide expression systems involve introducing a whole gene into the viral genome and expressing the gene efficiently in infected cells, usually as an unfused protein. Although purification of the expressed protein may be necessary, these expression systems are suitable for the production of immunogens that can be supplied orally as animal feed. A number of RNA viruses were proposed that can be packaged in the particles without the limitation on the size of RNA, such as filamentous viruses (Yusibov *et al*., 2006). However, there are still size limitations for filamentous vectors for some reason (e.g., TMV, PRV and PVX). TBSV and CPMV are icosahedral and theoretically are more limited than the filamentous viruses (Johnson *et al*., 1997).

Tobacco Mosaic Virus as an Expression Vector

Tobacco mosaic virus (TMV) is the leading vector commercially for the transient production of recombinant proteins in plants. Belonging to the tobamovirus group, TMV is a positive strand RNA virus consisting of 6395 nucleotides (Goelet *et al*., 1982). The TMV genome consists of two coterminal replicase proteins, a movement protein and a coat protein (CP) (Hunter *et al*., 1976).

The 5' open reading frame (ORF) is translated directly from the genomic RNA, yielding the 126kDa replicase. The genome of TMV employs two distinct strategies for protein expression: read-through of an amber stop codon and production of sub-genomic RNAs (Fig 1) (Dawson and Lehto, 1990). An amber stop codon at the end of the 126 kDa replicase undergoes a translational read-through to produce the 183 kDa replicase

(Fig 1). Only the 183 kDa replicase has the RNA-dependent RNA polymerase (RdRp) domain. The negative strand copy of the genome is the template for replication of the positive genome and subgenomic mRNAs (Beachy and Zaitlin, 1977). The movement and coat proteins are translated from 3' coterminal subgenomic mRNAs (Fig 1). The movement protein is a 30kDa protein produced early in the infection cycle. It is for cell to cell movement of progeny viral RNA genomes. It modifies the size limit of plasmodesmatal junctions when viral RNA move from cell to cell (Deom *et al*, 1992).

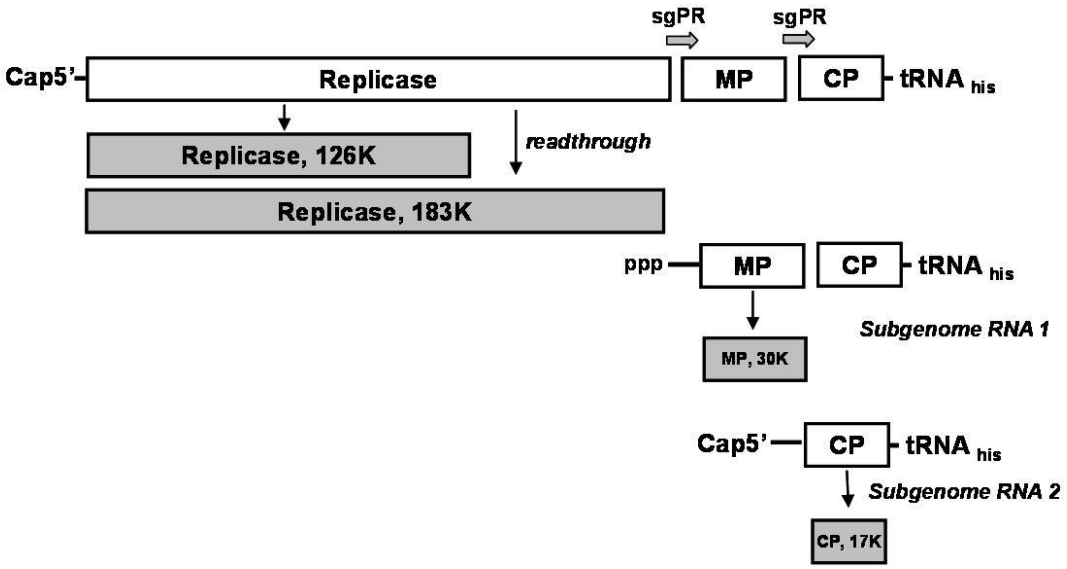


Figure 1. Genome organization of tobacco mosaic virus. Tobacco mosaic virus (genus *Tobamovirus*) has a positive-sense single-stranded RNA of 6395 nt, coding for 126 and 183 kDa proteins, replicases; 30 kDa protein; movement protein; 17.5 kDa protein; coat protein. The 126K protein contains the methyltransferase and helicase motifs of a replicase. The 183K protein (the complete replicase) is produced occasionally when the stop codon of the 126K gene is ignored. TMV produces two subgenomic RNAs from subgenomic promoters in negative strand genomic RNA. The movement protein, 30K (MP), and the coat protein, 17K (CP) are produced from subgenomic RNAs. sgPR, subgenomic promoter. In the vectors, heterologous sequences have been placed under a duplicated CP promoter either between the MP and CP genes, or in place of the CP gene.

Coat protein is a 17.5 kDa protein produced at very high levels later in the infection cycle. It encapsidates the viral genome and allows the movement of the virus throughout the vascular systems of the plant (Dawson *et al.*, 1988).

Vectors created from TMV include an early vector using duplicate coat protein promoters to drive the foreign gene and coat protein gene (Dawson *et al*, 1989). The heterologous chloramphenicol acetyl transferase (CAT) gene was cloned into the vector in place of the additional coat protein gene, and driven by the additional CP subgenomic promoter. The virus was able to replicate and CAT was expressed in active form. However, as this viral vector contained the duplication of the TMV coat protein promoter, the foreign gene was quickly deleted by homologous recombination between the identical subgenomic promoters, and was maintained only in the inoculated leaf itself (Dawson *et al*., 1989).

These stability issues were largely solved by Donson and colleagues in 1991 by the use of heterologous promoters from different tobamoviruses. TMV coat protein gene and its promoter were replaced by those of odonoglossum ring spot virus (ORSV) in the TB-2 hybrid vector (Donson *et al*., 1991). The subgenomic promoter from TMV controlled foreign gene expression of neomycin phosphotransferase, while the ORSV subgenomic promoter controlled the expression of the coat protein. This viral vector was able to replicate efficiently and maintain the inserted heterologous sequences over many passages (Donson *et al*., 1991).

The TB2 vector worked well for protein expression, but the expression level of protein was relatively low. Subsequently, the 30B hybrid vector containing the coat protein from tomato mild green mosaic virus (TMGMV) was created by Shivprasad and

colleagues in 1999 (Shivprasad *et al*., 1999). Three pseudoknot sequences from TMV were placed at the 3' untranslated region (UTR) of the vector to increase protein expression in a fashion analgous to a poly (A) sequence. A subgenomic promoter (TMV U5) and coat protein gene from tobacco green mild mosaic virus strain U5 replaced the ORSV gene because TMV U5 was able to allow long distance movement of the virus throughout the plant. This vector was very stable and successful in the production of high yields of GFP protein throughout the plant (Shivprasad *et al*., 1999). Further development of the TMV-based vectors was attempted to increase the yields of recombinant protein by modifying the virus to improve its normal function, such as increasing viral spread through DNA shuffling of the movement protein (Toth *et al*., 2002).

Another general expression vector design for TMV-based vectors is the coat protein fusion vectors. Coat protein (CP) of TMV accumulates to as much as 10% of the dry weight of an infected leaf and this property of TMV has been exploited for the expression of antigenic peptides on the surface of TMV virion. In CP fusion vectors, a ''read-through sequence'' is introduced immediately after the CP stop codon and before the ORF of interest (Hamamoto, 1993). Several epitopes have been fused to the CP and incorporated into soluble virions presenting the peptide on the surface of the virion (Turpen *et al*., 1995; Koo *et al*., 1999). Such epitope presenting virions may be better than crude extracts or the whole protein for the stimulation of immune system. Fusion to the CP may not be suitable for some peptides or proteins especially those requiring posttranslational maturation for active production and where the expression of the complete protein is required.

Size limitation of the foreign gene in the viral vector is the main disadvantage of TMV as an expression vector. The maximum size of the insert gene is 1.5 kb. Another limitation of TMV-based expression systems is bio-safety issue, which the virus including the foreign DNA might be able to spread to other crops. Furthermore, TMV-based vectors containing heterologous sequences lose the foreign gene insert after several rounds of replication. The deletion of most foreign genes after three to four passages leaves the hybrid virus, which is less competitive and less pathogenic than the wild type TMV (Rabindran and Dawson, 2001). However, this may be regarded as an advantage in terms of environmental safety, since the vectors quickly revert back to wild type.

PVX-based Expression Systems

Molecular Mechanism of Potexvirus

Potexvirus is a large group of flexous and filamentous plant viruses with length range from 470 to 580nm (Koenig & Lesemann, 1978). *Potexviruses* consist of a single-stranded, positive-sense genomic RNA, with a cap structure at the 5' terminus and a poly-(A) tail at the 3' terminus (Fig 2) (Dolja *et al*., 1987; Sonenberg *et al*., 1978). Potato virus X (PVX) is the type species of the genus *Potexvirus*. The genome of PVX contain five open reading frames (ORFs), and two subgenomic promoters directing transcription of subgenomic RNAs 1 and 2 (sgRNA1 and sgRNA2) (Huisman *et al*., 1988; Mackie *et al*., 1988). The genomic RNA allows the expression of ORF1 (4371 nt) encoding for the RNA-dependent RNA polymerase (RdRP) with methyltransferase, helicase, and polymerase motifs (Fig 2) (Davenport *et al*., 1997). ORF2 to 5 are expressed through several subgenomic RNAs. ORF2, 3 and 4 codes for the overlapping triple gene block

(TGB) proteins TGB1, TGB2 and TGB3, which are required for virus cell-to-cell movement (Bancroft *et al*., 1991). ORF2 codes for a multifunctional protein that has RNA helicase activity, promotes translation of viral RNAs, increases plasmodesmal size exclusion limits, and acts as a suppressor of RNA-mediated post-transcriptional gene silencing (PTGS) (Verchot, 2005). The triple gene block (TGB) 2+3+4 is found in other

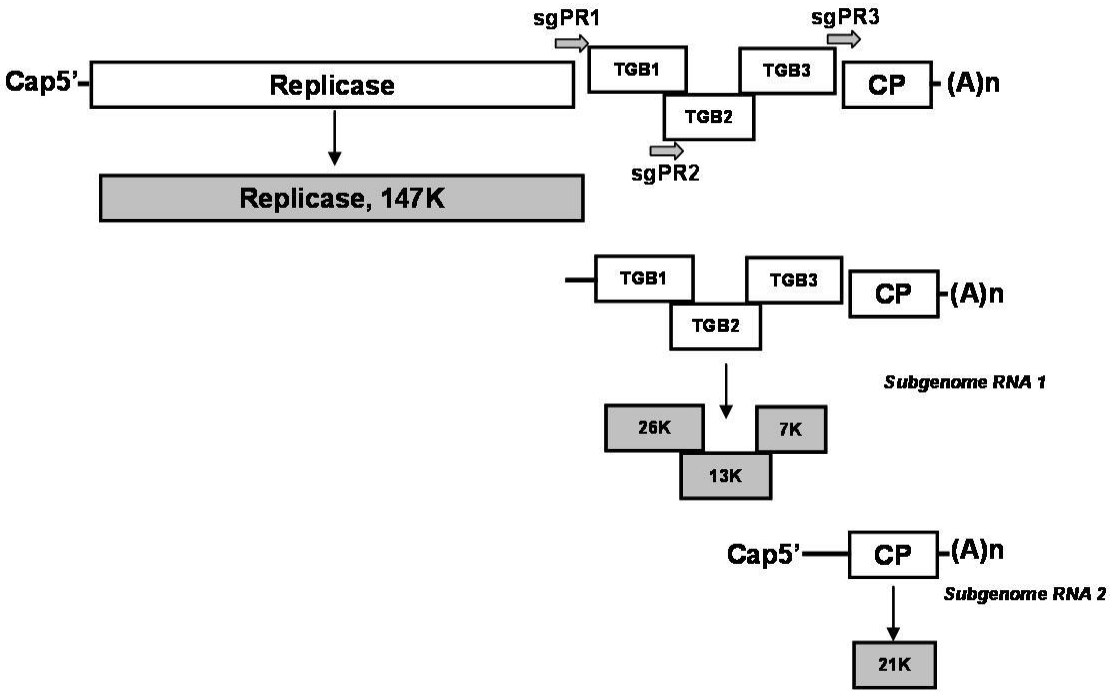


Figure 2. Genome organization of potato virus X. Potato virus X (PVX) (genus Potexvirus) has a (+)-sense single-stranded RNA of 6.4kb, coding for 147 kDa proteins, replicase; 26 kDa, 13 kDa and 7 kDa triple gene block (TGB) protein; 21 kDa protein; coat protein. The 147K protein contains has three replication-associated domains: including an N-terminal methyltransferase-like domain, an NTP-binding/helicase-like domain, and a C-terminal RNA dependent RNA polymerase (RdRp) domain. The 26K, 13K and 7K triple gene block (TGB) proteins are expressed through two subgenomic RNAs, which were produced from negative strand genomic RNA. The 26K protein is a multifunctional protein that has RNA helicase activity, promotes translation of viral RNAs, increases plasmodesmal size exclusion limits, and acts as a suppressor of RNA-mediated post-transcriptional gene silencing (PTGS). The 13K and 7K proteins are involved in cell-to-cell movement. The 21K coat protein (CP) is produced from subgenomic RNAs. (A)n, poly A tail, sgPR, Subgenomic promoter.

groups of virus. ORF5 (714nt) encodes the coat protein, which is required for viral encapsidation and long distance movement (Cruz *et al*., 1998).

RNA Elements Regulating Potexvirus Replicaion

The 5' non-translated region (NTR) regulates genomic and subgenomic RNA synthesis and encapsidation, as well as virus plasmodesmal transport. The 5'NTR contains two RNA-stem loop structures within the first 182 nt of PVX genome, named 5'SL1 and 5'SL2, which are required for PVX replication (Kim and Hemenway, 1996; Miller *et al*., 1998, 1999). The length of 5' NTR varies slightly among potexviruses. The PVX 5'NTR is 84 nt in length, the 5'SL1 and 5'SL2 stem-loop structures extend into the replicase ORF. The 5' SL1 controls virus replication, cell-to-cell movement and virion assembly as the multifunctional element (Kwon & Kim, 2006; Miller *et al*., 1999; Lough *et al*., 2006). After virus uncoating, 5'SL1 can bind host proteins and start translation or replication of genomic RNA. Once subgenomic RNAs are synthesized, the 5'SL1 may release the host proteins and bind to the CP for movement to adjacent cells (Kwon & Kim, 2006). 5'SL1 might regulate CP production by complementary binding with elements in the subgenomic RNA promoter, and these long-distance interactions are essential for subgenomic RNA synthesis by the replicase (Kim and Hemenway, 1999).

The 3'-NTR region is required for recognition by the viral replicase and for the initiation of RNA synthesis. The PVX 3' NTR is 74 nt in length and contains three stem-loop structures, 3'SL1, 3'SL2, 3'SL3, which play roles in negative-strand and positive-strand RNA accumulation (Pillai-Nair *et al*., 2003). Two host factors, chloroplast phosphoglycerate kinase (CPK, p43) and p51, were able to bind to the 3'NTR of the potexvirus, BaMV, and regulate RNA accumulation (Lin *et al*., 2007). The interaction

between the 3'SL3 stem loop and an internal conserved sequence in the subgenomic promoter were also found to be required for negative-strand RNA synthesis (Hu *et al*., 2007). Conserved elements in both 5' and 3' termini interact with the same internal RNA elements for RNA synthesis. These observations indicate that long-distance *cis*-acting interactions regulate virus replication and gene expression.

Cell-to-cell Movement

Potexviruses use three movement proteins, TGBp1, TGBp2 and TGBp3, and the viral coat protein for virus cell-to-cell movement. TGBp1 has the function of an RNA-silencing suppressor and TGBp3 has a single transmembrane domain. Plant virus movement proteins pass through plasmodesmata to allow virus cell-to-cell movement. Virus movement proteins interact with plasmodesmata to expand the pore to allow selective transportation of viral molecules. The most recent models of potexvirus movement show that TGBp1, CP and viral RNA genome form a transportation complex to transfer to the plasmodesmata. The viral complex of ribonucleoprotein (vRNP) interacts with cellular proteins at the mouth of plasmodesmata, and expands the pore to allow trafficking between cells (Lucas, 2006; Verchot-Lubicz, 2005). Coat proteins of potexvirus reside in plasmodesma (Oparka *et al*., 1999). However, TGBp1, but not coat protein, can trigger plasmodesmal gating. It suggests that these proteins act together with cellular protein to promote plasmodesmal gating and virus cell-to-cell transportation and the TGBp1-CP-RNAs complex can traffic between cells through plasmodesmata (Lough *et al*., 2000). Potexvirus TGBp1 protein has RNA-binding and RNA helicase activities and may unwind RNA secondary structure while forming the vRNP complex (Kalinina *et al*., 2002; Leshchiner *et al*., 2006).

As a component of the vRNP complex, TGBp1 can expand the pore of plasmodesmata and allow transfer of viral RNA between cells. It has RNA helicase activity and is a suppressor of RNA silencing (Voinnet *et al*., 2000). TGBp1 interacts with RDR6, which is a factor in short interfering RNA (siRNA) production and promotes the gene silencing of some viruses (Qu *et al*., 2005; Xie&Guo *et al*., 2006). RDR6, DCL4 and HEN1 are factors required for initiation or maintenance of virus-induced gene silencing in growing tissues. DCL4 produces 21nt viral siRNAs, which are amplified by RDR6 (Blevins *et al*., 2006; Dunoyer *et al*., 2005). The amplified 21 nt siRNAs then target viral RNA for silencing and restrict virus infection. Other viral gene silencing suppressors, such as potyvirus HC-Pro, tombusvirus P19 and closterovirus p21, bind siRNAs and prevent protein microRNA methylation by HEN1 (Blevins *et al*., 2006; Merai *et al*., 2006; Yu *et al*., 2006).

Two different formats of PVX-based viral vectors were developed to create a systemically moving overexpression vector (Chapman *et al*., 1992). In one the heterologous protein encoding sequence was placed under a duplicated subgenomic promoter of CP which was located between the triple gene block 3 and CP genes. Expression of GUS was detected in systemically infected leaves at 13 days post-inoculation (dpi) (Hendy *et al*., 1999). However, complete and partial deletions of the sequence encoding GUS were observed in a northern blot analysis, attributable to recombination between the homologous sequences of the duplicated subgenomic promoters (81 nt each) (Ziegler *et al*., 2000). The second type of PVX vector fused the foreign protein to the N-terminus of the CP genes. The 2A catalytic sequence (FMDV) promotes cotranslational cleavage between the CP gene and the foreign insert gene

(O'Brien *et al*., 2000). This system was used to express the rotavirus VP6 sequence and VP6-2A-CP was cleaved to produce typical VP6 virus-like particles (VLP). However, the cleavage is not 100% efficient, and some CP subunits still have the inserted protein forming uncleaved VP6-2A-CP.

Other Plant Viruses Used as Expression Vectors

Cowpea Mosaic Virus

Cowpea mosaic virus (CPMV), the type species of the *Comovirus* group, contains two separately encapsidated, positive single-stranded RNAs of 5889 (RNA-1) and 3481 (RNA- 2) nucleotides, in which each RNA contains a single open reading frame and is expressed as a major precursor polyprotein that is subsequently processed into mature proteins (Figure 3). RNA-1 is able to replicate independently in plant cells, and RNA-2 encodes the viral movement protein (MP) and coat proteins L and S (CP). First generation infectious CPMV-based vectors were constructed and the plants were inoculated with a mixture of RNA-1 and RNA-2 transcripts generated by T7 RNA polymerase (Usha *et al*., 1993, Porta *et al*., 1994). Subsequently, infectious CPMV-based vectors were created as the 35S promoter-driven cDNA clone of the RNA genome (Dalsgaad *et al*., 1997). Most recently, an agroinoculation system has been developed (Liu and Lomonossoff, 2002). Through passaging of CPMV virion to healthy plants *N. benthamiana*, the yields of modified viral particles are similar to the yields obtained with wild-type CPMV (Porta *et al*., 2003).

CPMV was developed as the first epitope display plant viral vector (Usha *et al*., 1993). CPMV has well organized particle structure in which 60 subunits, each with two

coat protein L and S, are arranged to form an icosahedral capsid packaging the bipartite RNA genome. There are several peptide loops on the surface of both L and S coat protein subunits, which allow for the insertion of foreign peptides. This feature makes CPMV suitable for epitope display on viral particle surface. The most favored peptide insertion site is the βB-βC loop on the subunit coat protein of CPMV (Porta *et al*., 1994). A more detailed description about the development of CPMV as the epitope display viral vector system can be found in the review article by Canizares (Canizares *et al*., 2005). The epitope display system of CPMV can elicit strong immune responses, which is an important advantage of this viral vector. Many CPMV vecotors have been used to immunize and protect against pathogens including canine parvovirus, mink enteritis virus, *P. acruginosa* and hepatitis B virus (Langeveld *et al*., 2001, Dalagaard *et al*., 1997, Brennan *et al*., 1999, Mechtcheriakova *et al*., 2006). CPMV-based viral vectors have been developed to express full-length proteins. Since increasing the size of RNA-2 should not affect its encapsidation, the CPMV expression systems have be focused on modifying RNA-2. However, all the RNA-2 encoded products have to be retained for moving both locally and systemically in plants. Heterologous proteins are expressed either as coat protein or movement protein fusions with a proteolytic cleavage site to allow the target protein to be released (Verver *et al*., 1998). The use of duplicated CPMV proteinase cleavage sites was studied to achieve release of GFP from the fusion polypeptides, but duplication of the proteinase cleavage sites tended to cause loss of insert through homologous recombination (Gopinath *et al*., 2000). Most work on the expression of valuable target proteins has been investigated by fusing the sequence of the

foreign protein with the S protein at the C-terminal via the foot and mouth disease virus (FMDV) 2A catalytic peptide (Gopinath *et al.*, 2000).

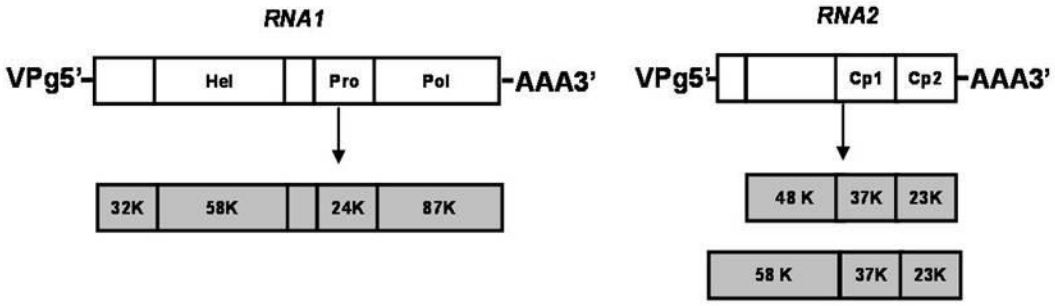


Figure 3. Genome organization of cowpea mosaic virus. Cowpea mosaic virus (family *Comoviridae*) has two positive-sense single-stranded RNAs. The protein encoding regions from each RNA molecule are first translated as a major polyprotein that is subsequently processed into mature proteins, a strategy similar to that of members of the family Potyviridae. RNA1: 32K, 32 kDa cysteine proteinase; 58K, 58 kDa protein of unknown function; VPg, viral genome-linked protein; 24K, 24 kDa main viral proteinase; RdRp, RNA dependent RNA polymerase. RNA2: 58K/48K(MP), two proteins with overlapping cistrons – 58 kDa protein of unknown function and 48 kDa movement protein; CP (L), large coat protein subunit; CP (S), small coat protein subunit.

Potyviruses: Potato virus A (PVA); Plum Pox Virus (PPV); Tobacco Etch Virus (TEV)

Potyviruses have a positive single-stranded RNA genome encoding a single polyprotein, from which all viral proteins are released by proteolytic cleavage (Fig 4). Potyviruses vectors differ from most other plant viruses by having no subgenomic promoters for heterologous genes expression. The target protein is translated as part of the polyprotein and cleaved from it using the recognition sites for the virally-encoded proteinases. The first potyvirus vector was developed based on tobacco etch virus (TEV), which expressed GUS gene between the P1 and HC-Pro encoding sequences (Dolja *et al.*, 1992). Since then, at lease eight other potyvirus species have been used to produce heterologous proteins. Three insertion sites in potyvirus-based virus vectors

have been investigated, including the cloning site between P1 (protein 1, proteinase) and HC-Pro (helper component proteinase), the cloning site between NIb (replicase) and CP (coat protein), and the cloning site within the P1 encoding region. The infectious clone of potato virus A (PVA) was engineered as an expression vector for production of heterologous proteins between the NIb and CP sequences of PVA (Ivanov *et al*., 2003). The PVA genome encodes for a large polyprotein, which is subsequently cleaved into up to ten mature proteins. Foreign genes are inserted into the infectious cDNA clone of PVA and translated as part of the polyprotein in plants. Three insertion sites in the genome of PVA were used for foreign sequences expression. Proteolytic cleavage sequences for the viral proteinases were added to separate the heterologous protein from the viral polyproteins (Kelloniemi *et al*., 2008). Plum pox virus (PPV), a member of Potyvirus) has been developed both as an expression vector for full-length proteins and as an epitope display system (Lopez-Moya *et al*., 2000). There has been one vaccine report in PPV expression vector, in which the VP60 protein of rabbit haemorrhagic disease virus (RHDV) was inserted between the NIb and CP sequences of PPV (Fernández-Fernández *et al*., 2001). Rabbits immunized with recombinant VP60 demonstrated specific immune responses that protected the rabbits against the challenges of rabbit haemorrhagic disease virus. Since the two termini of the coat protein are exposed on the surface of PPV virions, PPV was studied as a good epitope display system to present antigenic epitopes of canine parvovirus VP2 protein (Fernández-Fernández *et al*., 1998). Zuchini yellow mosaic virus (ZYMV) is a member of the *Potyvirus* genus. ZYMV has been used to express two plant proteins, MAP30 and GAP31, with activity against HIV (Arazi *et al*., 2002).

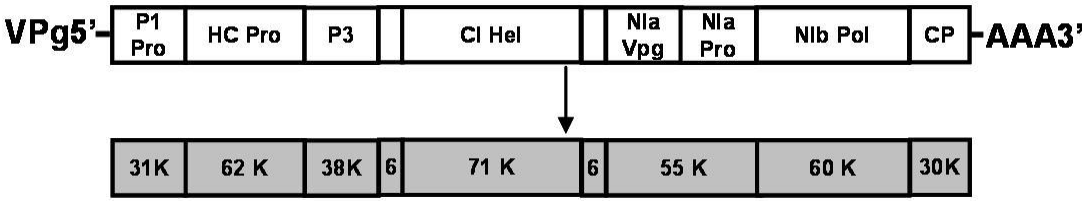


Figure 4. Genome organization of *Potyvirus*. One RNA molecule (9.7kb) comprising a single ORF translated into a polyprotein of 3063 amino acids, which undergoes a complex proteolysis achieved by three viral proteins: P1-pro, HC-pro and NIa. The final products are P1-pro (31K), HC-pro (62K), P3 (38K), CI-Hel (71K), 6kDa, NIa (55K), NIb (60K), and CP (30K).

Tomato Bushy Stunt Virus (TBSV)

Tomato bushy stunt virus, the type species of *Tombusvirus*, has been developed as a virus vector for full-length heterologous protein production and epitope display. TBSV has size limitation on foreign gene inserts due to its isometric morphology. Since the coat protein of TBSV is not essential for infection, the coat protein in the TBSV-based expression vectors has been replaced by a target gene (Scholthof *et al*., 1993). Subsequently, one new version of a TBSV-based vector was constructed in which the CP gene was substituted with a polylinker (Scholthof, 1999). With the technical improvement of virus infection, heterologous genes were permitted to express in the inoculated leaves of plants. TBSV-based vectors have been used to express p24 protein of HIV-1 as a fusion protein with the CP gene, but no report is available about the immune characteristic of this fusion protein (Zhang *et al*., 2000).

Tobacco Rattle Virus (TRV)

Tobacco rattle virus (TRV), the type species of the genus *Tobravirus*, contains two separately positive single-stranded RNAs, called RNA1 and RNA2. The RNA molecules are capped at the 5'-end, and the 3'-end folds into a tRNA-like structure. TRV

is often used in functional genomics studies via its special capacity as a virus induced gene silencing (VIGS). Although several plant viruses have been modified to produce VIGS vectors, the most widely used VIGS vectors are based on the *Tobacco rattle virus*. There are several advantages to use TRV-based VIGS vector to study gene function in plants. TRV has one of the widest host range of any plant virus. Over 100 plant species are infected in nature, and under laboratory conditions more than 400 species have been infected (Visser, 1999). TRV is able to spread vigorously throughout the entire plant, but the symptoms of infection are mild compared with other viruses (Burch-Smith *et al*., 2004). TRV infects growing points of plants, so it can be used to investigate the gene function in developmental biology studies (Ratcliff *et al*., 2001). A modified TRV VIGS system has been developed by Professor Dinesh-Kumar and co-workers at Yale University. TRV vectors were improved by adding a duplicated 35S promoter and a ribozyme at the C-terminus for more efficient production of viral RNA, as well as a number of amino acid changes in the viral sequence (Liu *et al.*, 2002). These VIGS vectors are not limited to infection in *N. benthamiana* and they were successfully used for silencing in tomato and other species (Liu *et al*., 2002).

Alfalfa Mosaic Virus (AIMV)

Alfalfa mosaic virus (AlMV), the type species of genus *Alfamovirus*, is a tripartite RNA virus with a broad host range. AIMV vectors are particularly useful for producing target protein fused to the viral coat protein. The N-terminus of the coat protein is located on the surface of the virion and can be used for the insertion of peptides. The AlMV coat protein gene has been used in TMV vectors for epitope display (Staczek *et al.*, 2000). The ability to create and maintain a peptide insertion of up to 50 amino acids

in length as a coat protein fusion makes this system very suitable for the development of vaccines. The AlMV replicase and DNA copies of RNA1 and RNA2 were integrated in the plant genome to produce p12 transgenic tobacco plants in which the viral infection can be initiated by the inoculation of RNA3 alone (Sanchez-Navarro *et al.*, 2001). The viral particles cannot infect non-transgenic plants, improving the containment of the virus. Two peptides of respiratory syncytial virus G protein were presented on the surface of AlMV in this way. Strong B-cell and T-cell immune responses were generated by the viral particles (Belanger *et al*., 2000). AlMV is a very efficient tool to present target gene in the form of coat protein fusions.

Cucumber Mosaic Virus (CMV)

Cucumber mosaic virus (CMV), the type species of the genus *Cucumovirus*, contains a tripartite RNA genome, which is closely related to AlMV. A recombinant CMV was engineered with the RNA-3 of CMV-S strain, containing the movement and coat protein genes, and the RNA1 and RNA2 of the CMV-D stain (Natilla *et al.*, 2004). In this study, the R9 mimotope of hepatitis C virus envelope protein E2 were introduced into the insertion sites in the coat protein of chimeric CMV particles. Patients with chronic hepatitis C showed significant immune responses to crude extracts of these chimeric CMV particles. These chimeric CMV virions also elicited the HCV specific humoral immune reaction in rabbits (Piazzolla *et al.*, 2005). Recently, this epitope presentation system was developed further to display double copies of R9 mimotope on the coat protein of chimeric CMV (Nuzzaci *et al.*, 2007). It was strongly recognized by sera of HCV-infected patients compared with R9-CMV. The high-level immunogenic

responses with 2R9-CMV suggest that this chimeric CMV may be useful in the development of oral vaccines against HCV.

New Approaches for Increased Efficacy and Biosafety

Agroinfection

To increase infectivity, it was thought that viral genomes could be directly inserted into the plant host genome. Yamaya *et al* (1988) was the first to accomplish this, constructing a full-length cDNA copy of the TMV genome, and creating transgenic plants using the *Agrobacterium* system. They found the transformed plants showed symptoms of TMV infection. The next step involved a technique known as agroinfection, in which whole plants can also be locally transformed by *Agrobacterium* without tissue culture. Turpen *et al.* inserted a 35S-driven cDNA of the TMV genomes into the Ti-plasmid of *Agrobacterium*, and infiltrated the *Agrobacterium* into whole leaves using a syringe ('agroinfiltration") to greatly increase infectivity of the TMV cDNA, since every cell in the infiltrated area now contained the TMV transgene in its chromosomes (Turpen *et al*., 1993). For RNA viruses, agroinfection provides an inexpensive and larger scale alternative approach to the *in vitro* transcription methods, which are used to transcribe from the DNA viral vector to produce infectious RNA, which is subsequently rubbed onto the leaves to create an infection. In addition, the co-agroinoculation with multiple components from a mixed suspension of agrobacteria cultures, each harboring different gene of viral vectors or proteins, can deliver multiple target genes into plant cells simultaneously. This new approach for plant RNA viruses held great promise for its speed, high expression levels of foreign proteins, and ease of

use. However, the authors noted very few infection points per leaf even though every cell in the inoculation zone was putatively transformed with an expressed copy of the TMV genome.

Based on the agroinfiltration strategy, Icon Genetics, a German plant biotechnology company, has developed a powerful expression platform of agroinfiltration of TMV vector (Marillonnet *et al.*, 2004, 2005). Echoing the findings of Turpen, *et al*., their work showed that only 1% of the agroinfiltrated cells were infected, even though all cells were transformed. The problem was thought to be getting viral transcripts out of the nucleus to allow natural viral replication in the cytoplasm to commence. How can these viral transcripts be exported from the nucleus to the cytoplasm when the positive RNA virus genome never goes into nucleus natively? The Icon group solved this problem by inserting 12-18 introns into the viral sequence. Previously, Johansen (1998) had added introns to a plant viral sequence cloned in a plasmid to stabilize plasmid expression in *E. coli* (plant viruses apparently contain toxic genes that are cryptically expressed in bacteria). Unexpectedly, infectivity also increased dramatically. The Icon group reasoned that this was due to a coupling of splicing to the nuclear export machinery. They found that the added introns increased infectivity from 1% to over 70% of the infiltrated cells. The Icon vectors demonstrated very high protein expression rates as a systemically infectious virus expression system (Marillonnet *et al.*, 2005). Furthermore, several deconstructed RNA viral vectors also have achieved high-level expression of recombinant proteins (Refer to Chapter 3 of this dissertation; Lindbo, 2007; Komarova *et al*., 2006). The tobacco mosaic virus (TMV)-based TRBO/G viral vector (Lindbo, 2007) showed 100-fold better expression of GFP than a cauliflower mosaic virus 35S promoter-

driven construct. In potexviral replacement virus vectors, the TGB and CP viral genes were removed and replaced with heterologous sequence, and around 100% of plant cells were infected by viral vectors with 40% TSP for the expression of GFP (Refer to Chapter 3 of this dissertation; Komarova *et al*., 2006). As the most efficient approach, they presently deliver the highest protein expression of any plant expression system.

Biosafety

Over the last decades, plant virus-based expression systems have been developed and successfully utilized for high-yield production of heterologous proteins in plants. However, widespread application of recombinant viruses raises concerns about possible risks to the environment. One of the primary safety issues that must be considered is the uncontrolled spread of the genetically engineered virus from experimental plants to susceptible wild plants. Intact viral vectors have the potential to spread and infect non-target plants, but replication-defective or movement-defective viruses will avoid viral dissemination. As well, some of the symptoms of viral infection can be lessened or eliminated (Porta *et al.*, 2003; Arazi *et al*., 2001).

Agroinfection allows the replacement of the MP and/or CP genes of vector viruses with heterologous sequences, at least in some virus species. TMV lacking the CP gene has been used to produce large amounts of foreign proteins (Marilloinnet *et al*., 2005, Gils *et al*., 2005, Dorokhov *et al*., 2007). In the PVX- replacement virus vector, both the TGB and CP viral genes were removed and replaced with heterologous sequence, leaving the replicase and 5' and 3' ends as the only remaining viral sequence (Komarova *et al*., 2006). The expression levels of GFP from this vector were 2.5-fold higher than with the full PVX vector, in which the GFP encoding sequence was placed

between the TGB and the CP genes. Removal of the CP prevents systemic movement of TMV and PVX (Takamatsu *et al*., 1987, Chapman *et al*., 1992, Marilloinnet *et al*., 2005, Komarova *et al*., 2006) and inhibits the spread of the genetically modified virus, which is positive from the biosafety point of view.

Gene Silencing Suppressors

Expression of heterologous proteins from the viral vectors can be further enhanced by co-inoculation with RNA-silencing suppressor proteins, which increases the quantity of intact mRNAs (Lindbo 2007). Expression of GFP was enhanced 100-fold from a vector-TMV when *Tomato bushy stunt virus* silencing suppressor p19 was coexpressed in the plants.

PVX was the first system in which small RNAs were related to gene silencing in plants. PVX-specific 25 nt short interfering RNAs (siRNAs) were found in tobacco leaves inoculated with PVX (Hamilton & Baulcombe, 1999; Hamilton *et al*., 2002). Later, 21 nt and 25 nt siRNAs were found in PVX-infected plants. PVX TGBp1 gene silencing suppressor can control the accumulation of the 25nt siRNA. When TGBp1 was deleted form the PVX genome, the 25nt siRNA accumulated and systemic silencing occurred (Voinnet *et al*., 2000). TGBp1 was determined as a gene silencing suppressor of systemic RNA silencing by regulating the 25nt siRNA. The shorter 21 nt siRNAs regulate local RNA silencing, and the 25 nt siRNAs regulate the systemic gene silencing (Hamilton & Baulcombe, 1999; Hamilton *et al*., 2002; Voinnet *et al*., 2000).

Potexvirus TGBp1 is a multi-functional protein, which has RNA helicase activity and is also a suppressor of RNA silencing (Voinnet *et al*., 2000). TGBp1 interacts with RDR6, which is a factor in siRNA production and promotes the gene silencing of some

viruses (Qu *et al*., 2005; Xie & Guo *et al.*, 2006). RDR6, DCL4 and HEN1 are factors required for initiation or maintenance of virus-induced gene silencing in growing tissues. DCL4 produces 21 nt viral siRNAs, which are amplified by RDR6 (Blevins *et al*., 2006; Dunoyer *et al*., 2005). The amplified 21 nt siRNAs then target viral RNA for silencing and restrict virus infection. Other viral gene silencing suppressor, such as potyvirus HC-Pro, tombusvirus P19 and closterovirus p21, bind siRNAs and prevent protein microRNA methylation by HEN1 (Blevins *et al*., 2006; Lakatos *et al*., 2004, 2006; Merai *et al*., 2006; Yu *et al*., 2006). CaMV has a silencing suppressor that stabilizes double-stranded RNA (dsRNA) products of RDR6 and interferes processing of long dsRNAs by DCL4 (Blevins *et al*., 2006).

Edible Vaccines

In the past few years, plant virus vectors have significantly improved in the production of immunogenic vaccines in plants. Recombinant proteins and peptides expressed in plants have been shown to provide protective immune responses against various animal diseases (Dalsgaard *et al*., 1997; Langeveld *et al*., 2001; Fernandez-Fernandez *et al*., 2001). Immunity has been stimulated by both parenteral immunization and mucosal immunization (Koo *et al*., 1999; Yusibov *et al*., 1997). There have been some striking successes with edible vaccines composed of unprocessed transgenic plants. For example, a vaccine against immunobursitis disease virus (IBDV) was produced in the model system mustard plant, *Arabidopsis,* under the control of a 35S promoter. Ground up transgenic plant material was fed to chickens, followed by inoculation with IBDV virions 2 weeks later. Complete protection was achieved, while 100% of the nonvaccinated chickens died (Wu *et al.*, 2004).

The next generation plant-based edible vaccine may have a much higher level of antigen if the target gene is carried by a viral vector. For edible vaccines, viral vectors should be developed for developing countries that can infect suitable host plants, including cereals and legumes, in order to take advantage of the assets of low cost and scale afforded by plant systems. Several candidate viral vectors may allow the edible vaccine systems to be more achievable: 1. cowpea mosaic virus (CPMV), clover yellow vein virus (CIYVV), sunn hemp mosaic virus (SHMV), all of which infects legumes; 2. wheat streak mosaic virus (WSMV), foxtail mosaic virus (FoMV), all of which infects cereals; 3. zucchini yellow mosaic virus (ZYMV), which infects cucurbits (Gopinath *et al*., 2000; Masuta *et al*., 2000; Choi *et al*., 2000; Arazi *et al*., 2001). For plants susceptible to agroinfection, one might consider *Agrobacterium* to overcome restrictions of host range caused by the inability of the virus to spread. Many plant viruses can be delivered and replicate in a large range of plant species by agroinfection, which can deliver the viral genome to high percentage of cells in target plants. In addition, defective or deconstructive viral replicons for the expression of foreign proteins allow reducing the risk of environment spread (Sanchez-Navarro *et al*., 2001; Mori *et al*., 2001). Ultimately, a transgenic version of a deleted virus, perhaps driven by an inducible promoter, would be the least expensive and highest efficiency system for the production of edible vaccines.

In some cases, though, it may be desirable to purify vaccines and immunogenic chimeric viral particles for further application. In the laboratory, small scale extractions are sufficient for the experiments of material characterization, but industrial-level scale purification will be essential for the widely practical uses. Recombinant TMV virions

expressing a 12 amino acid malarial peptide were studied by large-scale growth and purification, and the yield of purified particles was more than 1 kg/hectare under the field conditions (Pogue *et al*., 2002). The industrial-scale production of plant-based vaccines could make an important improvement to world health.

Virus Induced Gene Silencing (VIGS)

Some of the early discoveries in RNA silencing were made in the study of a plant virus vector. When a PVX-GUS expression vector was inoculated to GUS-transgenic plants, GUS expression was silenced (Baulcombe, 1996; English *et al*., 1996). Recombinant PVX viruses expressing phytoene desaturase (PDS) or GFP proteins induced gene silencing of the transgenic GFP or the PDS genes in plants (Ruiz *et al*., 1998; Voinnet *et al*., 1998). These studies led to the development of VIGS technology. VIGS uses plant viral vectors carrying the host gene inserts to silence the expression of the specific host gene in the inoculated plant (Angell and Baulcombe, 1997). Plants are infected with the recombinant viruses carrying the target sequences, inducing the degradation of the target host gene mRNA. Viral ssRNA form hairpin-like structures that are recognized by the host (Baulcombe 1999). The structures are cut into small interfering RNA (siRNA) fragments of 21-24nt by a cellular RNase (Dicer) and one strand of siRNA is incorporated into an RNA-induced silencing complex (RISC) (Baulcombe 2005, Voinnet 2005). The ssRNA is recognized through homologous siRNA and then degraded by the RISC.

Virus-induced gene silencing (VIGS) offers a rapid and high throughput technique platform for the analysis of gene function in plants. It can be used by plant breeders to identify genes coding for useful agronomic traits. VIGS has obvious

advantages over other known approaches for gene function analysis. For instance, it does not require development of stable genetic plants, and thus is rapid and less laborious. It is applicable for the functional analysis of genes for which only partial sequences are available, such as expressed sequence tags (ESTs). siRNA are formed from both viral sequences and inserted heterologous sequence that targets the homologous host gene for silencing. Inserting the targets sequence in an antisense orientation in the vector-virus also triggers silencing by forming double-stranded RNA hybrids with the mRNA from the target gene (Kumagai *et al*., 1995).

Tobacco rattle virus (TRV) has been the most commonly used vector-virus for VIGS study so far. The potential applications of the TRV vector are not restricted to *Nicotiana* and *Arabidopsis*. TRV has a reported wide host range of over 400 species from monocots and dicots families, including tomato, potato and oats (Robinson and Harrison, 1989). Thus it may be possible to use TRV for VIGS by infection of a wide range of important crop plants. Unlike other RNA virus vectors that have been used for VIGS, the TRV construct is able to target host RNAs in the growing points of plants (Ratcliff *et al*., 2001). The silencing of PDS has been used as a marker for the effectiveness of VIGS in several instances. The silencing of PDS produces a typical white color that is the result of photobleaching, which occurs in the absence of the gene product.

PVX was developed as VIGS viral vectors to study host gene expression in entire plants. PVX viruses expressed host genes were shown to silence target genes uniformly throughout the entire plants (Ruiz *et al*., 1998). Adding viral suppressor protein by agroinfiltration can restore silenced protein expression. With viral vectors and

agrodelivery of silencing suppressors, it provides the capacity to switch target genes off and on for analysis of gene function (Mallory *et al*., 2002).

Recombinant Antibodies that are Expressed in Plants

Antibodies are complex glycoproteins that recognize and bind to target antigens with specificity. This specific antibody-antigen binding allows to be used for diagnosis, prevention and treatment of disease. The production of antibodies in plants represents a special challenge because the peptides must fold and assemble correctly to recognize the antigens. Typical antibodies are tetramers of two identical heavy chains and two identical light chains. There are more complex forms, such as dimers of typical antibodies for secretory antibodies. In mammalian cells, antibody assembling requires two types of cell involved, but plants could assemble the antibodies in one single cell (Ma *et al*., 1995). Antibodies produced in plants appear to have the same activity as the one produced in mammalian cell cultures. Functional full size antibodies can be expressed as heterologous proteins on in mammalian or plant systems, not in bacteria, yeast or insect cells.

Plants have been used to express many different antibody forms: full-length immunoglobulins, full size variable fragment (Fab), single-chain variable fragments (scFvs), antibody-fusion proteins, single-chain antibodies (Schillberg *et al*., 2003; Stoger *et al*., 2002). Several different types of immunoglobulin (Ig) have been produced successfully in plants, including IgG (Hiatt *et al*., 1989), IgA (Larrick *et al*., 2001) and the chimeric IgA/IgG (Ma *et al*., 1998). All full-length immunoglobulin molecules contain two identical heavy chains and two identical light chains, encoded by separate genes. For transgenic plants, the genes are introduced into separate plant lines and then

progeny with the hybrid is produced by crossing these two transgenic parents. The first antibody expressed in plants was the full length serum IgG produced by crossing plants that expressed heavy and light chains (Hiatt *et al.*, 1989). The first secretory antibody expressed in plants, the chimeric IgA/IgG, was achieved by sequential crossing of four lines carrying individual components (Ma *et al*., 1998). Secretory IgAs are dimers of the typical serum-type immunoglobulins and include two extra components: the secretory component and the joining chain. Four separate transgenes are required to produce such molecules (Larrick *et al*., 2001). For transient expression, the single-chain Fv epitopes have been produced rapidly from co-infection of *N. benthamiana* plants with two viral vectors that each expresses a separate chain. A particular advantage of this system is the short development time and rapid protein production. It takes weeks rather than months to establish the virus-based expression platform (McCormick *et al.*, 1999).

Antibodies have been expressed in many different plant systems. Tobacco was used for antibody production in the early studies, but antibodies can also be produced in cereal seeds (Stöger *et al*., 2000), suspension culture cells (Fischer *et al*., 1999), algae (Mayfield *et al*., 2003), alfalfa plants (Khoudi *et al*., 1999) and potato (Artsaenko *et al*., 1998). Proteins accumulated in dry seeds are good for long-term storage without degradation or loss of activity (Azzoni *et al*., 2002). Stable transgenic plants are able to express correctly folded and functional antibodies, but yields are very low in the range of 1-40ug/g of fresh biomass. In addition, it takes more than two years to generate the transgenic plants to produce antibody material (Schillberg *et al.*, 2003; Stoger *et al.*, 2005; Ma *et al*., 2005). Some plant-based recombinant antibodies have been progressed to clinical trial, for example, the anti-*Streptococcus aureus* full-length antibody used to

prevent tooth decay. Transient expression systems allow for faster production, but they could not provide high yield coexpression of the two or several polypeptides needed for the assembly of immunoglobulins (Verch *et al.*, 1998; Rodriguez *et al.*, 2005) until the recent development of a dual virus system. Full-length and fully functional antibodies were expressed rapidly by coinfection of two viral vectors, TMV and PVX, each expressing a separate chain. This expression technology yielded as high as 0.5g of mAb per kg of fresh biomass (Giritch *et al.*, 2006). However, since immunoglobulins are glycoproteins, concerns have been raised about different glycosylation pattern between plant and animal cells.

Post-translational Modification

Although the protein synthesis pathway is the same in plants and animals, there are some differences in post-translantional modifications. The difference in protein N-glycosylation patterns between animals and plants is seen as one of the main problems in production of mammalian glycoproteins in plants, especially immunoglobulins. Plant-derived proteins have the glycan groups, α-1,3-fucose and xylose, which are absent in mammalians glycosylation and might be immunogenic when injected to humans. On the other hand, the mammalian specific glycosylation modification such as sialic acid and β-1,4-galactose are missing from proteins expressed in plants.

The possibility of plant-specific glycans inducing immune responses in humans has been considered. However, these plant-specific glycan residues are also associated with every normal plant glycoprotein in our diet. Mice were administered a plant expressed recombinant antibody containing plant-specific glycans, but showed no anti-glycan immune response (Chargelegue *et al.*, 2000). Nevertheless, the foreign glycan is

one the most important issues that might affect the application of plant-derived recombinant antibodies. Recent attention has focused on the development of humanized glycosylation patterns of recombinant proteins. Humanizations of N-glycosylation in plant-based recombinant proteins have been attempted recently. To humanize the glycosylation of immunoglobulins in plants, a transgenic tobacco line that expressed the heavy and light chains of a murine antibody was crossed with a line that expressed human β-1,4-galactosyltransferase. The progeny produced antibodies with about 30% partially galactosylated N-glycans. This provided a useful approach for the 'humanization' of plant glycans (Bakker *et al*., 2001). Human immunoglobulins produced in tobacco plants with transgenic human β-1,4-galactosyltransferase had galactose residues and low levels of xylose and fucose residues, but immunoglobulins produced in wild type tobacco plants had no galactose and contained high levels of xylose and fucose (Bakker *et al*., 2006). Meanwhile, gene silencing has been used to disable the plant-specific fucosyltransferase and xylosyltransferase enzymes. RNA silencing with lower expression of the plant enzymes for incorporating xylose and fucose to proteins was used to produce human antibodies with low amount of xylose and fucose incorporation in duckweed (Cox *et al*., 2006).

There is natural variation in glycan patterns, and many proteins have several glycosylation sites and many glycoforms in mammalian cells. Rodent cell lines can recognize differences in glycan patterns (Raju *et al*., 2000). Some classes of glycosylation forms might be more important for immune responses. There are not enough data to know whether the method of delivery of recombinant proteins (oral versus injection) make a difference in the immune response.

The KDEL endoplasmic reticulum retention signal was demonstrated as the necessary retention in endoplasmic reticulum for proper assembly and accumulation of full-length antibody in plants, as in mammalian systems (Hiatt *et al.* 1989). However, it was found that the KDEL sequence on occasion could improve accumulation levels and in others accumulation levels are independent of endoplasmic reticulum retention (Peeters *et al.*, 2001). Icon Genetics tried to express antibodies with a KDEL ER retention signal in either the LC or the HC. However, they did not see any increase in expression compared with the same antibodies without KDEL sequence (S. Marillonnet, personal communication).

Allergens and Allergy

Defining Allergens and Allergies

Allergenic diseases, such as asthma, rhinoconjunctivitis, eczema, and anaphylaxis, are increasing dramatically and affect more than 25% of the population in industrialized countries. In the USA, the annual cost of treating asthma is estimated to be $6 billion (Galli, 2000). Type I hypersensitivity is the clinical manifestation of an immune response against foreign protein molecules, commonly known as allergens, which leads to stimulation of T cell helper type 2 (Th2) responses and subsequent bias towards the IgE synthesis. The allergen triggers the activation of IgE-binding mast cells in the exposed tissue, leading to a series of responses that are characteristic of allergy (Janeway, 2001). The term allergen is used to describe three distinct molecular features: the property to bind IgE antibodies; the property to sensitize (promote the immune system to produce high-affinity IgE antibodies); and the property to elicit allergic

reactions (i.e., to trigger allergic symptoms in a sensitized person). Allergens can be divided into: 1. complete allergens which have all these features, 2. incomplete allergens which can elicit allergic symptoms but do not sensitize (Jankov, 2005).

An allergen is an antigen which gives rise to hypersensitivity. Most allergens are relatively small, the size of which range from 10-70kDa in molecular weight, and highly soluble proteins that are carried on desiccated particles such as pollen grains or mite feces (Aalberse, 2000). Allergens are typically presented to the immune system at very low doses. Although a large number of allergens have been characterized, the structural, functional, and biochemical features that these molecules ability to elicit powerful IgE antibody responses, are still uncertain (Aalberse, 2000). Molecular-level information on the structure of allergen indicates that allergens are considerably heterogeneous protein structures and that there is no particular amino acid sequence which is responsible for the allergenicity (Ferreira *et al*., 2004). Allergen sequences are homologous to diverse proteins, such as enzymes (such as cystein protease), profilin (acrosomal protease), lipid transfer protein, and thaumatin-like proteins (Ferreira *et al*., 2004).

Immunology of Allergy

Sensitization. The primary response to allergen is known as sensitization. In the first few years after birth, minute amounts of soluble allergens (for example, respiratory allergies, in the nanogram range) are released from allergen bearing particles (for example, pollen) on mucosal surfaces (Grunewald *et al*., 1998). The antigen-presenting cells (for example, dendritic cells; DCs) take up allergens and present peptides (T-cell epitope) of allergen to allergen-specific T cells through the MHC, and the naive T cell will be activated and differentiated to become an allergen-specific Th2 cell. Th2 cells

can then help to activate allergen-specific B cells by means of T-cell-B-cell interactions through same peptides (T-cell epitope). Th2 cells produce cytokines, such as interleukin-4 (IL-4) and IL-13, which induce the immunoglobulin-class switching of B cells to IgE. As a result of sensitization, allergic patients produce allergen-specific IgE antibodies, although low levels of allergen-specific IgG antibodies could be found (Grunewald *et al*., 1998). After sensitization, allergen-specific T cells form long lived memory T cells that reactive to repeated allergen contact (Chakir *et al*., 2000). The primary response also leads to the production of an allergen-specific IgE antibody memory. Long lived memory B cells that produce allergen-specific IgE antibody are found in the mucosa of the respiratory tract and also in the peripheral blood of allergic patients. When seasonal allergen contact with patients, IgE positive memory B cells are strongly activated by contact with the allergen to produce increased levels of antibody. These allergen-specific IgE memory B cells must receive help from allergen-specific T cells to be activated (Naclerio *et al*., 1997).

The immediate-phase reaction. IgE binds tightly to mast cells and basophils by the high-affinity receptor FcβR I. Cross-linking of receptors by allergen-bound IgE induces the release of inflammatory mediators (for example, histamine and leukotrienes), then cause the immediate symptoms of disease in minutes, such as rhinitis, conjunctivitis and asthma (Turner and Kinet, 1999).

The late-phase reaction. This is caused by the presentation of allergens to T cells; then the T cells become activated, proliferate and release proinflammatory cytokines (for example, IL-4, IL-5 and IL-13) (Larch, 2000). This process might be enhanced by the

IgE-mediated presentation of allergens to T cells. Th2 cytokines (for example, IL-5) induce tissue eosinophilia and the release of inflammatory mediators from eosinophils. Therefore, the symptoms of Type I hypersensitivities fall into two categories, immediate and late-phase reaction. Immediate symptoms are characterized by allergic rhinitis, conjunctivitis, asthma, dermatitis, and in severe cases, anaphylactic shock. The immediate symptoms occur within minutes of exposure to the allergen. However, the late-phase symptoms occur hours and even days later and remain with the atopic individual as chronic conditions such as asthma and dermatitis (Larch, 2000).

Cedar Allergens

In central Texas from late December through February, mountain cedar (*Juniperus ashei*) pollen is the principal allergen responsible for allergic rhinitis in hypersensitive individuals. Cedar hypersensitivity is one of the most frequent causes of seasonal allergic disease. Mountain cedar pollen causes severe seasonal allergic rhinitis in North America (central Texas, New Mexico, and northern Mexico) (Goetz *et al*., 1995; Cross *et al*., 1978). Mountain cedar is a member of the *Cupressaceae* family, which also includes common cypress (*Cupressus sempervirens*) and Arizona cypress (*Cupressus arizonica*), the cause of pollinosis in the Mediterranean region (France, Italy, and Israel) and Australia (Panzani *et al.*, 1986; Mari *et al*., 1996). Each member of this family has cross-reactivity with the pollen of all the other members of this group (Reid *et al*., 1992; Takahashi *et al.*, 1993).

The major allergen from mountain cedar pollen, termed Jun a 1, was isolated and characterized by the Goldblum group at UTMB Galveston. The predominance of Jun a 1 in the soluble proteins of mountain cedar pollen and its high degree of homology with the

know allergens Cha o 1 (the major allergen of *Chamaecyparis obtusa*) and Cry j 1 (the major allergen of *Cryptomeria japonica*) , which have pectate lyases, make it likely to be the major allergen of this pollen. Amino acid sequence conservation also makes Jun a 1 a potential target for cross-reactivity between these pollen allergens. IgE from the sera of Japanese cedar–sensitive patients has immunoreactivity with Jun a 1.

Full-length Jun a 1 cDNA has been cloned by the Goldblum group (Midoro *et al.*, 1999). The high degree of sequence identity between this allergen and those of other conifers and even ragweed may explain some common IgE epitopes among plants. This group also maped four IgE binding sites on Jun a 1. In the study, synthetic, overlapping peptides of Jun a 1 and sera from patients allergic to mountain cedar pollen were used to identify linear epitopes. Two of the three dominant linear epitopes map near the highly conserved PL active site, and are likely to be shared by the group 1 allergens of other cedars and cypresses, and perhaps PLs from other plants and even microorganisms. This may help to explain the high degree of cross-reactivity between cedar pollen allergens and might represent a pattern of reactivity common to other allergens with catalytic activity (Midoro *et al*., 2003).

Allergen Therapy and Recombinant Allergens

The basis for antigen targeted immunotherapy of allergy depends on two phenomena: 1. allergen-specific T cells are controlled by various allergen-specific factors, including antigen dose, antigen conformation and the site of antigen exposure; 2. the profile of allergens and epitopes that are recognized by IgE does not change substantially during the natural course of allergic disease (Constant *et al*., 2000; Akdis *et al*., 1998; Ball *et al*., 1999). Currently, immunotherapy mainly involves desensitization.

It involves repeated subcutaneous injections of increasing doses of adjuvant-bound allergen extracts. Desensitization switches the Th2 response to the Th1 response, increasing the IgG to IgE ratio, and inhibits the release of chemical mediator (Larche, 2000; Valenta, 2002).

Although injection immunotherapy has improved the quality of life for many people, it still leaves a lot to be desired as an allergen therapy, with only 30% of the patients responding after two years of weekly injections (Midoro *et al*., 1999). A major disadvantage of injection therapy is that it uses natural allergen extracts, which are hard to standardize because they contain mixtures of allergenic and non-allergenic compounds. Injection is not specific to a patient's reactivity profile (Liu *et al*., 2003). Even worse, the patients become sensitized to other components in the injection. Another strong disadvantage of current desensitization therapy is that the preparations contain active allergens, which cause local and systemic allergic reactions ranging from acute allergic response such as urticaria to more severe responses like asthma attacks and anaphylactic shock (Valenta *et al*., 1999). Demand of recombinant allergen is increasing for diagnostic applications, as well as allergen-specific immunotherapy using recombinant allergens. An impressive number of allergens from different allergenic sources have been expressed and characterized. In many cases natural allergenic extracts are mixtures of several proteins with non protein contaminants which are sometimes very difficult to purify. Such mixtures may give inaccurate results if used for diagnosis of allergic diseases. Recombinant allergens can improve the specific diagnosis of type I allergy, for example recombinant profilin (Bet v 2) (Bohle *et al*., 2004; Susani *et al*., 1995).

Therefore, in addition to applications in component-resolved allergy diagnosis, genetically engineered allergens may also be used for the treatment of most common forms of allergy and even prophylactic vaccination. The latest advancement in allergen vaccination is the genetic engineering of allergens in order to modify the allergen so that they retain T cell epitopes while minimizing IgE binding (i.e., timothy grass pollen allergen Phl p 5b) (Schramm *et al*., 1999; Holm *et al*., 2004).

Recently published results from a multicenter clinical trial on the development and evaluation of allergen-specific immunotherapy based on genetically engineered allergens suggest that immunotherapy with genetically engineered allergen derivatives have the potential to induce an allergen-specific, mixed Th2/Th1-like immune response. This immune response was characterized by an initial induction of IgE antibodies, followed by strong IgG1, IgG2, and IgG4 responses recognizing new epitopes as well as epitopes defined by the disease-eliciting IgE antibodies (Larsen *et al*., 1996). Recombinant allergens are also important tools in determining linear and conformational IgE epitopes and the effect of mutations on IgE binding. There is an increasing demand for recombinant allergens.

Plant Expression Systems

Plant expression systems offer a solution to many of the previous problems. Because more than 50% of allergens are of plant origin, it makes sense that one should use plants to express these proteins. Expression in plants causes fewer problems with correct folding and solubility; plants are able to glycosylate their protein products, and their proteins are soluble. This method of protein expression is easy and relatively

inexpensive, generating various source proteins at much higher levels (Breiteneder *et al*., 2001; Ma *et al*., 2003).

Foreign genes can be produced in plants by permanent insertion or by transient expression using virus-based vectors. Transgenic plant expression involves integrating the foreign DNA into the host plant's chromosomes. Transient expression is based on plant virus vectors expression systems. Both techniques would be useful in creating recombinant allergens, but, as mentioned earlier, transient expression has several advantages over transgenic plants. Creating transgenic plants is extremely time-consuming, requiring long time of tissue culture whereas transient expression system can result in gene expression within several days. The speed advantage of transient vectors is important when screening for mutants. Transient expression systems are cost-effective and allow large-scale production of proteins (Reiner *et al*., 2003; Simon *et al*., 1999).

Viral vectors are advantageous when high levels of gene expression are desired within a short time, although the instability of the foreign genes in the viral genome may present problems. Tobacco mosaic virus (TMV) and Potato virus X (PVX) based vectors were used for the transient expression of plant allergens in *Nicotiana benthamiana* plants. Protein expression involves either the inoculation of tobacco plants with infectious RNA transcribed *in vitr*o from a cDNA copy of the recombinant viral genome or the transfection of whole plants from wounds inoculated with *Agrobacterium tumefaciens* containing cDNA copies of recombinant plus-sense RNA viruses.

Several allergens have been successfully produced via a TMV expression system; specifically, Bet v 1, the major birch pollen allergen (Krebitz *et al*, 2000); Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*) (Krebitz *et al*, 2003); Art

v 1, pollen allergen of *Artemisia vulgaris* (Gadermaier *et al*., 2003); Der p 5, a mite allergen (Hsu *et al*., 2004); Hev b 1 and Hev b 3, two spina bifida associated latex allergens (Valenta *et al*., 1999); and Jun a 3, a mountain cedar allergen (Moehnke *et al*., 2008).

CHAPTER TWO

Efficiency Protein Expression in Legumes and *Nicotiana* from Agroinfection-Compatible Sunn Hemp Mosaic Virus Expression Vectors

Abstract

Plants viruses are increasingly being examined as alternative recombinant protein expression systems. The current generation of plant virus expression vectors is designed with a single host plant in mind, namely, the tobacco, *Nicotiana benthamiana*. Future development of viral vectors for oral delivery of vaccines or for functional genomics must focus on crucial crops such as cereals and legumes. Sunn hemp mosaic virus (SHMV) is a tobamovirus, which infects leguminous plants and is a good candidate for development as a vector.

In this work, SHMV-based viral vectors for high yield of target proteins in plants have been constructed. The SHEC vector series (sunn hemp elimination of coat protein) is a nonsystemic viral vector for agroinoculation or, potentially, for transgenic crops. The SHAC vector series (sunn hemp alternate coat protein) has the native coat protein ORF replaced with the homologous sequence from another tobamovirus. Both types of constructs are driven by a 35S promoter and can be delivered as a T-DNA to plant cells by *Agrobacterium tumefaciens*. Co-agroinfiltration with RNA silencing suppressor p19 or HcPro resulted in high levels of GFP expression in *N. bethamiana* by 6 days post inoculation (dpi). Interestingly, without suppressor co-inoculation, protein expression is very poor in *N. benthamiana*. Thus, an on/off switch is operational in this tobamoviral system, dependent on the presence of suppressor. This trait may be especially useful in

the development of inducible transgenic versions of SHEC vectors. Three lengths of subgenomic promoter we investigated, with fairly similar rates of GFP expression for each. Furthermore, strong GUS expression was obtained for several legumes with agroinoculated SHEC/GUS plus p19. A SHAC vector with a tobacco mild green mottle virus coat protein ORF produced almost no symptoms on *N. benthamiana* but was able to express GFP. This demonstrates the possible suitability of the SHAC series as VIGS vectors. This study reports the first SHMV agroinfection expression vectors.

Introduction

Plant expression systems have advantages compared to other methods of recombinant protein production - the comparatively low cost of large-scale production; cultivation is much cheaper and easier without sterile condition of cell culture; the eukaryotic protein modification machinery, allowing sub-cellular targeting, proper folding, and post-translational modifications; the absence of human pathogens (Streadfield, 2005). Stable transgenic plants are one approach, although their creation is time-consuming and labor-intensive (Kearney *et al*., 1994). Plant viral vectors represent another approach which delivers more rapid and higher level, but transient, expression of proteins. Compared with transgenic plants production systems, plant viral vectors are easier to manipulate and could produce much more recombinant proteins quickly. A number of different plant viruses have been developed into protein production vectors, the most commercially useful being tobacco mosaic virus (TMV) of the *Tobamovirus* family and potato virus X (PVX) of the *Potexvirus* family (Pogue *et al*., 2002; Richard *et al*., 2003; Yusibov *et al*., 2006).

Several innovations have led to dramatic improvements in plant viral vectors. The early versions of these vectors cited used *in vitro* transcription to create infectious RNA, which is expensive and not amenable to large scale production in contrast to a more recent method named "agroinfection" (Gleba *et al*., 2005). With agroinfection, an *Agrobacterium tumefaciens* suspension harboring T-DNA carrying the viral genome is syringe or vacuum infiltrated into plant leaves, resulting in almost 100% of the plant cells being transformed by *Agrobacterium* in the infiltrated zone (Liu and Lomonossoff, 2002). Agroinfection was developed originally for DNA plant viruses (Grimsley *et al*., 1986, 1987). As DNA viruses have disadvantages as far as insert size, RNA viruses were developed for the agroinfection system (Leiser *et al*., 1992) including a number of different RNA viruses.

Another development was the use of RNA silencing suppressors to increase expression. Virus replication elicits the protection responses of the plant cell posttranscriptional virus-induced gene silencing (VIGS). This inhibits viral vector infection and reduces target protein expression. Countering the RNA silencing antiviral mechanism, many plant viruses encode RNA silencing suppressors. HC-Pro, from *Potyvirus* (Potato virus Y and Tobacco etch virus), was the first identified RNA silencing suppressor) (Anandalakshmi *et al*., 1998), which is a powerful suppressor in all assays. P19, from *Tombusvirus* (Tomato bushy stunt virus and Cymbidium ringspot virus), blocks both local and systemic silencing in transient expression assays (Voinnet *et al*., 1999). Both HcPro and p19 have been used to enhance transient expression systems in plants based on suppression of gene silencing (Voinnet *et al*., 2003). A recently developed TMV vector, driven by a 35S promoter in a binary vector, was delivered via

agroinfection along with an *Agrobacterium* culture carrying a 35S-driven p19 suppressor. This system produced 0.6-1.2 mg of recombinant protein per gram of infiltrated plant tissue, which is 10-25 times higher than the 35S promoter driven transient expression systems without p19 (Lindbo, 2007).

By far, the current emphasis in commercial and scientific use of viral vectors lies with the tobacco host *Nicotiana benthamiana*. Future development needs to focus on the creation of new vectors that can be used in different hosts, such as hosts amenable to oral delivery of vaccines, including cereals and legumes. Sunn hemp mosaic virus (SHMV) is a good candidate for such a vector, since it is closely related to the commercially robust TMV but infects a wide range of legumes as well as *N. benthamiana*.

SHMV, being a member of the *Tobamovirus* group, is a plus-strand RNA virus of 6483 nucleotides with the same genomic arrangement as TMV. The 3' 1.8 kb of sequence were determined in initial reports (Meshi *et al*., 1981; Meshi *et al*., 1982), while the final 4.6 kb at the 5' end were given in a separate report, completing the sequence (Silver *et al*., 1996). Like TMV, the SHMV genome consists of genes coding for two replicase proteins, a movement protein and a coat protein (CP). The first two open reading frames (ORF) produce two replicase proteins, a 129 kDa and an 186kDa protein. An amber stop codon at the end of the 126 kDa replicase undergoes a translational read-through to produce the 183 kDa replicase. The movement and coat proteins are translated from 3' coterminal subgenomic mRNAs. The movement protein is a 31 kDa protein produced early in the infection cycle, which is required for virus cell to cell movement. The coat protein is 18 kDa protein produced at high levels later in the infection cycle, and is required for virus long distance movement.

By deletion of the CP gene, we have constructed a viral vector, SHEC (Sunn Hemp Elimination of Coat protein), that is incapable of long distance movement. The coat protein subgenomic promoter was used in this vector to drive foreign genes, using three different potential promoter lengths. The use of co-agroinoculation with silencing suppressor genes was found to be critical for successful marker gene expression. To create a full length vector, a subgenomic promoter from a different tobamovirus was needed to drive the coat protein gene (in addition to the SHMV coat protein promoter driving the foreign gene), since recombination between identical subgenomic promoters has be shown to severely destabilize TMV vectors (Dawson *et al*., 1989). This chimeric viral vector with an Alternate Coat protein (SHAC) systemically infected *N. benthamiana* and expressed GFP.

Materials and Methods

Plants

Seeds of *Nicotiana benthamiana*, *Vigna unguiculata* (cowpea) and *Phaseolus vulgaris* (Pinto bean), *Lens culinaris* (lentils), and *Pisum sativum* (peas) were sown in Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada) and plants were grown in 4" pots at 23°C with 24 hour illumination. *N. benthamiana* plants were agroinoculated at the 5-10 cm stage. Legumes were agroinoculated at the two to four week stage with fully expanded primary or secondary leaves.

SHMV cDNA Constructs

All SHMV clones used in this study are the derivatives of a wild-type SHMV cDNA clone (Silver *et al*., 1996) and were constructed with standard recombinant DNA

techniques. The high fidelity polymerase Phusion (NEB, Beverly, MA) was used according to company protocols for PCR. Recombinant DNA was introduced into *E.coli* NEB l0-beta Electrocompetent cells (NEB, Beverly, MA) and screened by PCR using Taq polymerase (NEB, Beverly, MA) or restriction digest and then sequenced using a CEQ capillary sequencer (Beckman Coulter, Fullerton, CA).

pSHMV. The binary vector, pJL22 (Lindbo, 2007), has the mini binary plasmid, pCB301 (Xiang *et al*., 1999), as backbone. JL22 has a multiple cloning site (MCS) flanked by a CaMV 35S promoter and a CaMV 3' polyA signal/transcription terminator. SHMV full length viral cDNA (Silver *et al*., 1996) and a ribozyme sequence from JL24 (Lindbo, 2007) were inserted into the MCS of pJL22. SHMV *in vivo* transcripts should be processed by the ribozyme to generate authentic SHMV 3' ends. This full viral expression cassette, including promoter and terminator, is flanked with the left and right borders of the T-DNA (Fig 5).

pSHacgCP. The use of overlap PCR allowed the double start codons at the beginning of coat protein ORF in pSHMV to be functionally eliminated by mutation to a pair of ACGs. The two primers (dCP-SHMV Up and dCP-SHMV Down) (Table 1) in the initial PCR of the overlap strategy were used to mutate ATGATG to ACGACG without a change in protein sequence of the movement protein ORF in pSHMV. These were mated with the downstream primer, JL22 RB Down, and the upstream primer, SHMV 4658 Up, respectively, in the intial PCR and were used as a pair for the second (overlap) PCR. The overlap PCR product was cloned back into *Kpn*I/*Pme*I cut pSHMV to make pSHacgCP.

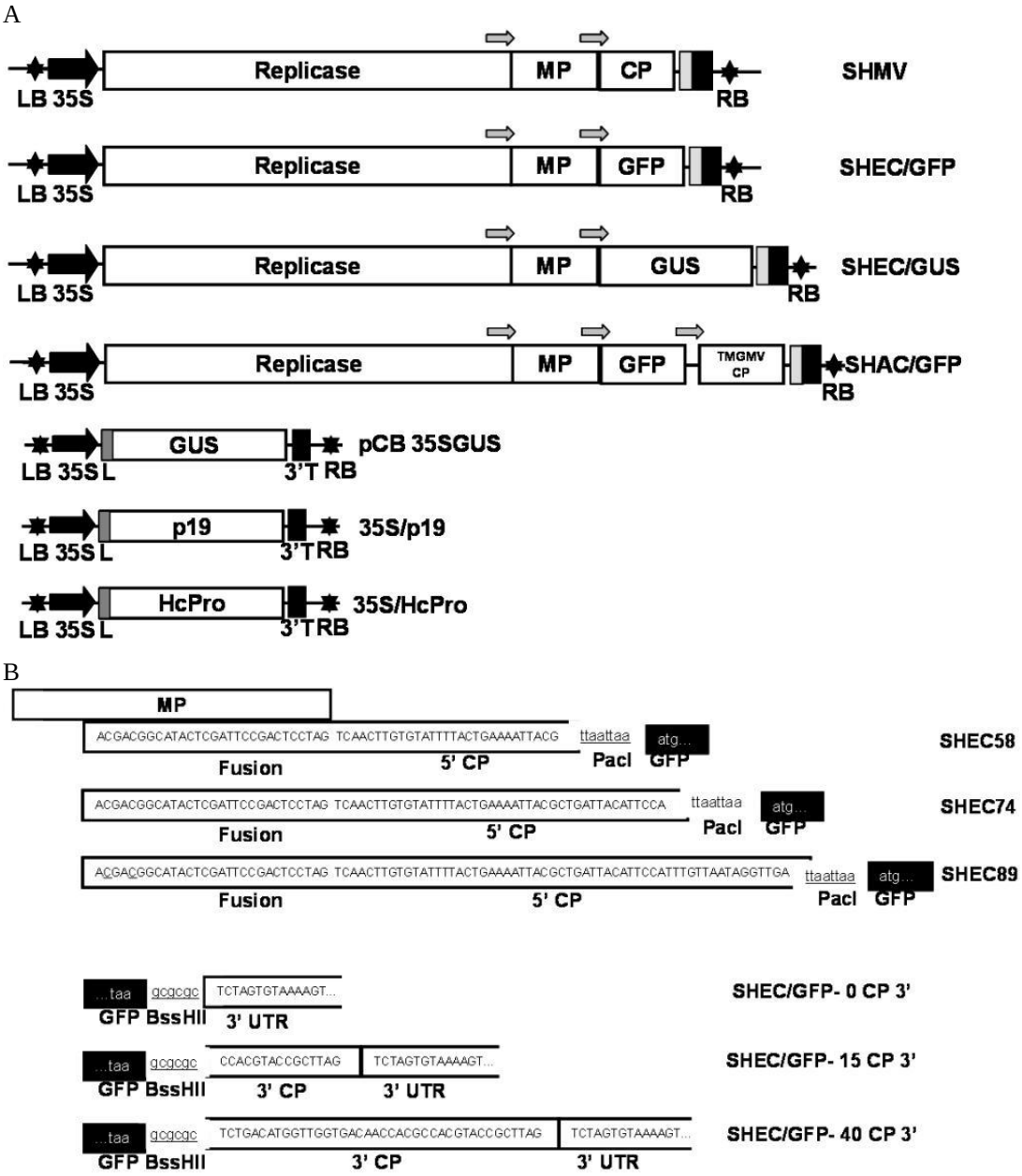


Figure 5. Schematic diagram of binary plasmid in SHMV study. Schematic diagram of (A) the T-DNA regions of binary plasmid used in this study and (B) the amount of 5' and 3' CP ORF sequence included in various constructs. (A). 35S promoter-driven versions of sunn hemp mosaic virus (SHMV) vector cDNAs and 35S driven GUS, p19 and HcPro were constructed. All plasmids were based on the binary vector pCB301 backbone. Open boxes represent open reading frames; black stars: left border and right border of T-DNA; block arrows: CaMV duplicated 35S promoter; light grey box: ribozyme; black boxes: CaMV 3' terminator sequence; gray arrows, subgenomic promoters; dark grey boxes: tobacco etch virus 5' non-translated leader sequence (L); RB: T-DNA right border sequences; LB: T-DNA left border sequences. SHMV transcripts are processed by a ribozyme to generate authentic TMV 3' ends. (B). All CP deletion mutants have the root "SHEC" (Sunn Hemp Elimination of CP). In the start coden of 5'CP, two ATGs were mutated to ACGs, shown as underlined. Restriction sites *Pac*I and *Bss*HII were introduced at the flank of GFP ORF as cloning sites for other foreign inserts. The numbering in each construct name indicates the number of nucleotides of the CP ORF retained to increase GFP expression. For example, SHEC58 remains the 5' end 58nts of SHMV CP to reserve the subgenomic promoter to drive the expression of GFP, and SHEC/GFP- 40 CP 3' contains the 3' end 40nts of SHMV CP.

pSHEC. Mutagenic PCR was performed to delete the CP ORF and introduce *Pac*I and *Bss*HII cloning sites for foreign genes. These sites were introduced using two primers, SHMV PacBss3UTR Up and JL22 RB Down, effectively. The PCR product (comprising the 3' UTR and the terminator, ribozyme and T-DNA right border) was cut with *Pac*I/*Pme*I and cloned into the generic cloning vector pNEB193. Three different lengths of subgenomic promoter were retained to drive foreign genes instead of the CP gene. Specifically, three downstream primers (SHMV Pac5838 Down, SHMV Pac5854 Down and SHMV Pac5869 Down) (Table 1) were designed to retain 58 nts, 74 nts and 89 nts of the 5' end of the CP ORF sequence, respectively. These were each paired with the upstream primer, SHMV 4658 Up, as above and the PCR products were each cloned into the *Kpn*I/*Pac*I site of pNEB293 already carrying the 3'UTR and downstream elements. Finally, the *Kpn*I/*Pme*I fragment was transferred from pNEB193 to pSHacgCP to create SHEC58, SHEC74 and SHEC89.

pSHEC/GFP and pSHEC/GUS. The Cycle 3 green fluorescent protein (GFP) gene was PCR amplified to add *Pac*I and *Bss*HII sites and cloned into the *Pac*I/*Bss*HII sites of pSHEC58, pSHEC74 and pSHEC89 to obtain pSHEC58/GFP, pSHEC74/GFP and pSHEC89/GFP. In the same way, GUS (the *E. coli uidA* gene) was subcloned into pSHEC74 to form pSHEC74/GUS construct.

pSHAC/GFP. pJL24 (Lindbo, 2007) is a TMV vector with the CP subgenomic promoter, CP ORF, and 3'UTR of the *Tobamovirus*, tobacco mild green mosaic virus (TMGMV) to prevent recombination with the TMV subgenomic promoter driving the foreign gene. The GFP, CP subgenomic promoter and CP ORF of pJL24 were PCR

Table 1. Primers used for plasmid construction in SHMV study.

Plasmid	Primer	Oligonucleotide sequence (5'–3')	Purpose
pSHacgCP	SHMV 4658 Up (pSHMV nt. 4597-4621)	CAGATATCCAATCGGTCTCCAACAA	To generate a mutation in the initiation codon of the CP cDNA and eliminate the double ATG's start codon within pSHMV. The mutation is in underline.
	dCP-SHMV Down (pSHMV nt. 5769-5801)	TCGGAATCGAGTATGCCGTCGTCAAATACAGAC	
	dCP-SHMV Up (pSHMV nt. 5769-5801)	GTCTGTATTTGACGACGGCATACTCGATTCCGA	
	JL22 RB Down (pSHMV nt. 6874-6901)	TCTAATAAACGCTCTTTTCTCTTAGGTT	
pSHEC58 pSHEC74 pSHEC89	SHMV Pac5838 Down (pSHMV nts.5851-5822)	TGGAATTTAATTAACGTAATTTTCAGTAAA	Three downstream primers (containing the rare-cutting PacI) were paired with the primer - SHMV 4658 Up - as above to save 58 nts, 74 nts and 89 nts 5' end of CP DNA sequence from mutated start codon of coat protein respectively. *Pac*I and *Bss*HII cloning sites were introduced by PCR amplified with two primers (SHMV PacBss3UTR Up and JL22 RB Down). Once each pair of PCRs is completed, they are cut with *Kpn*I/*Pac*I or *Pac*I/*Pme*I and cloned into pSHacgCP vector backbone cut with *Kpn*I and *Pme*I to create pSHEC58, pSHEC74 and pSHEC89.
	SHMV Pac5854 Down (pSHMV nts.5865-5836)	AACCTATTAATTAATGGAATGTAATCAGCG	
	SHMV Pac5869 Down (pSHMV nts.5880-5850)	CTGCGTTAATTAATCAACCTATTAACAAATG	
	SHMV PacBss3UTR Up (pSHMV nts 6275-6292)	TGCTCGTTAATTAAACTGCGCGCTCTAGTGTAAAAGTTTGGTC	
pSHEC58/GFP pSHEC74/GFP pSHEC89/GFP	PacGFPUp	TTGTCATTAATTAAGCTAGCAAAGGAGAAGAAC	To clone the GFP ORF into the pSHEC vector. Primer PacGFPUp adds a *Pac*I site (underline) at the 5' end, and primer GFPBssDown adds a *Bss*HII site (underline) to the 3' end.
	GFPBssDown	TTTACTCCTAGGTTATTTGTAGAGCTCATCCA	
pSHEC74/GUS	PacGUSUp	GGATGGTTAATTAAATGTTACGTCCTGTAGAAAC	To clone the GUS ORF into pSHEC vector. Primer PacGUSUp adds an *Pac*I site (underline) at the 5' end, and primer BssGUSDown adds a *Bss*HII (underline) site at the 3' end of the ORF.
	GUSBssDown	TTTACTGCGCGCTCATTGTTTGCCTCCCTGC	
pSHAC/GFP	PacGFPUp	TTGTCATTAATTAAGCTAGCAAAGGAGAAGAAC	GFP gene and coat protein (CP) gene of Tobacco Mild Green Mosaic Virus (TMGMV) were amplified together with JL24 as the template. The amplified DNA fragment was cloned into the *Pac*I/*Bss*HII sites of the pSHEC74 to generate pSHAC74/GFP constructs.
	TMGMVCPBssDown	TTTACTGCGCGCCTAAGTAGCCGGAGTTGTG	
pSHEC74/GFP 15CP3'	BssSHMVUp15 (pSHMV nts 6235-6255)	TTTACTGCGCGCCCACGTACCGCTTAGTCTAG	To add a 15bp or 40bp more to the 3' end of CP in pSHEC74 vector. The PCR product was generated using primer BssSHMVUp15 or BssSHMVUp40 with a *Bss*HII site (underline) at the 5' end and primer JL22 RB Down at downstream of *Pme*I site in T-DNA right border of pSHMV. The amplified DNA fragment was cloned into pSHEC74/GFP vector backbone cut with *Bss*HII and *Pme*I.
pSHEC74/GFP 40CP3'	BssSHMVUp40 (pSHMV nts 6235-6255)	TTTACTGCGCGCTCTGACATGGTTGGTGACAAC	
pCB/GUS	XbaGUSUp	GGATGGTCTAGAATGTTACGTCCTGTAGAAAC	To clone the GUS ORF into pCB302 vector. Primer XbaGUSUp adds a *Xba*I site (underline) at the 5' end, and primer SpeGUSDown adds a *Spe*I (underline) site at the 3' end of the ORF.
	SpeGUSDown	TTTACTACTAGTTCATTGTTTGCCTCCCTGC	

amplified and the PCR product was cloned into the *Pac*I/*Bss*HII sites of the pSHEC74 to generate pSHAC74/GFP construct.

35S/GUS. To clone the GUS ORF into the generic binary vector, pCB302 (Xiang *et al*., 1999), primer XbaGUSUp was used to add a *Xba*I site at the 5' end, and primer SpeGUSDown was used to add a *Spe*I site to the 3' end of the GUS ORF. The PCR product was cloned into the *Xba*I/*Spe*I sites of the pCB302 to generate pCB/GUS.

pSHEC74/GFP 15CP3' and pSHEC74/GFP 40CP3'. In these constructs, 15 or 40 bp of the 3' end of the ORF of CP were reintroduced in an attempt to improve translation, following the design of the TMV TRBO vector (Lindbo, 2007). A PCR product was generated using upstream primer BssSHMVUp15 or BssSHMVUp40 and downstream primer JL22 RB Down. The amplified DNA was cloned into pSHEC74/GFP cut with *Bss*HII and *Pme*I.

Agroinoculation of Plants

Binary constructs were transformed into *A. tumefaciens* EHA105 by electroporation at 1.44 kV and 129 Ω for 5 ms using a BTX 600 Electro Cell Manipulator (BTX Inc., San Diego, CA, USA). *A. tumefaciens* transformants were selected with 10 µg/ml rifampicin and 50 µg/ml kanamycin (Phytotechnology lab, Shawnee Mission, KS). Initially, three transformants per binary vector construct were tested for agroinoculation of plant leaves. The gene expression or virus activity was tested at 6 and 8 days post-inoculation and one of three transformants was used for further experimentation. Agroinoculation was carried out as described (Kapila *et al*., 1997) with modifications. A two-day colony of *A. tumefaciens* was transferred to 5 ml LB media supplemented with

10 mM MES (Fisher Scientific, Fair Lawn, NJ), 20 uM acetosyringone (Phytotechnology lab, Shawnee Mission, KS), 10 ug/ml rifampicin and 50 ug/mL kanamycin, and grown overnight at 24°C. The cells of the overnight culture were collected by centrifugation, resuspended in induction media (10 mM MES, 10 mM $MgCl_2$, 150 uM acetosyringone) for a final OD_{600} of 1.0 and incubated for 2 h to overnight at room temperature. The cultures of *A. tumefaciens* were infiltrated with a 3 ml syringe without needle at the abaxial leaf surface.

RT-PCR

Total RNA was extracted from leaves after seven days post-inoculation using Tri-reagent (Sigma, St. Louis, MO) according to the manufacter's protocol. RT-PCR reactions were performed using an RT-PCR kit (NEB, Beverly, MA), as described by the supplier, using SHMV-specific primers.

Electron Microscopy

Virus samples were prepared for transmission electron microscopy using a leaf dip method. Hexagonal 300 mesh copper grids (Electron Microscopy Sciences, Hatfield, PA) were coated with a film made from a 1% (w/v) aqueous formvar solution. A drop of a saturated aqueous uranyl acetate stain was placed on the coated grid. The cut edge of the leaf was pulled through the drop of stain several times to release virus particles. After 1 minute, the stain was removed with filter paper and the grid was allowed to dry. Samples were viewed using a JEOL JEM 1010 Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan) operated at 60kV.

Protein Extraction and SDS-PAGE

Inoculated *N. benthamiana* leaf tissue (1 g) was ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder was resuspended by vigorous mixing in 2 ml of protein extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 0.1% β-mercaptoethanol). Extracts were centrifuged for 15 min at 13,000×g at 4°C. Clarified supernatant was stored at 4°C. Clarified extracts of protein samples were mixed with 3× SDS-PAGE sample buffer (NEB, Beverly, MA) and PAGE analyzed on a 5% stacking gel and 15% separation gel. Gels were stained with Coomassie brilliant blue R-250 (Sigma, St Louis, Mo) to visualize proteins.

Detection of GFP Fluorescence

Plants were examined under long-wave UV light using a hand-held UV device (UVL-56, UV Products, Upland, CA) and photographs were taken with Canon digital EOS Rebel camera equipped with a Hoya yellow (K2) filter (Hoya Corporation, Japan) . For GFP-positive plants, samples from infiltrated tissues were mounted with water on a glass slide. Images were obtained with a Nikon TE2000-U inverted microscope, captured using a CoolSnap *cf* camera (Roper Scientific, Tucson, AZ) and analyzed with Metavue imaging software (version 5, Molecular Devices Co, Downingtown, PA).

Histochemical GUS Assay

X-Gluc substrate solution was made with 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Gold Biotechnology, St. Louis, MO), 100 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferricyanide (Acros Organics, Morris Plains, NJ) and 0.5 mM potassium ferrocyanide (Acros Organics, Morris Plains, NJ).

Seven dpi leaves were placed in X-Gluc solution and were subjected to a 700 mm Hg (93 kPa) vacuum for 5 min to improve the penetration of the substrate. The samples were incubated in the dark at 37°C from 1 h to overnight until the staining was satisfactory. After staining, the leaves were cleared with 70% ethanol to improve contrast by removing photosynthetic pigments. GUS-stained leaves were viewed under a light microscope. Micro and macrophotography was performed as above but without UV light.

Spectrophotometric GUS-assay

Transient GUS activity was determined using the spectrophotometric GUS assay (Jefferson *et al*., 1987). Inoculated leaves were harvested and ground as frozen tissue in liquid nitrogen. Freshly prepared 1 ml of GUS lysis buffer (50 mM $NaPO_4$ pH 7.0, 10 mM β-mercaptoethanol, 0.1% Triron X-100) was added to ground tissue powder. Crude extracted homogenate was centrifuged in a microcentrifuge at 12,000 x g for 5 min and the extract supernatant was collected. Lysis buffer (100 ul of 10 mM p-nitrophenyl beta-D-glucuronide (PNPG, Sigma)) was added and the reaction was allowed to proceed at 37^oC for 15 h. The reaction was terminated by the addition of 0.4 ml 2.5 M 2-amino-2-methyl propanediol (Sigma), followed by measurement of the absorbance at 415 nm.

Results

Full Length Infectious SHMV cDNA Clone

Full-length SHMV cDNA under transcriptional control of the 35S promoter was inserted into the T-DNA of binary vector JL22 to construct pSHMV (Fig 5). *Agrobacterium* stain EHA105 containing pSHMV was agroinoculated in *Nicotiana benthamiana*, *Vigna unguiculata* (California Blackeye, cowpea), *Phaseolus vulgaris*

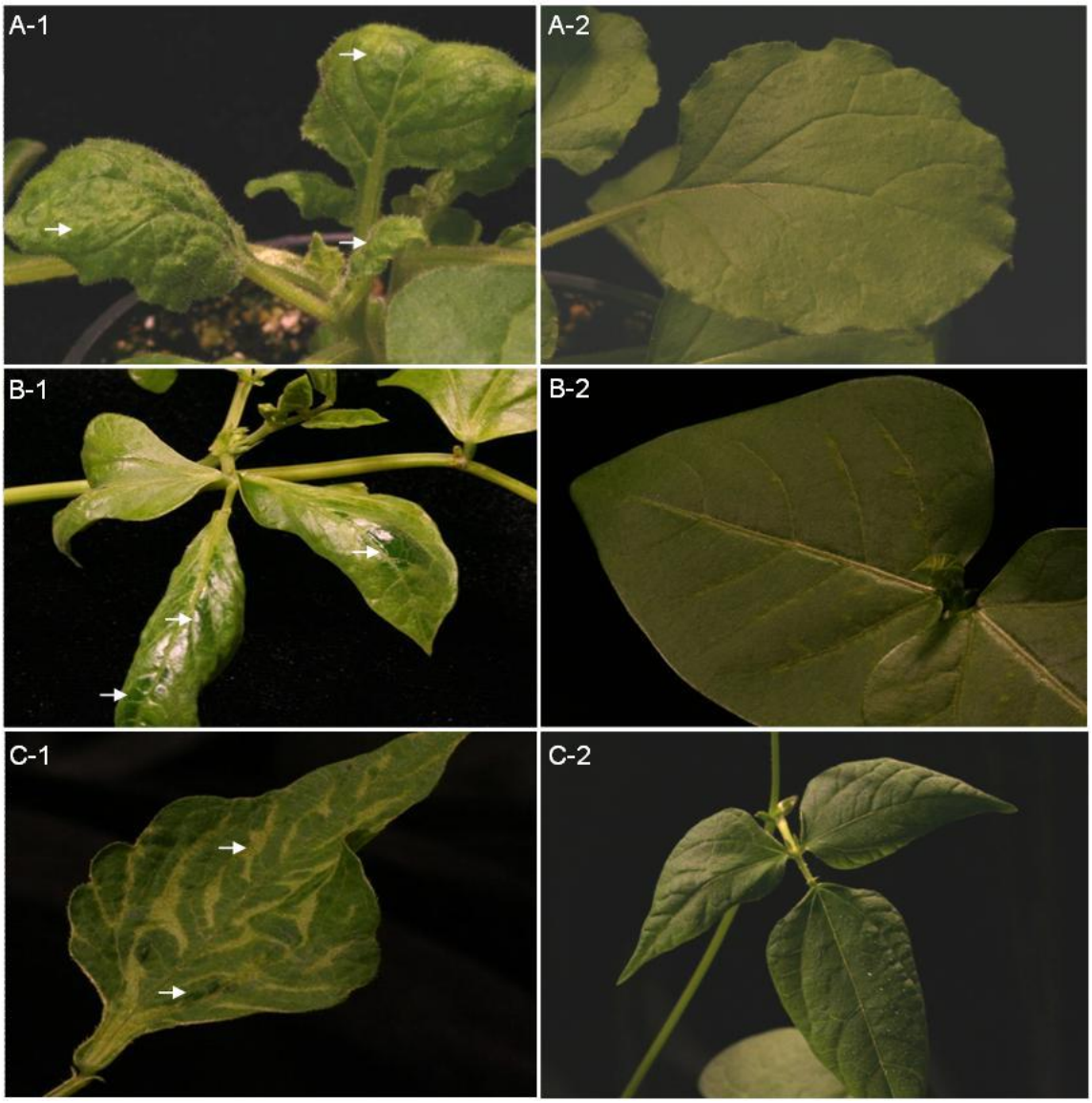


Figure 6. Infection with wild type SHMV via agroinoculation. *Nicotiana benthamiana* (A-1); Cowpea (B-1), Pintobean (C-1) leaves showed systemic mosaic, mottling symptoms (indicated with white arrows) after infection with wild type SHMV via agroinfection. Leaves were agroinfiltrated with *A. tumefaciens* (*A.t.).* Cells (O.D.600 1.0) containing the binary plasmid pSHMV. pSHMV has the full length SHMV cDNA driven by a 35S promoter as the T-DNA in the binary plasmid. When SHMV RNA is transcribed from the T-DNA, the SHMV initiates self-replication. The SHMV can move cell-to-cell and systemically in plants. *Nicotiana benthamiana* (A-2); Cowpea (B-2), Pintobean (C-2) leaves demonstrated the healthy plants control without agroinfection of *A. tumefaciens* (*A.t.)*/pSHMV.

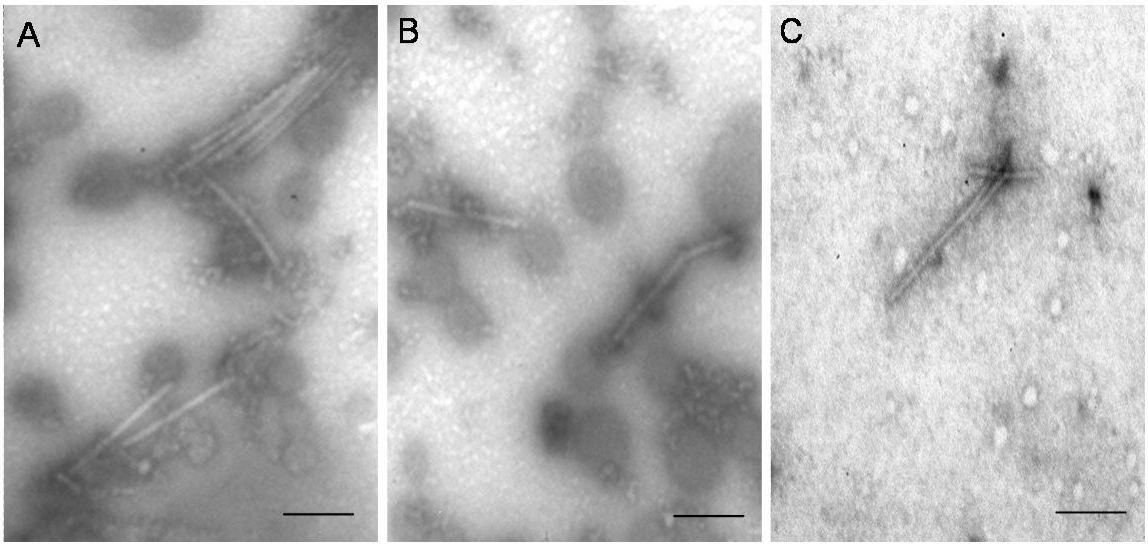


Figure 7. Electron Microscopy of SHMV virion. *Nicotiana benthamiana* (A.and B.) and Pinto bean (C.) leaves were agroinfected by *A.t.*/SHMV and electron micrographed at 30 days post-inoculation. Rod shape viral particles were visualized by negative staining. Scale bar corresponds to 100nm. (EM courtesy of Dr. Ann Rushing, Baylor University.)

(Pinto bean) to look for symptoms and evidence of viral replication. Signs of SHMV infection were apparent 10 days post-inoculation not only in locally inoculated leaves but in systemic leaves as well. Symptoms of viral infection were visible as systemic mosaic, mottling and vein yellowing in *Vigna unguiculata* and *Phaseolus vulgaris* and necrotic or chlorotic local lesions for *Nicotiana benthamiana* (Fig 6). In local infections of chlorotic lesions, SHMV RNA was detected (by RT-PCR) 10 days post-inoculation. RNA was extracted from systemic leaves and RT-PCR was carried out to look for evidence of systemic SHMV RNA derived from pSHMV. Systemic movement of viral RNA derived from pSHMV was demonstrated in inoculated plant. These symptoms and SHMV RNA were not seen in control uninoculated plants. As confirmation of the systemic spread of SHMV, transmission electron microscopy of systemically infected leaves revealed the presence of rigid rods typical of SHMV virions (Fig 7). These analyses suggested that

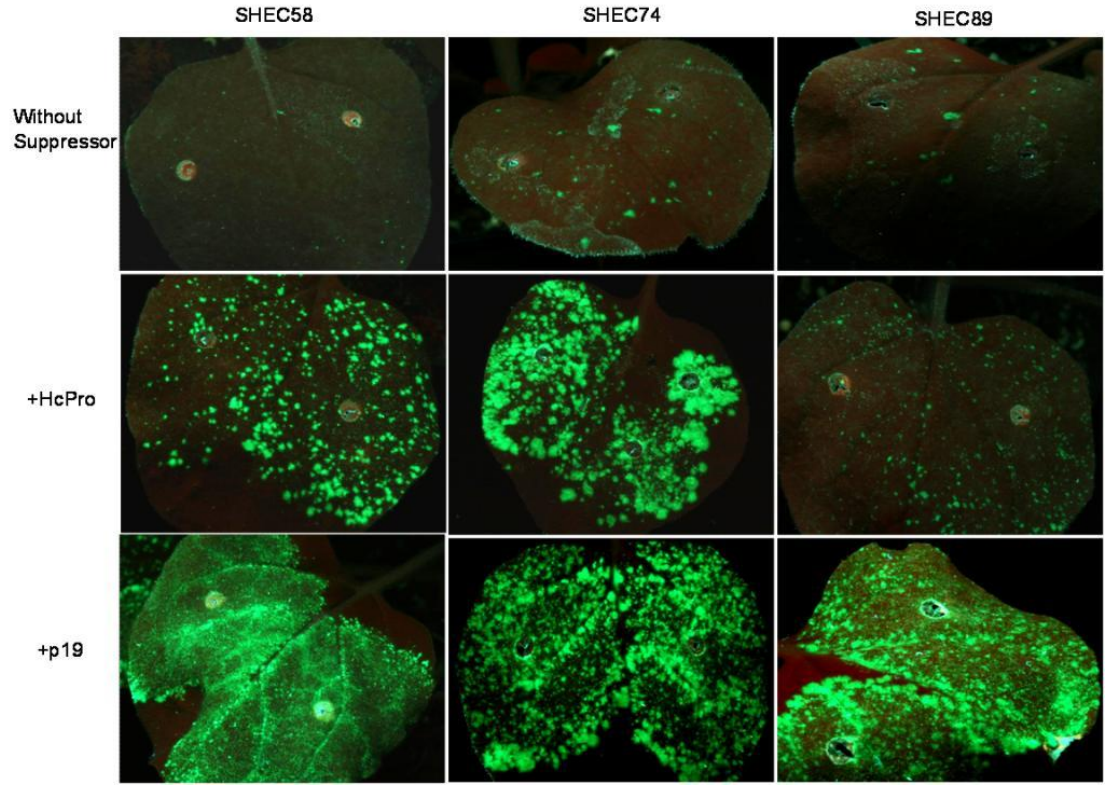
agroinfection with SHMV clones resulted in replication, movement, and production of virions.

Local Infection of pSHEC Expression Vector

Construction of pSHEC. A CP-deletion vector was constructed to allow the environmentaly safe expression of protein via agroinoculation or in transgenic plants. The coat protein (CP) subgenomic promoter would be used to drive foreign gene expression and we expected it to comprise sequence both upstream and downstream of the start codon of CP ORF. Since there was an additional AUG next to the start codon, we mutated both of them to ACGs (SHacgCP construct) so that the start codon of the foreign gene would be used. Next, we created constructs with varying lengths of putative subgenomic promoter, including 58, 74 and 89 nts of CP ORF in constructs SHEC58, SHEC74 and SHEC89, respectively. Thus, these vectors differed from pSHMV only in the mutated AUGs, a *Pac*I/*Bss*HII cloning site, and the elimination of the CP ORF except for the upstream bases noted. GPF was inserted into the cloning site of each construct to create the SHEC/GFP series.

To test the expression of the SHEC/GFP vectors, cultures of the EHA105 strain of *A. tumefaciens* with SHEC/GFP were prepared and *N. bethamiana* plants were agroinoculated. Four days post-inoculation (dpi), GFP-expressing cells could be seen using a hand-held UV lamp and fluorescence microscopy. There are many green spots shown on inoculated leaves and no significant differences of GFP expression between these SHEC/GFPs (Fig 8).

I.



II.

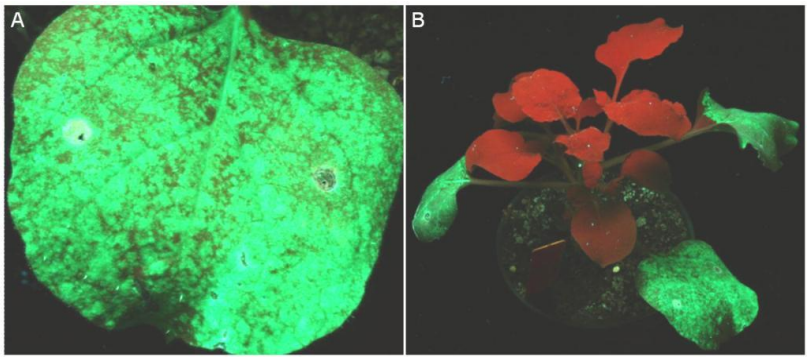


Figure 8. Effect of gene silencing suppressor on the agroinfection efficiency of SHEC vector. Agroinfection induced local expression of GFP by SHEC vectors (ΔCP) on *Nicotiana benthamiana.* The SHEC can move cell-to-cell, but not systemically in plants. Fig 8-I. Three SHEC vector variants (SHEC58, SHEC74, and SHEC89) were examined on *N. benthamiana* by agroinfection with three co-inoculation treatments: *A. tumefaciens* (*A.t.*, O.D.600 1.0) mixture of *A.t.*/SHEC:GFP and *A.t*/35S:p19; mixture of *A.t.*/SHEC:GFP and *A.t.*/35S:HcPro; *A.t.*/SHEC:GFP alone without silencing suppressor. Photograph at 6 days post-infiltration (dpi). Fig 8-II. After 8 days post infiltration, the green fluorescent protein was produced at the highest yield on *N. benthamiana* inoculated with mixture of *A.t.*/SHEC74:GFP and *A.t.*/35S:p19.

Co-inoculation with Gene Silencing Suppressors P19 and HcPro. Co-inoculation of RNA-silencing suppressor proteins has enhanced the expression of heterologous proteins from the vectors (Komarova *et al*., 2006; Lindbo 2007; Lindbo 2008). Tomato bushy stunt virus silencing suppressor p19 increased 100 times the expression of GFP in TMV vectors (Lindbo 2007). HC-Pro, from *Tobacco etch virus*, serves a similar function in suppressing gene silencing. To test two gene silencing suppressors, *N. benthamiana* plants were agroinoculated with a 1:1 mixture of 35S/p19 or 35S/HcPro and pSHEC/GFP cultures. The accumulation of GFP was followed and imaged by hand-held UV light and fluorescence microscopy at 3-7 days post-inoculation. When plants were infiltrated with

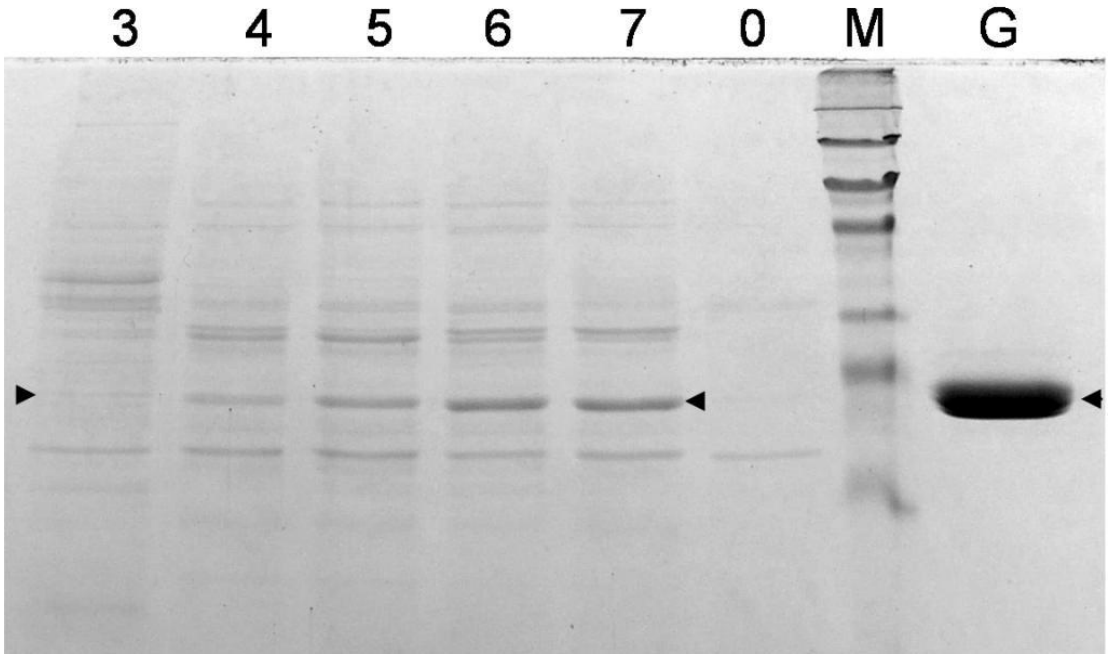


Figure 9. Time course analysis of GFP expression from SHEC74. *N. benthamiana* leaves were agroinfiltrated with the mixture of *A.t.*/SHEC74:GFP and *A.t.*/35S:p19 cell suspension. Total soluble proteins were extracted from infiltrated leaf tissue from 3–7 days post infiltration. Protein extract samples were analyzed on a 15% SDS PAGE gel and subsequently stained by Coomassie brilliant blue. Lanes: M, MW marker; 3–7, extracts from *A.t.*/SHEC74:GFP infiltrated leaves, 3–7 days post-inoculation, respectively; 0, Non-inoculated *N.benthamiana* leaf extract; G, purified GFP. Marker band sizes (in kDa) are listed. Arrow: 27 kDa GFP.

virus and either of these two gene silencing suppressors, the amount of GFP expression was dramatically increased (Fig 8-I). At 7 dpi, the great majority of the cells fluoresced (Fig 8-II).

A. tumefaciens/pSHEC78/GFP + *A. tumefaciens*/35S:p19 co-infiltrated leaves from 3–7 dpi were extracted and the relative GFP expressions were examined by SDS-PAGE electrophoresis and coomassie blue staining. Recombinant GFP expression in infiltrated leaves increased up to the maximal GFP expression level of up to 25% total soluble protein (TSP) at 7 dpi (Fig 9).

3'-terminal sequences of coat protein gene as the downstream UTR. The 3'-terminal 22 nt of the TMV CP ORF as a UTR downstream of the GFP ORF have a positive effect on GFP accumulation (Man and Epel, 2004; Lindo 2007). However, with the last 3'-terminal 50 nt of the CP ORF as a UTR downstream of the GFP ORF, GFP accumulation decreased by 70%. To further elucidate possible up-regulatory roles of specific sequences from the 3' end of CP ORF, constructs were made adding either 15nt or 40 nt sequences derived from the 3' termini of the CP ORF of SHEC74/GFP, creating 5' GFP-15 3' or 5' GFP-40 3' constructs respectively. However, these 3' ORF additions did not significantly increase the GFP accumulation level in *N. benthamiana* compared with SHEC74, which lacked any 3' CP ORF sequence (data not shown).

Cell-to-cell movement of SHEC expression vector. Because the coat protein of tobamoviruses is necessary for systemic movement (Donson *et al*., 1991), pSHEC expression vector did not move systemically in plants without the help of the coat protein. When the CP genes of TMV, PVX, Brome mosaic virus (BMV), and Cowpea mosaic

virus (CPMV) were replaced with the GFP encoding sequence, the chimeric viruses were restricted to local GFP expression (Lindbo 2008; Komarova *et al*., 2006; Schmitz and Rao 1996, Verver *et al*., 1998). Thus CP was essential for cell-to-cell movement of these viruses. With the SHEC vectors, lacking a CP ORF, we also found no green spots in systemic leaves, only in the inoculated leaves (Fig 8). Fluorescence was seen to move into the petioles and stems over several weeks, presumably by cell-to-cell movement through the parenchyma rather than through the phloem since no vein banding patterns typically seen with systemic infections were ever seen in SHEC/GFP infected plants.

In the process of agroinfection, the binary plasmid containing viral vector cDNA is replicated in *A. tumefaciens* and the T-DNA in the binary plasmid is transferred into the nucleus of plant cells during *Agrobacterium* infection. The transcription of viral cDNA was driven by plant promoter 35S in plant nucleus, resulting viral genome is exported into cytoplasm. The transcripts of viral RNA initiate viral replication and gene expression in the cytoplasm. The RNA dependent RNA polymerase (RdRp) – replicase is synthesized in the procession of SHEC/GFP viral expression. Replicase leads to the replication of viral vector genome and the synthesis of positive and negative strand viral RNAs. Subsequently, two subgenomic RNAs containing the gene of GFP and MP are synthesized, and GFP and MP are expressed. Movement protein of SHMV provides cell-to-cell spread of SHEC viral vector. Individual GFP-expressing foci can be observed on a leaf, and they can be enlarged into green spots (Fig 8). Because the SHEC/GFP replicon cannot express the SHMV CP, it cannot move long distance systemically. The SHEC/GFP replicon was never observed to move systemically in agroinoculated plants in our experiments.

Co-inoculation with pSHMV. To test if wild-type SHMV CP could be used to rescue the SHEC infectivity, SHEC was agroinoculated along with wild type SHMV. Theoretically, SHEC should be able to replicate its genome and should be able to use wild-type CP to package its genome and form viral particles. GFP were produced from this mixed inoculation successfully. However, systemic leaves failed to show the presence of GFP (data not shown).

SHEC expression in legumes. The viral vector SHEC infected *N. benthamiana* and legumes locally. When *N. benthamiana*, cowpea and pinto bean were co-inoculated

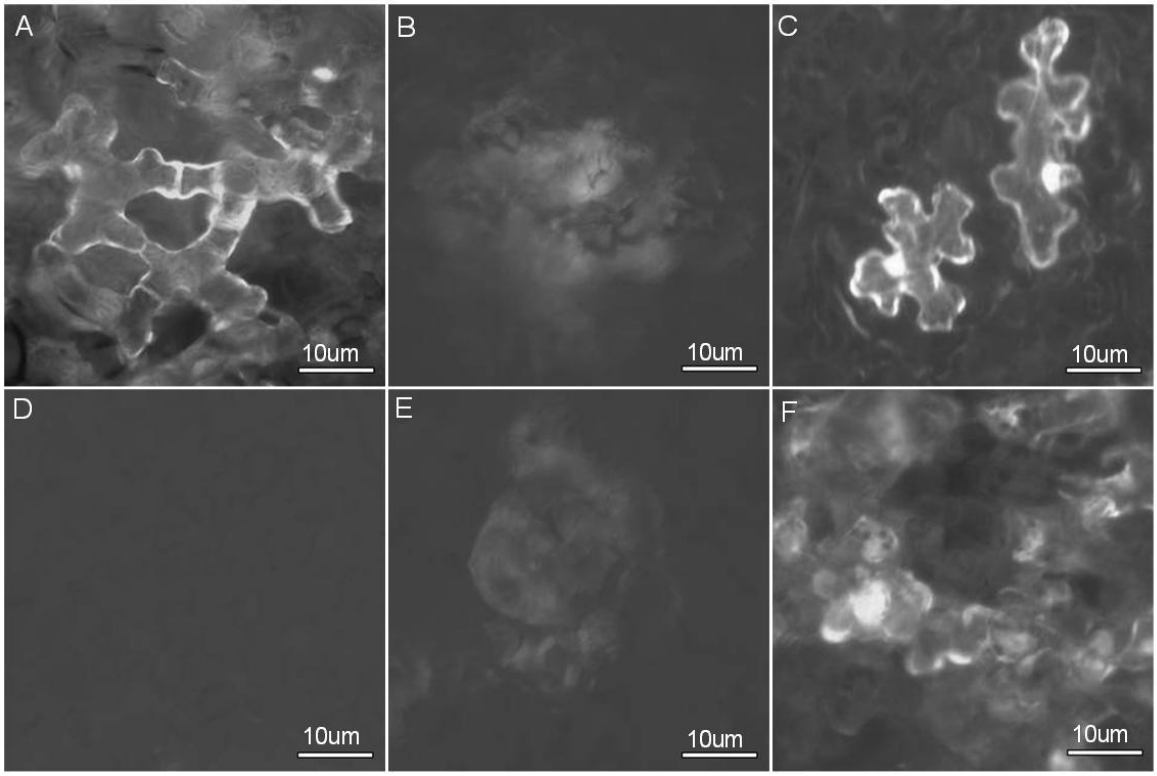


Figure 10. Fluorescence microscopy of plants infected with SHEC/SHAC:GFP. SHEC:GFP or SHAC:GFP transient expression on plants after 10 days post-inoculation as visualized by fluorescence microscopy. A. SHEC74:GFP expressed in *N. benthamiana*; B: SHEC74:GFP in cowpea; C: SHEC74:GFP in pinto bean; D. uninoculated cowpea; E. SHAC74:GFP in cowpea; F. SHAC:GFP in pinto bean.

with the mixture culture of *Agrobacterium* containing SHEC74:GFP and 35S:p19, green fluorescence was examined in the inoculated leaves of plant by fluorescent microscopy. Green fluorescent cells were bright and could be seen easily through the microscope in inoculated *N. benthamiana* and legumes (Fig 10). The uninoculated controls were uniformly dark. In legumes the green fluerescence was not as bright as *N. benthamiana*, but very different from control (Fig 10).

SHEC containing the β-glucuronidase (GUS) gene, in co-agroinoculation with 35:p19, yielded legume infections that showed strong GUS expression macroscopically for pinto bean and cowpea as well as for *N. benthamiana* (Fig 11) and somewhat less so for pea and lentil (data not shown). When pinto bean, cowpea and *N. benthamiana* infections were examined microscopically, all showed large numbers of dark blue cells (data not shown). To verify the absence of endogenous GUS activity and the necessary of p19 for the expression, *A.tumefaciens*/pSHEC78/GFP was used as the negative control.

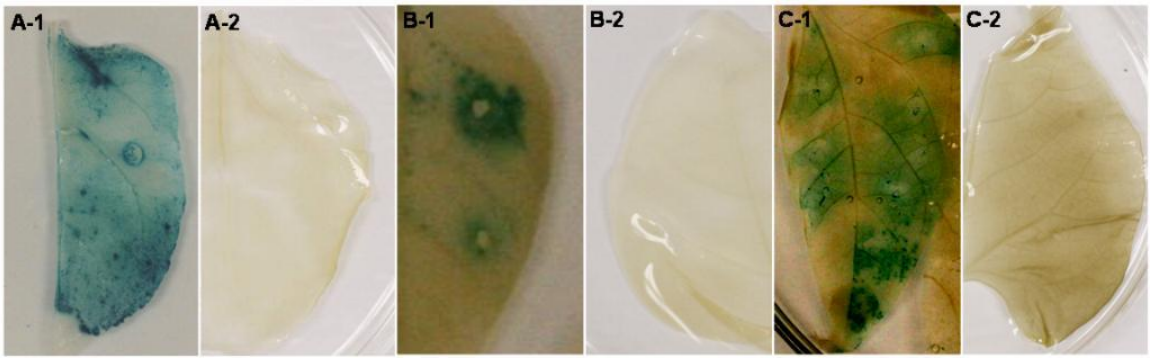


Figure 11. Histochemical GUS assay for plant leaves agroinfected with SHEC:GUS. Histochemical GUS assay for *Nicotiana benthamiana* (A-1); cowpea (B-1), pintobean (C-1) leaves agroinfected with SHEC:GUS. Leaves were agroinfiltrated with *A. tumefaciens* (*A.t.).* Cells (O.D.600 1.0) mixture of *A.t.*/SHEC74:GUS and *A.t*/35S:p19. *Nicotiana benthamiana* (A-2); cowpea (B-2), pintobean (C-2) leaves demonstrated the staining control of uninoculated plant leaves.

To compare SHMV expression vector with other transient expression systems, *A. tumefaciens* cultures with plasmids pCB/GUS or 35S/p19 were mixed and co-infiltrated into *N. benthamiana* and pintobean leaves. The activity levels of GUS in extracts were determined by a spectrophotometric GUS assay. SHEC/GUS expression was five times that of the 35S:GUS in the pCB/GUS construct co-infiltrated with the 35S::p19 gene in both *N. benthamiana* and legumes (Fig 12).

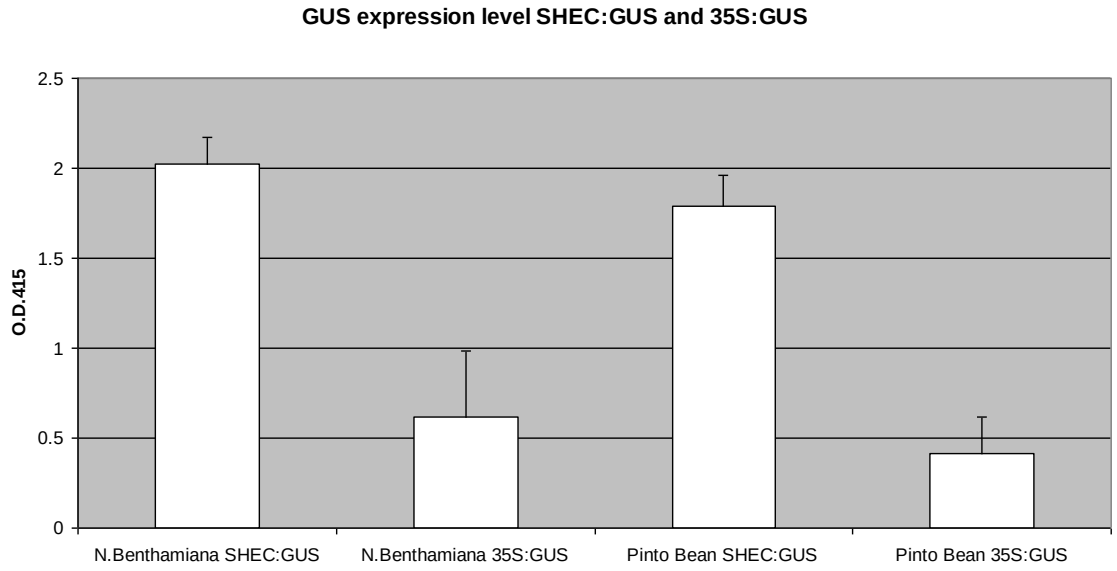


Figure 12. β-Glucuronidase expression level of SHEC:GUS and 35S:GUS. β-Glucuronidase expression of SHEC74:GUS and 35S:GUS when in the presence of post-transcriptional gene silencing suppressor p19 in *N. benthamiana* and pinto bean.

Systemic Infection of Rescued SHMV Expression Vector: pSHAC/GFP

To allow systemic movement, the duplicated subgenomic promoter for CP was used for the heterologous sequences inserted between the MP and CP genes of TMV (Dawson *et al*., 1989). However, recombination between the duplicated homologous CP promoters caused the deletion of insert when vector viruses inoculated on plants. To avoid this problem, the subgenomic promoter of another virus was used to drive the

foreign protein gene sequence in TMV-based p30B vector (Donson *et al*., 1991). To rescue SHEC vector for systemic movement, the pSHAC78/GFP was constructed. The CP gene of the TMV and SHMV relative, TMGMV, together with its subgenomic promoter, was inserted upstream of the 3' untranslated region (UTR) of pSHEC74/GFP to compensate the function of SHMV CP. The additional sequences of 5' end of SHMV CP ORF were reserved as the subgenomic promoter to drive GFP expression as in SHEC74. Using a different tobamoviral CP subgenomic promoter was expected to prevent homologous recombination between duplicated homologous subgenomic promoters which would lead to deletion of the foreign gene (Dawson *et al*., 1989; Donson *et al*., 1991). *A.tumefaciens*/pSHAC78/GFP was agroinfiltrated into *N. benthamiana* leaves. Plants were observed under UV illumination to visualize GFP expression from SHAC vector. Green fluorescent spots were observed in locally inoculated leaves at three dpi, and moved systemically to uninoculated leaves at about seven days post-inoculation. To monitor the stability of foreign gene expression in recombinant viral vector, SHAC74/GFP was serially passaged in *N. benthamiana* every 2-3 weeks using sap inoculation. This chimeric SHMV had no disease symptoms at all during the infection of plants (Fig 13-A) and the GFP expression was stable for twelve weeks over four serial passages using sap inoculation (Fig 13-B).

The viral vector SHAC systemically infected *N. benthamiana* efficiently. However, SHAC/GFP did not move and express GFP systemically in legumes. When pinto bean was co-inoculated with the mixture culture of *Agrobacterium* containing SHAC/GFP and 35S:p19, green fluorescence was observed only in the inoculated leaves of the plant by fluorescent microscopy (Fig 10). GFP expression was not able to be

found in upper leaves of legumes, and the genomic RNA of SHMV was not detected in systemic leaves by RT-PCR (data not shown).

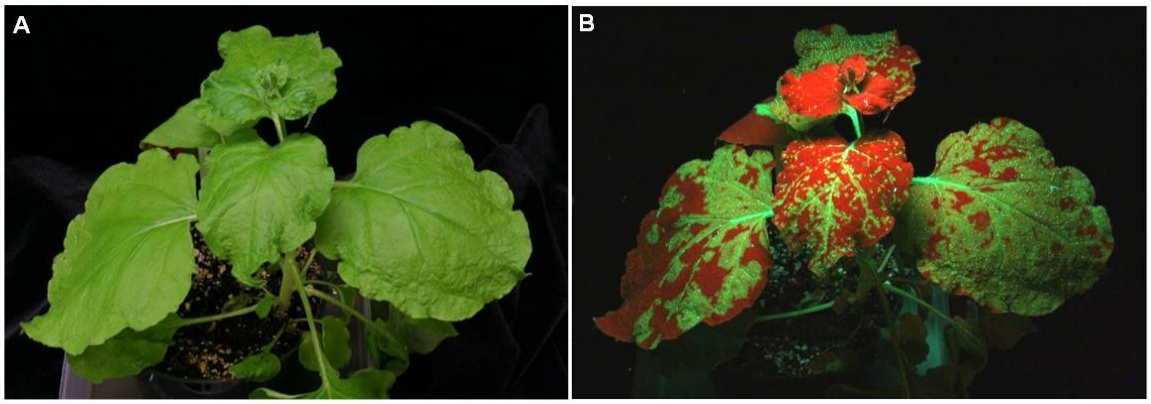


Figure 13. Agroinfection induced systemical expression of SHAC vector without viral symptoms. *Nicotiana benthamiana* leaves were agroinfiltrated with *A. tumefaciens* (*A.t.*, O.D.600 1.0) containing the binary plasmid *A.t.*/SHAC74:GFP. The SHAC can move cell-to-cell and systemically in plants. A. *N. benthamiana* plant inoculated with SHAC and photographed under visible light; B. the same plant photographed under ultraviolet light. The plant was photographed at the second passage.

Discussion

Most of the plant viral vectors for expression of heterologous proteins in plants are based on RNA viruses. Constructing the infectious cDNA clone of the RNA viruses was the first step for the invention of viral vectors. Sunn hemp mosaic virus (SHMV) is a tobamovirus which infects legume plants and causes disease. Isolates of SHMV have been sequenced, and the full-length infectious clone based on agroinfection was successfully developed. Infectious SHMV cDNA caused viral infection symptoms on both legumes and *N. benthamiana.* Development of full-length infectious clones builds the foundation to progress towards new viral vectors for the heterologous proteins expression.

Realizing that the production of an excellent full length vector will be a long term process, as it was for TMV, we focused on creating a deleted vector suitable for agroinoculation and for use in stable transgenics. For these purposes, the coat protein gene is not necessary, freeing up a subgenomic promoter for expression of a foreign gene and simplifying the vector development process. Since the coat protein (CP) is the most abundant protein produced in virus's infection, the CP-replacement viral vectors should produced similar high amount of heterologous proteins. The local infectious viral vector SHEC constructed in this study involved the replacement of the coat protein of SHMV with a green fluorescent protein (GFP) or a β-glucuronidase (GUS). This vector was constructed to test whether the CP deletion vector could be used to express a foreign protein downstream of the CP subgenomic promoter.

The effects of additional bases from the 5' and 3' ends of the CP ORF on expression of GFP were examined to improve the viral vector. A subgenomic promoter comprising 58 bases of CP 5' ORF was as effective as longer promoter constructs in the expression of GFP. This is in accordance with previous studies on TMV. The minimal TMV CP subgenomic promoter was mapped between -69 and +12, whereas the boundaries of the fully active promoter were between -157 and +54 (Grdzelishvili *et al.*, 2000). Also, we found that the addition of 15 or 40 bases from the 3' end of the CP ORF did not improve GFP expression, in contrast to the report for the TMV-based TRBO vector (Lindbo, 2007). These were the only two parameters that we sought to optimize with the SHEC vector in this work.

Since the leaves of some legumes can be used as edible food for human and animals, the SHEC expression system proposed here can be used for transgenic edible

vaccines and for production of functional foods/feeds. The technology of developing effective vaccine using legume systems has many good points for producing oral vaccine from plants. Transgenic legume work can take advantages of simplified expression screening by SHEC construct.

1. Before making transgenic legumes, expression can be tested and screened via agroinoculation with SHEC systems on legumes. SHEC has been shown herein to strongly infect a variety of legumes in the presence of silencing suppressor and induce much greater expression of GUS than a comparable 35S-driven nonviral vector. Transient expression work can be undertaken in *N. benthamiana* and then easily transferred to legumes using the same *Agrobacterium* preparation. With transient expression screening on broad host range of legumes, the best candidate of target gene and the most suitable plant host will be identified to enter further time-consuming transgenic work.

2. SHEC systems have potentially high expression. The percentage of GFP-positive cells per leaf varied among the leaves. This variation might reflect differences in the transcriptional efficiency of viral RNA and in the post-transcriptional processing of viral RNA in virus infection. The addition of extra introns in a modified tobacco mosaic virus significantly improved the infection efficiency of the virus transcribed from the cDNA (Marillonnet *et al*., 2005). Such modification should improve the efficiency of virus infection and the expression of foreign protein in SHEC and SHAC viral vectors.

3. SHEC replicons without CP can be employed as a tool for expression heterologous proteins in legumes to ensure the safety of the environment. One of the alternative strategies of producing a plant-based vaccine is to infect the plants with full

size viruses carrying the desired antigen gene. However, full length viral genome will produce infectious virion and involve the bio-safety issue. SHEC replicons without CP can not move systemically and enable to limit its movement in individual plants without leaking into environment.

4. SHEC can be controlled via on/off switch dependent upon the presence of a gene silencing suppressor, which could be put under the control of an inducible promoter along with SHEC. In this way, gene silencing suppressor (such as p19) may be useful to prevent leakiness in future inducible transgenic plants. Co-expression viral vector and VIGS-suppressor protein can be co-agroinfected into plant leaves in transient expression study and the production of target protein can be dramatically increased (Linbo, 2007). Inducible promoter could be used to control the viral vector replication and the yield of recombinant protein, and gene silencing suppressor might be used to further prevent leakiness in inducible transgenic plants.

Systemic infection of the rescued SHMV expression vector, SHAC, produced no disease symptoms at all in *N. benthamiana* plants and GFP expression can stably maintained over several passages. SHAC produces mild symptoms, so it could show promise as a VIGS vector in *N. benthamiana*. SHAC vectors, carrying the host gene to be silenced, could be used for host gene characterization by investigating the virus induced gene silencing (VIGS) study. Unfortunately, SHAC cannot systemically infect on legumes. Many variations of SHAC could be made, such as using other tobamovirus CP genes, or simply replacing the subgenomic region without replacing the CP ORF, that would hopefully restore infectivity in legumes.

Tobamovirus vectors in tobacco provide the highest yield for commercial proteins produced in plants. Our study demonstrated that SHEC and SHAC vectors are able to replicate in plant cells, and they provides relatively high levels of target protein expression by agro-infection in the plant leaf of *N.benthamiana*. Also, we have produced the first tobamovirus vector for legumes. This vector can facilitate the screening and expression of proteins for fast and efficient pharmaceuticals production. It is also a good research system for molecular biology and molecular virology studies.

CHAPTER THREE

Highly Efficient Suppressor-dependent Protein Expression in Plants with A Foxtail Mosaic Virus Vector

Abstract

A new viral vector based on foxtail mosaic potexvirus (FoMV) was constructed by eliminating the triple gene block and coat protein genes, reducing the viral genome by 29%. The resulting FECT vector (Fomv Elimination of Coat protein and Triple gene block) is driven by a CaMV 35S promoter in a binary vector and was delivered via syringe agroinoculation of *Agrobacterium tumefaciens* to whole plants of *Nicotiana benthamiana*. Interestingly, agroinoculation of the vector alone results in only slight transient expression, whereas co-inoculation with silencing suppressor genes (carried in a separate agrobacterial strain) allows for highly efficient GFP expression of up to 40% TSP. Thus, the FECT vector provides high capacity expression coupled with a tight on-off switch which could be utilized in permanently transgenic plants. It is also a good model system for molecular biology and molecular virology studies.

FECT transient gene expression system are especially useful to rapidly confirm that the foreign molecule of interest is correctly assembled and retains its biological activity before generating stably transformed transgenic plants. Full-sized HC and LC components of an anti-langerin IgG, each carried by a separate FECT vector, were able to produce immunologically functional antibody upon co-inoculation. This vector also addresses many environmental safety concerns: i) its genome is reduced by almost one-third, ii) it does not replicate efficiently unless the plant immune system is suppressed, iii)

it lacks a coat protein and cannot form a virion, and iv) it is derived from a virus that in most hosts causes only mild infections (no symptoms observed in *N. benthamiana*).

Introduction

Plants expression systems have been developed as a production platform for therapeutic proteins in the past two decades. Plants have advantages over other expression systems, such as mammalian cell culture and bacterial fermentation. The application of plant systems means a lower cost of production and large-volume production. Like mammalian systems, plant expression systems have the advantage of being able to produce active forms of complex proteins with post-translational modifications, such as glycosylation, which are necessary for human therapeutic proteins for correct function in vivo (Gomord *et al*., 2004). Plant systems are also free of human pathogens potentially associated with mammalian cell cultures. Although much work has been done with transgenic plants, plant viral vectors have emerged as the most efficient approach to achieving high-level expression of recombinant proteins (Lindbo, 2007, Marillonnet *et al.*, 2005). Viral vectors systems take advantage of high levels of replication and maximum levels of foreign gene expression in a short time period from an engineered viral genome, with results within a week or two post-inoculation.

Over the last two decades, plant virus-based expression systems have been successfully developed and utilized for high-yield production of heterologous proteins in plants (Dawson *et al*., 1989). Viral vectors as transient gene expression systems provide increased speed and flexibility during early phases of experimentation. Several construction strategies of viral vectors were developed as a systemically moving overexpression tool. However, the potential widespread use of recombinant viruses

raises concerns about possible risks to the environment. The bio-safety issues have to be considered to control the spread of the genetically engineered virus from experimental plants to susceptible wild plants (Prins *et al*., 2008; Manske and Schiemann, 2005; Teycheney and Tepper, 2007). Intact viral vectors have the potential to spread and infect non-target plants, but replication-defective or movement-defective viruses avoid these problems. These deleted viral vectors can be safely used in the laboratory and, in large scale application, are even being used to inoculate an entire greenhouse at once (Marillonnet *et al*., 2005). In the field, it might be possible to achieve high expression in transgenic plants carrying an inducible virus as a transgene (Zuo *et al*., 2000; Tremblay *et al*., 2007). In all of these cases, deleted virus vectors would be greatly preferred over full virus vectors for them a far lower degree of environmental risk.

An obvious disadvantage to the deleted virus approach is that the vector cannot spread past the originally inoculated cells. However, this weakness can be successfully overcome by the agroinfection technique, which can deliver an inoculation of virus to every cell in the infiltration portion of the leaf of a whole plant (Marillonnet *et al*., 2005). Agroinoculation also gives a preview as to how expression would look in a permanently transgenic plant. For both agroinoculation and transgenic use, systemic spread becomes an unnecessary property. Agroinfection involves the local transformation of the infiltrated leaf with the cDNA form of the virus as a part of the T-DNA of the Ti plasmid. *Agrobacterium* infects each cell in the inoculated zone and inserts its T-DNA into the plant chromosome of each cell. A plant promoter placed upstream of the viral cDNA induces the transcription of viral genome in the plant nucleus and viral RNA is transported to cytoplasm for viral replication. Agroinfection provides an efficient

transient gene-transformation strategy for the movement-deficient plant virus expression systems, in which whole plants can be locally transformed by *Agrobacterium* and most cells become infected when *Agrobacterium* suspension is infiltrated into a plant leaf.

Agroinfection allows the replacement of the MP and/or CP genes of vector viruses with heterologous sequences in some virus species. Tobacco mosaic virus (TMV) lacking the CP gene has been used to produce large amounts of foreign proteins and agroinfection greatly increased infectivity of the TMV cDNA, since every cell in the infiltrated area contained the TMV transgene in its nucleus (Lindbo, 2007, Marilloinnet *et al*., 2005, Gils *et al*., 2005). In the potato virus X (PVX) replacement virus vector, both the triple gene block (TGB) and coat protein (CP) viral genes were removed, leaving only the replicase gene, and were replaced with GFP (Komarova *et al*., 2006). The expression levels of GFP from this vector were about 2.5-fold higher than that of full-length PVX vector with the GFP encoding sequence between the triple gene block and the CP genes. Removal of the movement proteins prevents systemic movement of TMV and PVX in above examples and inhibits the spread of the genetically modified virus, which is positive from the biosafety point of view.

Agrobacterium infiltration-mediated transient expression can be greatly enhanced by suppression of gene silencing. The level of protein expression peaks at two or three days post-infiltration and declines rapidly thereafter (Vaquero *et al*., 1999), because the transcription of T-DNA induces the posttranscriptional gene silencing (PTGS) (Voinnet *et al*., 2003). In order to overcome PTGS in the expression system, RNA silencing suppressor (such as P19 encoded by tomato bushy stunt virus or HcPro expressed by potato virus A) was coexpressed with target gene to suppress the gene silencing and

greatly increase the level of recombinant protein expression (Voinnet *et al*., 2003). Expression also persisted for much longer, up to 12 days post inoculation and longer. Production of recombinant protein in viral vector can also be enhanced by co-inoculation with gene silencing suppressor proteins. Using this approach, highly efficient production of GFP from a TMV-based vector was achieved with up to 100-fold increase of the overexpression level (Lindbo, 2007). As well, potexvirus expression was greatly increased (Komarova *et al*., 2006).

Foxtail mosaic virus (FoMV) is a member of the genus *Potexvirus*. *Potexvirus* is a large group of flexous and filamentous plant viruses with a single-stranded, positive-sense genomic RNA, with a cap structure at the 5' terminus and a poly-(A) tail at the 3' terminus (Dolja *et al*., 1987; Huisman *et al*., 1978). The FoMV genome structure resembles that of PVX, the type species of the genus *Potexvirus*, and the gene functions are presumed to be similar as well (Bancroft *et al*., 1991; Bruun *et al*., 2008). The genome of FoMV contains five open reading frames (ORFs), and two subgenomic promoters directing transcription of subgenomic RNAs 1 and 2 (sgRNA1 and sgRNA2) (Huisman *et al*., 1988; Mackie *et al*., 1988). The genomic RNA allows the expression of ORF1 encoding for the RNA-dependent RNA polymerase (RdRP) with methyltransferase, helicase, and polymerase motifs (Davenport *et al*., 1997). ORF2, 3 and 4 code for the triple gene block (TGB) proteins TGB1, TGB2 and TGB3, which are required for virus cell-to-cell movement (Bancroft *et al*., 1991). ORF2 codes for a multifunctional protein that has RNA helicase activity, promotes translation of viral RNAs, increases plasmodesmal size exclusion limits, and acts as a suppressor of RNA-mediated post-transcriptional gene silencing (PTGS) (Verchot, 2005). ORF5 encodes the

coat protein, which is required for viral encapsidation and long distance movement (Cruz *et al*., 1998). FoMV has a broad host range, infecting 56 species of the Gramineae and at least 35 dicot species and in a large number of cases without symptom expression (Short & Davies, 1987). The sequence of FoMV genomic RNA was first published in 1991 (Bancroft *et al*., 1991). Infectious full-length clones were constructed based on the same FoMV isolate and some corrections to the published sequence were noted (Robertson *et al*., 2000). The significant difference between the gene organizations of FoMV and PVX is the presence of ORF 5A upstream of the CP gene in FoMV. ORF 5A initiates 143nts upstream of the CP and extends the reading frame of CP gene. The 5A protein is produced in vivo, but it is not required for either replication or productive infection of plants (Robertson *et al*., 2000). Recently, the revised full-length sequence of foxtail mosaic virus clone was published in 2008, and reveals a triple gene block structure similar to potato virus X (Bruun-Rasmussen *et al*., 2008).

Foundational potexvirus vector work was done first not with FoMV but with PVX, the type species of the genus *Potexvirus*. PVX was engineered to express reporter proteins such as GFP and GUS, which were cloned just upstream of the CP gene and expressed from a duplicated copy of the coat protein (CP) subgenomic promoter. The reporter protein is translated from a sgRNA separate from the other viral proteins. Because PVX has a linear helical capsid, rather than an icoshedral capsid, the longer than wild type recombinant viral genome can still be encapsidated into infectious virus particles. However, both the complete and partial GUS encoding sequence were deleted, because of recombination between the homologous sequences of the duplicated subgenomic promoters (81 nt) (Chapman *et al*., 1992).

The potexvirus replicase is the only protein translated directly from the full-length genomic RNA, but other viral proteins are translated from 3' coternimal subgenomic RNAs (sgRNAs) (Grama and Maior, 1990; Morozov *et al*., 1990). The two sgRNAs of approximately 2.1 and 0.9kb in length have their 5' termini upstream of the TGB and CP genes, respectively (Dolja *et al*., 1987). The integrity of subgenomic promoter is very important for the accumulation of subgenomic RNA and target protein. However, the boundaries of sgRNA promoters have not been delineated for FoMV.

In this study, a vector with the properties of high protein expression and greatly lowered environmental risk was constructed. The TGB and CP genes of FoMV were removed and replaced with heterologous sequences while the subgenomic promoter of one of the TGB genes was reserved to direct the transcription of the heterologous coding sequence. The FoMV expression vectors driven by the cauliflower mosaic virus (CaMV) 35S promoter were delivered as a T-DNA to plant cells by *Agrobacterium tumefaciens*. This viral vector was not able to infect *Nicotiana benthamiana* systemically and its genome size was reduced by 29%. The reporter protein such as GFP was able to yield up to 1.6ug/g fresh weight of plant leaf when the viral vector was co-infiltrated with a gene silencing suppressor gene into plant cells by agroinfection.

Methods and Materials

Plants

Panicum virgatum cv. Blackwell (switchgrass), *Setaria viridis* (foxtail grass), *Hordeum vulgare* (barley), *Triticum aestivum* (wheat), *Avena sativa* (oat) and *Zea mays* (corn) plants were germinated from seed and grown in growth pots with exposure to 24h

per day illumination from plant-adapted spectrum fluorescent bulbs at temperatures ranging from 22 to 24°C. Plants 2-3 weeks from seed, with fully expanded leaves, were used for agroinfiltration and inoculation experiments. *Nicotiana benthamiana* was grown from seed and then transplanted and grown under 400W metal halide lamps to 10-15 cm before inoculation.

Plasmid Constructs

All FoMV viral cDNA constructs used in this study are derivatives of a wild-type FoMV cDNA clone that was a gift from Nancy Robertson of the USDA (Robertson *et al*., 2000) and were constructed with standard recombinant DNA techniques. The binary vector, pJL22, provided by John Lindbo (Lindbo, 2007), has the mini binary plasmid, pCB301 (Xiang *et al*., 1999), as backbone. JL22 contains multiple cloning sites (MCS) flanked by a 35S promoter and 35S polyA signal/transcription terminator.

pFoMV/JL22. FoMV full length viral cDNA was inserted into MCS of pJL22 using standard cloning procedures. FoMV full length viral cDNA obtained from Robertson already had 70 adenosine residues at its terminus, followed by a *Xba* I site. The 5' end of FoMV was amplified by PCR with primers FoMV5'termUP and FoMV756NotDown and cut with *Not*I. The 5' end of FoMV was cloned into JL22 backbone cut with *Stu*I and *Not*I to create JL22/FoMV5'. The 3' end of FoMV was prepared by restriction digest with *Pml*I and *Xba*I and then cloned into the JL22/FoMV5' backbone cut with *Pml*I and *Xba*I (Table 2). JL22 contains a CaMV 35S 3' polyA signal to generate authentic FoMV 3' polyA ends. This full viral cassette including promoter and terminator is flanked with the Left Border and Right Border of T-DNA (Fig 14).

pFECT0, pFECT22 and pFECT40. Primers were designed to delete the TGB and CP genes and keep the subgenomic promoter of sgRNA1 and 3'-end of the CP gene in the FoMV genome. The start codon AUG of TGB1 was mutated to AUC. To ensure that we had fully cloned the sgRNA1 promoter of FoMV constructs to drive GFP, we created primers to include the first 0, 22 and 40 bases of ORF of TGB1 to create pFECT0, pFECT22 and pFECT40, respectively. Restriction enzyme sites *Pac*I and *Avr*II were placed right after the retained subgenomic promoter of TGB1. Primer FoMV+0sgpDown added both *Avr*II and *Pac*I sites at 3' end of subgenomic promoter TGB1 and primer FoMV3044Up was upsteam of unique native *Bam*HI site. With these two primers, the amplified PCR fragment was digested with *Bam*HI and *Avr*II and cloned into vector backbone cut with same restriction endonucleases to create pFECT0. An *Avr*II enzyme site was present at the 3' end of the native CP gene and was utilized, with *Pac*I, as the cloning site to insert foreign genes for expression. Subsequently, two primers, FoMV+22sgp and FoMV+40sgp, were paired with FoMV3044UP to generate two PCR fragments including 22 and 40 bases of TGB1 ORF respectively. Then, pFECT0 cut with *Bam*HI and *Pac*I was used as vector backbone to clone two DNA fragments to create pFECT22 and pFECT40.The 3' terminal part of CP FoMV gene between *Avr*II and 3'-UTR was retained in the viral vector, because it may be crucial for the efficient expression of foreign genes. Deletion of this region dramatically reduced the viral replication and accumulation in PVX (Komarova *et al*., 2006).

pFECT/GFP, pFECT/DsRed, pFECT/HC, pFECT/LC. The cycle 3 GFP gene was PCR mutagenized to be flanked with *Pac*I and *Avr*II restriction sites on 5'- and 3'-ends, correspondingly. The GFP gene was cloned into the *Pac*I/*Avr*II sites of the

Table 2. Primers used for plasmid construction in FoMV study.

Plasmid	Primer	Oligonucleotide sequence (5'–3')	Purpose
pFoMV/JL22	FoMV 5' term UP (pFoMV nt. 1-21)	P-GAAAACTCTTCCGAAACCGAA	The 5' end of FoMV was amplified by PCR with primers FoMV5'termUP and FoMV756NotDown and cut with *Pml*I. The 3' end of FoMV was digested with *Pml*I and *Xba*I. Both 5' and 3' end fragments of FoMV were cloned into the JL22 backbone cut with *Stu*I and *Xba*I.
	FoMV756 NotI DOWN (pFoMV nt. 737-757)	TTTTTTGCGGCCGCTTAGCCAGT TTAGGTCCTTA	
pFECT0 pFECT22 pFECT40	FoMV Up (pFoMV nt 3044-3063)	GTGGGCATGTGCAGATGAGG	To create ΔTGB/ΔCP mutants, *Pac*I and *Avr*II cloning sites were introduced by PCR amplified with two primers (FoMVUp and FoMV+0sgp Down). PCR with mutated start codon of TGB was cut with *Bam*HI and *Avr*II and cloned into pFoMV vector backbone to create pFECT0. Other two downstream primers (with *Pac*I site) were used to save 22nts and 40nts 5' end of TGB DNA sequence. PCR fragments were cloned in pFECT0 vector backbone cut with *Bam*HI and *Pac*I to generate pFECT22 and pFECT40.
	FoMV+0sgp Down (pFoMV nts.4114-4131)	AACCTACCTAGGACTTTAATTA ATGTTATTTAATTCGTCAGTG	
	FoMV+22sgp Down (pFoMV nts.4124-4153)	GCTTTTAATTAAGTTCAACTATT TCACTATCGATTGTTATT	
	FoMV+40sgp Down (pFoMV nts.4150-4169)	GTCTTTAATTAACCAAGCTTTGT TAGTCGTTC	
pFECT0/GFP pFECT22/GFP pFECT40/GFP	PacGFPUp	TTGTCATTAATTAAGCTAGCAA AGGAGAAGAAC	To clone the GFP ORF into the pFECT vector. Primer PacGFPUp adds a *Pac*I site (underline) at the 5' end, and primer GFPAvrDown adds an *Avr*II site (underline) to the 3' end.
	GFPAvrDown	TTTACTCCTAGGTTATTTGTAGA GCTCATCCA	
pFECT40/DsRed pFECT40/LC pFECT40/HC	PacDsRedUP	GGATGGTTAATTAAATGGCCTC CTCCGAGAACG	DsRed and anti-Langerin antibody light chain and heavy chain genes were PCR mutagenized to be flanked with *Pac*I and *Avr*II restriction sites on 5'- and 3'- ends, correspondingly. The three gene fragments were cloned into the *Pac*I/*Avr*II sites of the pFECT40 to generate pFECT40/DsRed, pFECT40/LC and pFECT40/HC constructs.
	AvrDsRedDN	TTTACTCCTAGGCTACAGGAAC AGGTGGTG	
	PacLangLCUP	GGATGGTTAATTAAATGAAGTT GCCTGTTAGGCT	
	AvrLangLCDN	AATACTCCTAGGCTAACACTCTC CCCTGTTG	
	PacLangHCUP	ATATGGTTAATTAAATGGAATG GAGGATC TTTCT	
	AvrLangHCDN	TTTACTCCTAGGTCAGCTAGCTT TACCCAGAG	
pFECT40/GFP/PnosTnos	ApaPnos UP	ATATGAGGGCCCAACTGAAGGC GGGAAACGACAATC	To add PnosTnos in pFECT40, and create *Bsi*WI and *Spe*I in between Pnos and Tnos. Inner primers PnosBsi-overlapDN and TnosSpe-overlapUP have overlap sequence and *Bsi*WI and *Spe*I sites. Two inner primers pair with outer primers ApaPnosUP (*Apa*I at 5' end) and SbfTnosDN (*Sbf*I at 3' end) to generate two PCR products. The two products were fused using outer primers and cloned into pFECT/GFP.
	PnosBsi-overlapDN	GACCACTTTATGGAGGTTCGTA CGTCTAGGGGATCCGGTGCAG	
	TnosSpe-overlapUP	AACCTCCATAAAGTGGTCACTA GTATCGTTCAAACATTTGGC	
	Sbf Tnos DN	ATTATGCCTGCAGGAGCTGGCA TGCAAGCTGTCGAGG	
pFECT40/GFP/p19	BsiWI/p19 UP	TAATAACGTACGATGGAACGAG CTATACAAG	To clone the p19 ORF into pFECT40/GFP/PnosTnos vector. Primer Bsip19UP adds a *Bsi*WI site (underline) at the 5' end and primer p19SpeDown adds a *Spe*I (underline) site at the 3' end of the ORF. The amplified DNA fragment was cloned into pFECT40/GFP/PnosTnos vector backbone cut with *Bsi*WI and *Spe*I.
	p19SpeI DOWN	TTTTTTACTAGTTTACTCGCTTTC TTTTTCGAAGG	
pCB/GFP	Xba/GFP UP	TAAGCATCTAGAATGGCTAGCA AAGGAGAAGAAC	To clone the GFP ORF into pCB302 vector. Primer XbaGFPUp adds a *Xba*I site (underline) at the 5' end, and primer SpeGFPDown adds a *Spe*I (underline) site at the 3' end of ORF.
	GFP/SpeI DOWN	TTTTTTACTAGTTTATTTGTAGA GCTCATCCA	

pFECT0, pFECT22 and pFECT40 to obtain pFECT0/GFP, pFECT22/GFP and pFECT40/GFP constructs. DsRed and anti-Langerin antibody light chain and heavy chain genes were subcloned into pFECT vector in the same manner but with only FECT40.

FECT/GFP/p19. Nos promter (Pnos) and nos terminater (Tnos) were designed to control the transcription of p19 gene in the FECT/GFP/19 binary vector. Pnos/HygR/Tnos in pER8 plasmid was used as template to create two restriction enzyme sites (*Bsi*WI and *Spe*I) in between Pnos and Tnos, which would serve as cloning sites for p19 insertion. To fuse Pnos and Tnos together, two PCR products were generated for overlap. The first PCR with the Pnos sequence was generated using primer ApaIPnosUP (adds an *Apa*I site at the 5' end, Table 2) and primer PnosBsiWI-overlapDN (adds a *Bsi*WI site and ovelap sequence at the 3' end, Table 2). The second PCR with Tnos sequence was generated using primer TnosSpeI-overlapUP (adds overlap sequence and a *Spe*I site at the 5' end) and primer SbfTnosDN (adds a *Sbf*I site at the 3' end, Table 2). The two products were fused using primer ApaPnosUP and primer SbfTnosDN by overlap PCR to create the Pnos-Tnos fragment (*Apa*I)-Pnos (*Bsi*WI)-(*Spe*I)/Tnos-(*Sbf*I). Pnos-Tnos PCR product was digested with *Apa*I and *Sbf*I restriction endonucleases, and then cloned into the FECT/GFP backbone cut with *Apa*I and *Sbf*I to create FECT/GFP/Pnos-Tnos. To clone the p19 ORF into the FECT/GFP/Pnos-Tnos vector, a p19 PCR product was generated using primer BsiWI/p19UP (adds a *Bsi*WI site at the 5' end, Table 2) and primer p19SpeI DOWN (adds *Spe*I site at the 3' end, Table 2). P19 PCR product was digested by *Bsi*WI and *Spe*I and cloned into the

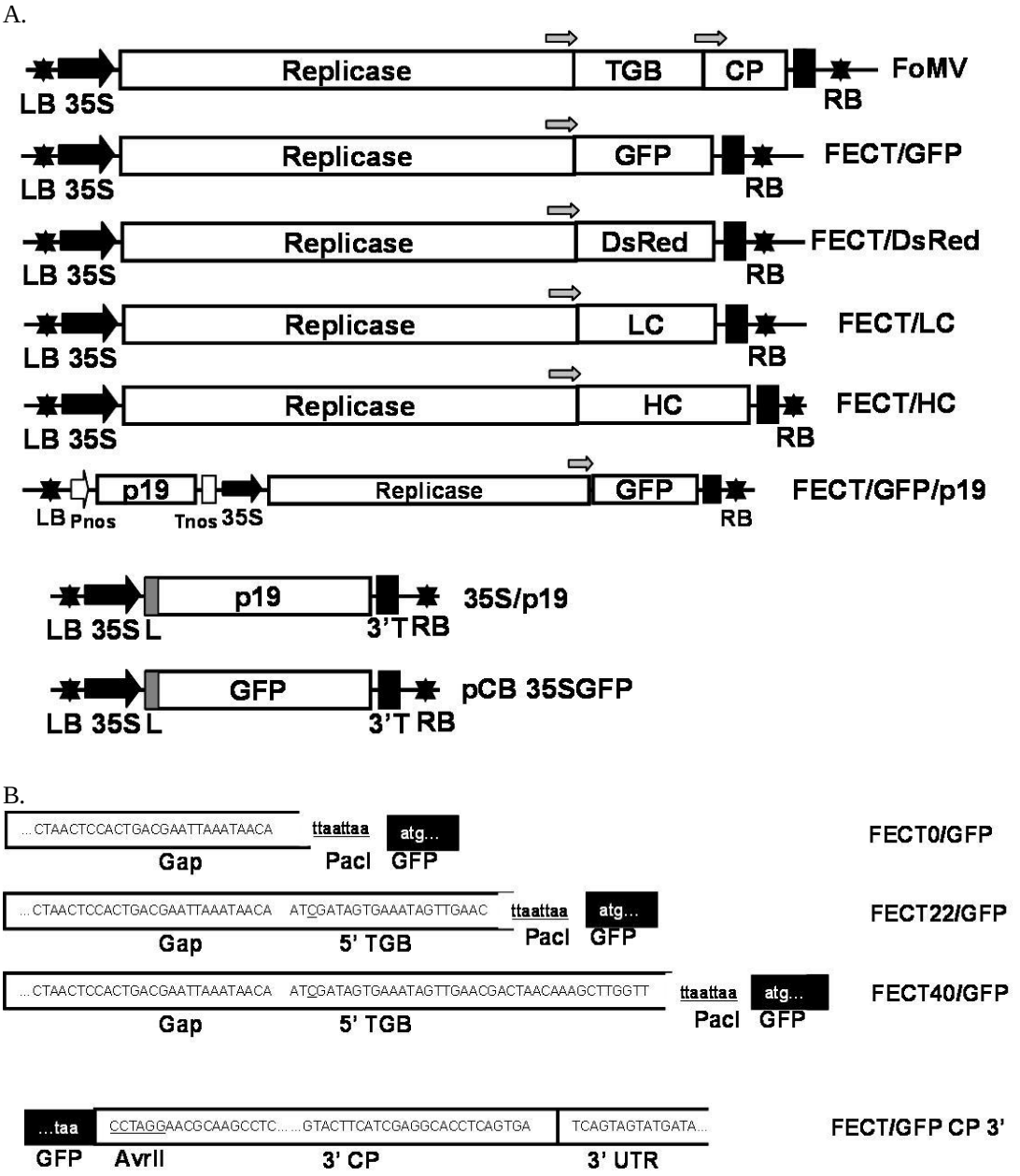


Figure 14. Schematic diagram of binary plasmids used in FoMV study. (A). Cauliflower mosaic virus (CaMV) 35S promoter driven versions of *Foxtail mosaic virus* (FoMV) vector cDNAs or FECT as viral vector to express GFP, GUS and antibody LC, HC and 35S driven versions of GFP, p19 were constructed. All plasmids were based on the binary vector pCB301 backbone. Open boxes represent open reading frames; black stars: left border and right border of T-DNA; block arrows: CaMV duplicated 35S promoter; black boxes: CaMV 3' terminator sequence; gray arrows, subgenomic promoters; white arrow: nos promoter (Pnos); white box: nos terminator (Tnos); dark grey boxes: Tobacco etch virus 5' non-translated leader sequence (L); RB: T-DNA right border sequences; LB: T-DNA left border sequences; TGB: triple gene block; CP, coat protein; LC, antibody light chain; HC, antibody heavy chain. (B). All TGB and CP deletion mutants have the root "FECT" (Foxtail Elimination of CP and TGB). In the start coden of 5'TGB, ATG were mutated to ATC, shown as underlined. Restriction sites PacI and AvrII were introduced at the flank of GFP ORF as cloning sites for other foreign inserts. The numbering indicates the number of nucleotides from the TGB ORF presented as upstream of the GFP ORF. For example, FECT40 remains the 5' end 40nts of FoMV TGB to reserve the subgenomic promoter to drive the expression of GFP.

FECT/GFP/Pnos-Tnos vector backbone cut with two restriction enzymes to generate FECT/GFP/p19 binary plasmid (Fig 14).

Cloning and Sequencing. The high fidelity polymerase, Phusion (New England Biolabs (NEB), Beverly, MA), was used according to company protocols in all constructions. Recombinant clones were introduced into *Escherichia coli* NEB l0-beta electrocompetent cells by electroporation at 1.44 kV and 129 Ω for 5 ms using a BTX 600 Electro Cell Manipulator (BTX Inc., San Diego, CA, USA) and colonies were screened by PCR using NEB Taq polymerase or by restriction digests of plasmid minipreps prepared by Wizard Plus Miniprep Kit (Promega, Madison, WI). Sequence verification was performed using a CEQ capillary sequencer (Beckman Coulter, Fullerton, CA).

Agroinfection

Agroinfiltration was performed as described (Kapila *et al*., 1997) with modifications. *Agrobacterium tumefaciens* stain GV3101 was used for the agroinoculation of *N. benthamiana* and cereals. *A. tumefaciens* was transformed with plasmid constructs using the same conditions as for *E. coli* above. *Agrobacterium* transformants were selected at room temperature on Luria-Bertani plates containing 10 μg/ml rifampicin, 25 μg/ml gentamycin and 50 μg/ml kanamycin. A colony of *A. tumefaciens* was inoculated to 5 ml of L-MESA medium (LB media supplemented with 10 mM MES, 20 uM acetosyringone (Phytotechnology Labs, Shawnee Mission, KS), a wound response compound that elicits *Agrobacterium* virulence, and the same antibiotics), and grown overnight at room temperature. The cells of the overnight culture

were harvested by centrifugation and resuspended in induction media (10 mM MES, 10 mM $MgCl_2$, 100 uM acetosyringone) for a final OD_{600} of 1.0 and incubated for 2 h to overnight at room temperature. The cultures of *A. tumefaciens* were infiltrated into the underside of leaves of plants with a 3 ml syringe without needle. For agroinoculation of two or more bacterium cultures at the same time, multiple cultures of *A. tumefaciens* were mixed in equal amounts and infiltrated together. The gene expression or virus activity was tested at 6 - 8 days post-infiltration and one of three plant replicates were analyzed per experiment.

RT-PCR

To detect FoMV (without GFP or DsRed) in the plant, total RNA was extracted after seven days post-inoculation using Tri-Reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. RT-PCR reactions were performed using the RT-PCR kit (NEB, Beverly, MA) as described by the supplier. To detect the presence of virus particles, FoMV specific primers were used to amplify the partial viral genome.

GFP and DsRed Photography

Plants were examined under long-wave UV light (UVL-56, UVProducts, Upland, CA). For macrophotography, a Canon Digital EOS Rebel XT camera (Canon Inc., Japan) equipped with a Hoya yellow (K2) filter (Hoya Corporation, Japan) was used. For microscopic analysis, samples from infiltrated tissues were mounted with water on a glass slide. Images were obtained with a Nikon TE2000-U inverted microscope, captured using a CoolSnap *cf* camera (Roper Scientific, Tucson, AZ) and analyzed with Metavue imaging software (version 5, Molecular Devices Co, Downingtown, PA).

GFP Quantification Assay

GFP fluorescence was analyzed and GFP protein was quantified using a standard curve determined from a purchased GFP standard (Vector Laboratories, Inc, Burlingame, CA), since the amount of GFP protein is directly proportional to the fluorescence intensity (Richards *et al*., 2003, Lindbo, 2007). Total soluble protein extracts were serially diluted in 50 mM carbonate/bicarbonate buffer, pH 9.6 and loaded on the 96-well Costar black plate with clear bottom (Costar, Cambridge, MA). Fluorescent activities were assayed with a Fluoroskan Ascent FL (Thermo Fisher Scientific Inc., Waltham, MA) using a 485 nm excitation and 538 nm emission filter set.

Protein Extraction, SDS-PAGE

Proteins were extracted by grinding agroinfiltrated leaves to a fine powder in liquid nitrogen and mixing 1:2 (w/v) with reducing protein extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 0.1% β-mercaptoethanol) or nonreducing protein extraction buffer without β-mercaptoethanol. The insoluble material was removed by centrifugation for 10 min at 16,000 x g in a benchtop centrifuge. The supernatant was collected and stored at 4°C. Clarified extract of protein samples were mixed with 3 × SDS-PAGE sample buffers (NEB, Beverly, MA) and analyzed by PAGE consisting of a 5% stacking gel and a 7.5% or 15% separation gel. Proteins in the gels were identified with Coomassie brilliant blue R-250 (Sigma, St Louis, Mo).

Western Blot

After electrophoretic separation, the proteins were transferred to a Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, NJ) using a semi-dry transfer

apparatus (Biorad, Hercules, CA) at 20 V for 30 min. The membranes were blocked for 1 h with TBST (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.05% (v/v) Tween 20) and 5% (w/v) skimmed milk. Blots were incubated for 1 h with anti-human IgG antibodies conjugated with alkaline phosphatase (Sigma, St Louis, MI), diluted 1:10000 in TBS with 1% skimmed milk to evaluate the production of the antibody in plants. The enzymatic reaction of alkaline phosphatase was developed with SIGMA *FAST* BCIP/NBT substrate solution (0.30 mg/ml nitroblue tetrazolium (NBT), 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 100 mM Tris and 5 mM $MgCl_2$) (Sigma, St. Louis, MI). Apparent molecular weight of proteins was estimated with prestained protein molecular weight markers (NEB, Beverly, MA). The anti-Langerin antibody was used as a control in the antibody studies.

Enzyme-Linked Immunosorbent Assays (ELISA)

ELISA 96-well plates (Costar, Nunc, Corning, NY) were coated for 1 h at 37^{o}C with 2 ug/mL of monoclonal anti-human IgG (Fc specific) (Sigma, St. Louis, MO), diluted in 50 mM sodium carbonate buffer (pH 9.6). After three washes with TBS-0.05% Tween 20, the wells were blocked for 1 h at 37^{o}C with 5% (w/v) skimmed milk in TBST. Plates were loaded with 50 ul of the protein extracts of two fold serial dilution and incubated for 1 h at 37^{o}C. After three washings, the bound recombinant IgG was detected with the specific antigen conjugated with alkaline phosphatase (provided by Gerard Zurawski of the Baylor Institute for Immunology Research, Dallas, TX), diluted 1:3000, for 1 h at 37^{o}C, and developed with p-nitrophenyl phosphate (Sigma, St. Louis, MO) as substrate. Optical densities were measured at 405 nm on a BIO-TEK ELx800 Universal Microplate Reader (Cole-Parmer, Vernon Hills, Illinois). A standard curve of

serial 1:2 dilutions of antibody positive control was included in the experiments, to assess the amount of antibody present in the samples.

Results

Full Length Infectious FoMV cDNA Clone

Full-length FoMV cDNA under transcriptional control of the CaMV 35S promoter was inserted into the T-DNA of binary vector JL22 to construct pFoMV (Fig 13). *Agrobacterium* strain GV3101 containing pFoMV was agroinoculated in *Nicotiana benthamiana* to look for symptoms and evidence of viral replication. There were no symptoms of viral infection of FoMV, but mild, nonsymptomatic infections are common with FoMV (Short & Davis, 1987). To detect the presence of FoMV infection, total RNA was extracted from leaves and screened by RT-PCR at 7 days post-inoculation. Systemic movement of viral RNA derived from pFoMV was demonstrated in inoculated plants, but was not seen in uninoculated controls (data not shown). Thus agroinfection with the pFoMV construct resulted in replication, movement, and production of virions and this FoMV sequence is so mild that no symptoms were produced.

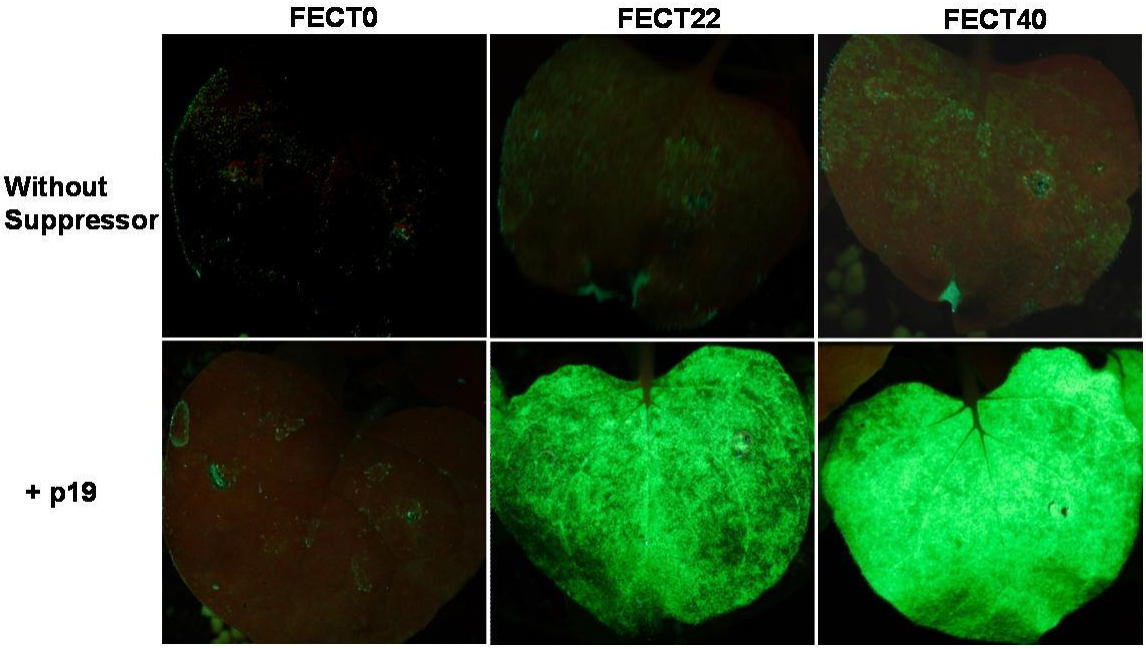
Local Infection of pFECT Expression Vector

Construction of the viral vector pFECT based on FoMV genome. The TGB and CP genes in a full-length cDNA clone of FoMV were removed and replaced with restriction sites amenable for inserting heterologous genes. However, the subgenomic promoter of subgenomic RNA1 and 3'-end of the CP ORF in the FoMV genome were retained to maintain the efficient viral replication in viral vectors, following the design of

Komorova *et al.* (2006) for PVX. The start codon of TGB1 ORF was mutated to prevent expression of TGB1 (Fig 14). The FoMV triple gene block (TGB) and coat protein (CP) are expressed from 3'-coterminal subgenomic RNAs (sgRNAs). The subgenomic promoters and the transcription start site of the sgRNA1 have not been mapped in FoMV. To ensure the inclusion of the entire functional region of the sgRNA1 promoter, which we expected to extend into the TGB1 ORF, the first 3 (start codon only), 22 and 40 bases of the TGB1 ORF were retained to create constructs named pFECT0, pFECT22 and pFECT40, respectively. Thus, the only difference between FECT0/GFP, FECT22/GFP and FECT40/GFP vectors is the promoter length (Fig. 14). FECT0 is the promoter length described in Komorova's paper (2006) for PVX.

To test viral replication and GFP expression of these FECT/GFP vectors, cultures of the GV3101 strain of *A. tumefaciens* carrying these constructs FECT/GFP were prepared. Leaves of 2-4 weeks old *N. benthamiana* were agroinfiltrated with each of the FECT/GFP cultures. At 2-4 days after agroinoculation, GFP-expressing cells could be seen faintly using a hand-held UV lamp and fluorescence microscopy. At this time, there were many faint green spots shown on inoculated leaves on leaves inoculated with FECT40/GFP, many fewer green spots shown for FECT20/GFP, but no green fluorescence could be detected on leaves inoculated with FECT0/GFP (Fig 15). However, the fluorescence was transient and, by eight days post-inoculation, the GPF spots on the FECT22/GFP and FECT40/GFP plants had disappeared (Fig 15). Apparently, the transcription of agroinfiltrated T-DNA induced posttranscriptional gene silencing (PTGS), which led to the inhibition of viral vector infection and the reduction of viral productivity (Voinnet *et al.*, 2003).

A.



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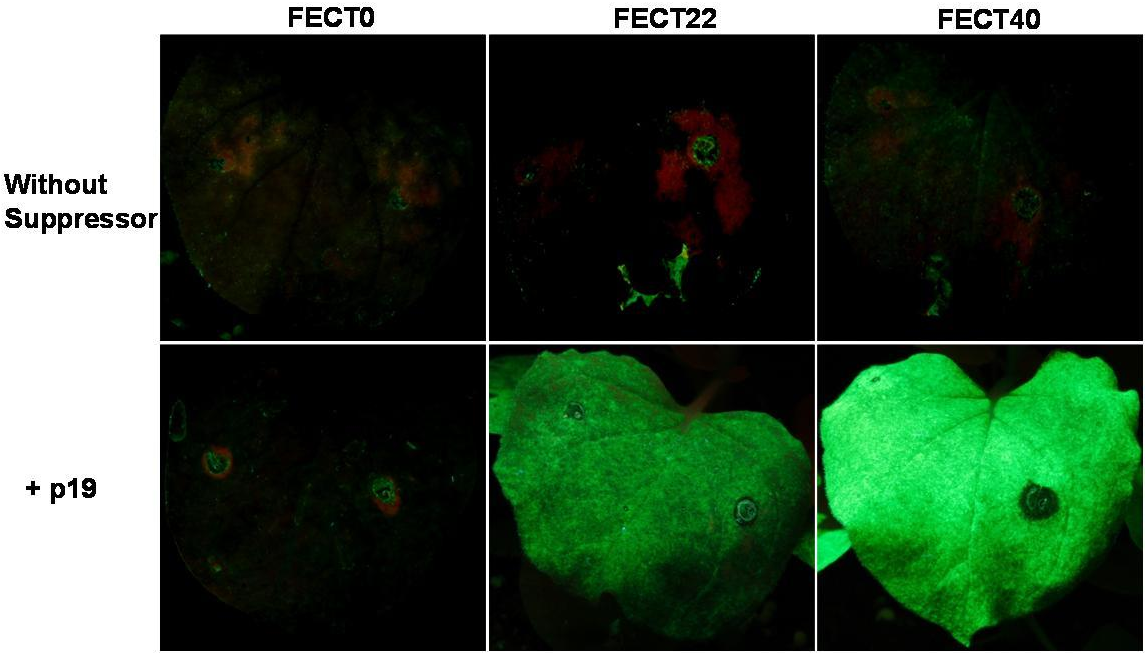


Figure 15. Effect of gene silencing suppressor on the agroinfection efficiency of FECT vector. Agroinfection induced local expression of GFP by FECT vectors (ΔTGB ΔCP) on *Nicotiana benthamiana.* The FECT can move cell-to-cell and systemically in plants. Fig 15-A. Three FECT vector variants (FECT0, FECT22, and FECT40) containing GFP driven by three different length promoters were agroinfiltrated with and without gene silencing suppressor: *A. tumefaciens* (*A.t.*, O.D.600 1.0) mixture of *A.t.*/FECT:GFP and *A.t*/35S:p19; or *A.t.*/FECT:GFP only without silencing suppressor. Photographed 4 days post-infiltration (dpi). Fig 15-B. After 8 days post infiltration, the green fluorescent protein was produced at the highest yield on *N. benthamiana* inoculated with mixture of *A.t.*/FECT40:GFP and *A.t.*/35S:p19. But the GPF spots on the FECT22/GFP and FECT40/GFP plants have disappeared.

Application of Gene Silencing Suppressors P19. It has recently been demonstrated that co-inoculation of RNA-silencing suppressor proteins enhances the expression of heterologous proteins from the viral vectors (Komarova *et al*., 2006; Lindbo 2007; Lindbo 2008). For example, *tomato bushy stunt virus* silencing suppressor p19 increased 100 times the expression of GFP in *tobacco mosaic virus* vectors (Lindbo 2007). To test these suppressors, *N. benthamiana* plants were agroinfiltrated with a 1:1 mixture of 35S/p19 and FECT/GFP cultures. The accumulation of GFP was followed and imaged by hand-held UV light and fluorescence microscopy at 3-7 days post-inoculation.

When plants were co-infiltrated with the suppressor, we were surprised at the level of fluorescence. The fluorescence of the inoculated zones of FECT40/GFP plants was very clearly seen under the UV lamp even with the room lights turned on. Though the amount of GFP-expression was dramatically increased in FECT20/GFP and FECT40/GFP inoculated plants, no fluorescence was seen with FECT0/GFP with or without suppressor co-infiltration (Fig 15). By four days post-inoculation, nearly 90% of the infiltrated area was fluorescent with FECT40/GFP. Also, the leaves with FECT40/GFP displayed stronger green color than the one with FECT20/GFP (Fig 16). Thus the subgenomic promoter includes at least up to 20 to 40 bases of the ORF.

The unusually high expression level led us to determine the percent of total soluble plant protein that the GFP represented in the inoculated zone. As replication of FECT/GFP replicon progressed, the amount of GFP expressed in the infiltrated leaf increased, so a time course was needed. *A. tumefaciens*/FECT40/GFP + *A.*

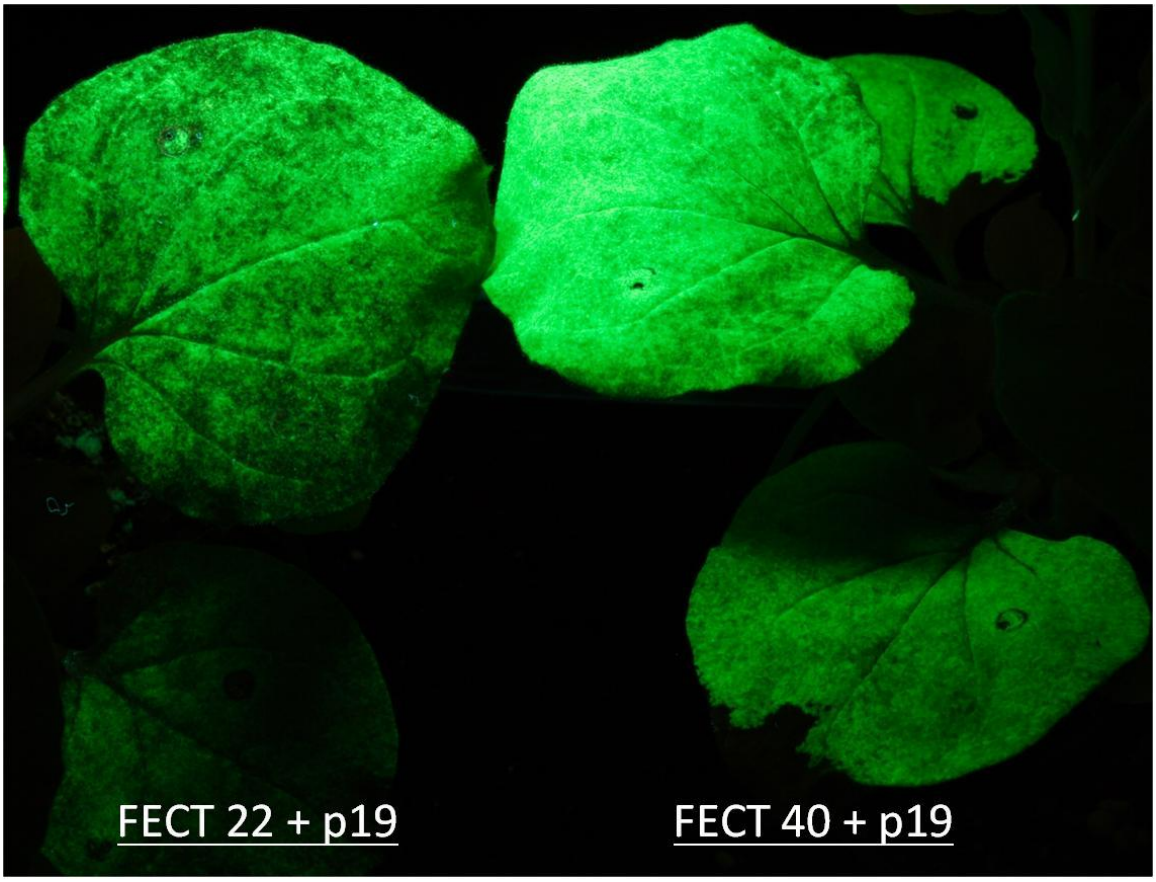


Figure 16. Comparing the expression efficiency of FECT22 and FECT40. The leaves with FECT40/GFP displayed much stronger green color than the one with FECT20/GFP Picture was took at four days post-inoculation, all at same exposure.

tumefaciens/35S/p19 co-infiltrated leaves from 2 to 7 dpi were homogenized and the relative amounts of GFP in extracts of total soluble protein were measured with SDS-PAGE electrophoresis and coomassie blue protein staining (Fig 17). The GFP was detected from the second day after inoculation (Fig 17). The expression level of fluorescent protein increased gradually, and reached a peak at 7 dpi (Fig 17). At this time it appeared that nearly 100% of the cells in the inoculated leaves were infected with FECT40/GFP mixed with suppressors. GFP accumulated to 30% to 40% of the total soluble protein extracted.

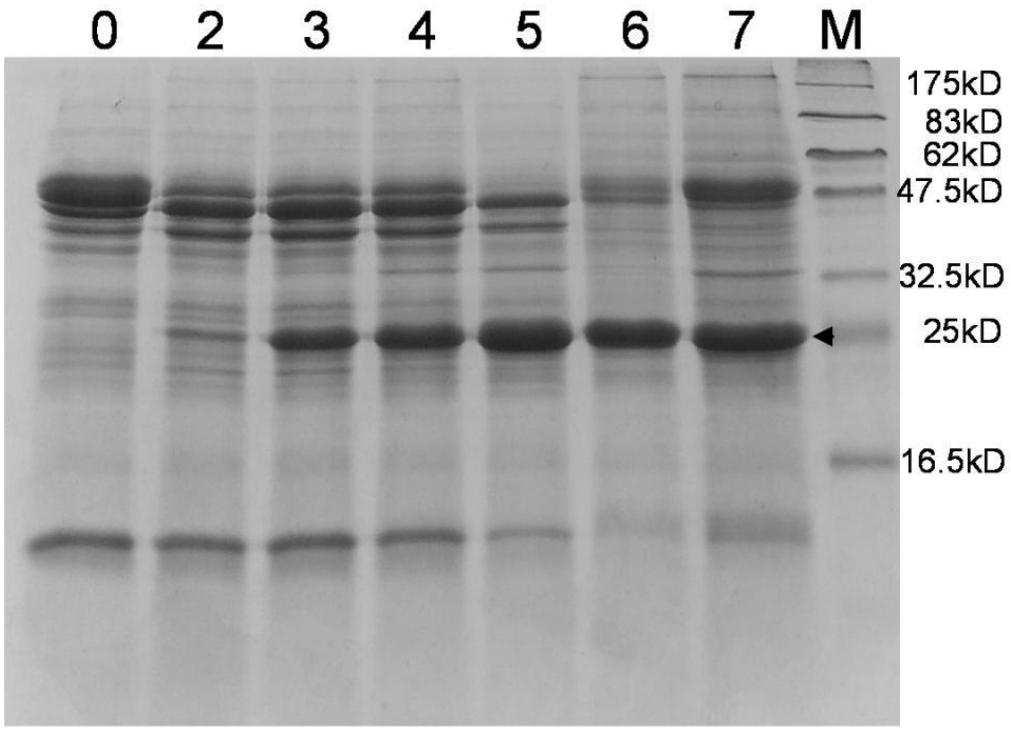


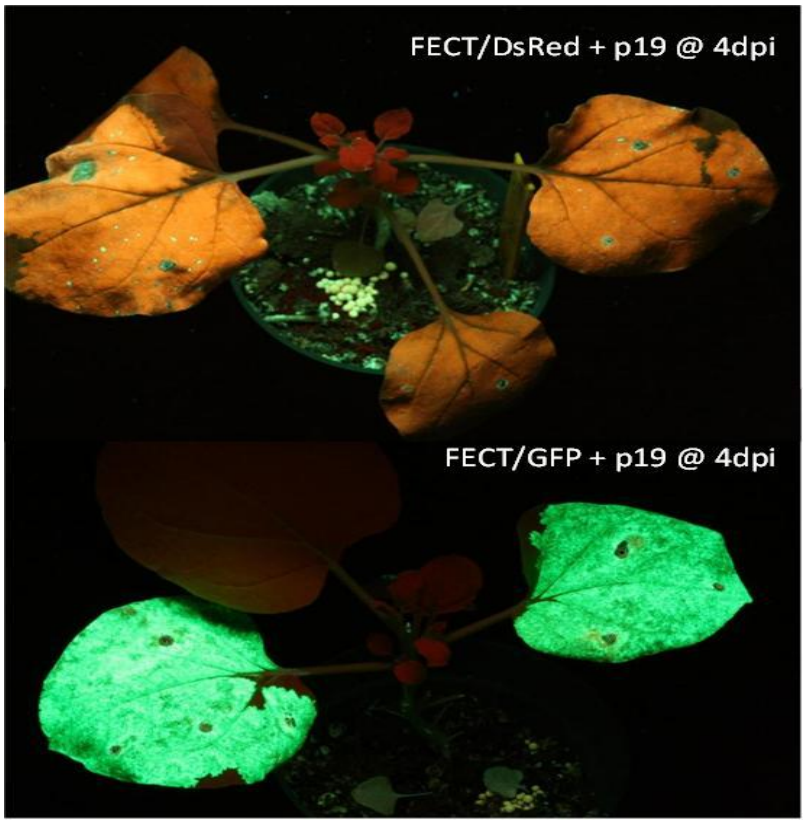
Figure 17. Time course analysis of GFP expression from FECT40/GFP. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* mixed culture of *A.t.*/FECT:GFP and *A.t.*/p19. Total protein extracts were prepared from infiltrated leaf tissue from 2 to 7 days post-inoculation. Protein extracts were analyzed by SDS-PAGE, subsequently stained with Coomassie Blue. Molecular weight of protein standards (kD) is noted. Lanes: M, protein marker; 0, protein extract from uninoculated leaf; 2 to 7, extracts from FECT agroinfiltrated leaves, 2 to 7 days post-inoculation, respectively.

At 7 days post-inoculation it appeared that nearly 100% of the cells in the inoculated leaves were infected with FECT40 mixed with suppressors (Fig 18). Green and red fluorescent cells were observed by fluorescence microscopy in plant leaves agroinoculated with FECT40/GFP or FECT40/DsRed. FECT vector was able to replicate in the majority of plant leaf cells when delivered by agroinfection. Target protein could be expressed only as the result of this replication and the subgenomic RNA synthesis, in which cell-to-cell movement is not required. Agroinoculation-mediated transformation has very high transformation efficiency and ensures FECT cDNA to be delivered almost

all cells in inoculated tissue. And T-DNA-mediated gene expression could produce proteins for an extended period. FECT vector is a non-pathogenic viral expression system and high levels of viral replication contributed to high yield protein production.

We next sought to compare FECT40/GFP expression to expression obtained by a nonviral 35S construct, since 35S expression is the standard used in plant biotechnology. Gene silencing suppressors can also be used in tandem with 35S promoters directly driving the gene expression of the ORF, without the use of viral vectors. This results in remarkably high protein expression (Voinnet *et al*., 2003). To make for an exact comparison, we placed the GFP ORF into the same binary plasmid backbone (pCB302) used for FECT40, using the same 35S promoter that drives the expression of the FECT40 viral transcript. *A. tumefaciens* cultures containing p19 and FECT40/GFP binary constructs were mixed and co-infiltrated into *N. benthamiana* leaves. Control plants were co-infiltrated with a mixture of *Agrobacterium* cultures containing either the pCB302/GFP or the 35S:p19 plasmids. Total soluble protein was extracted from infiltrated leaf tissue at seven days post-infiltration. The protein yield of GFP in plants was measured by specrofluorometry in fluorescence activity assay. The FECT40/GFP vector expressed 1.6 g/kg GFP of fresh-weight tissue, which represented up to 80 times more GFP than was obtained from co-infiltrating T-DNAs for 35S:GFP and 35S:p19 into plants (Fig 19). In multiple repetitions of this experiment, the FECT system consistently expressed significantly more GFP (1.58±0.13 g/kg) than the non-viral transient expression systems examined (0.02±0.002 g/kg).

1.



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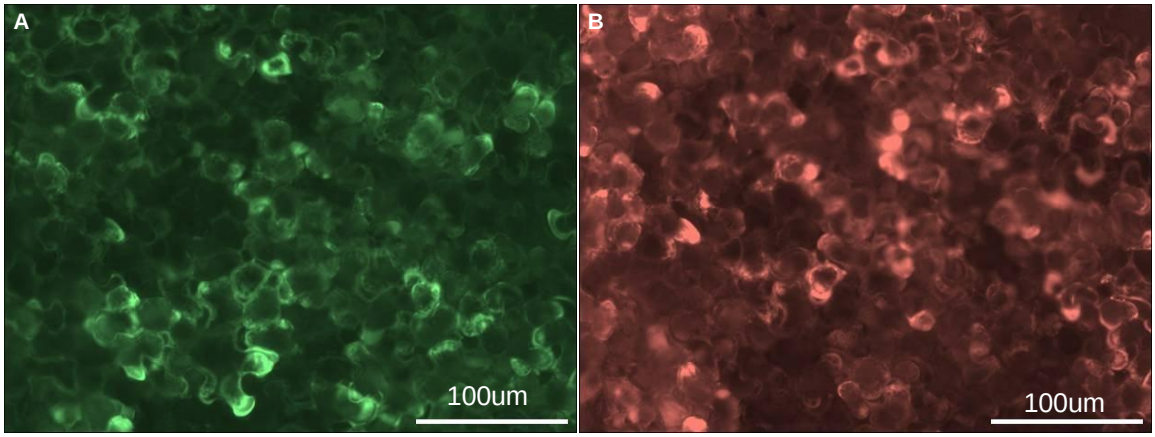


Figure 18. High efficient expression of FECT40 viral vector. Fig 18-1. Agroinfection induced expression of GFP and DsRed by FECT vectors (ΔTGB ΔCP) with co-inoculation of gene silencing suppressor 35S/p19on *Nicotiana benthamiana* at 4 days post-inoculation (4 dpi). The FECT can not move cell-to-cell and systemically in plants. Fig 18-2. Fluorescence microscopy of plants infected with FECT40 expressing GFP and DsRed. FECT40/GFP or FECT40/DsRed transient expression on plants after 4 days post-inocualtion (4 dpi) was visualized by fluorescence microscopy. A. FECT40/GFP was expressed in *N. benthamiana*; B: FECT40/DsRed in *N. benthamiana*.

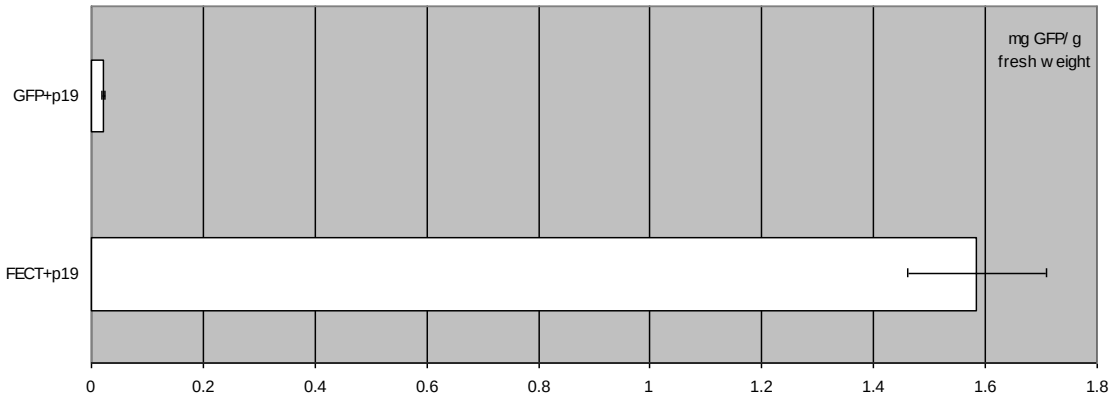
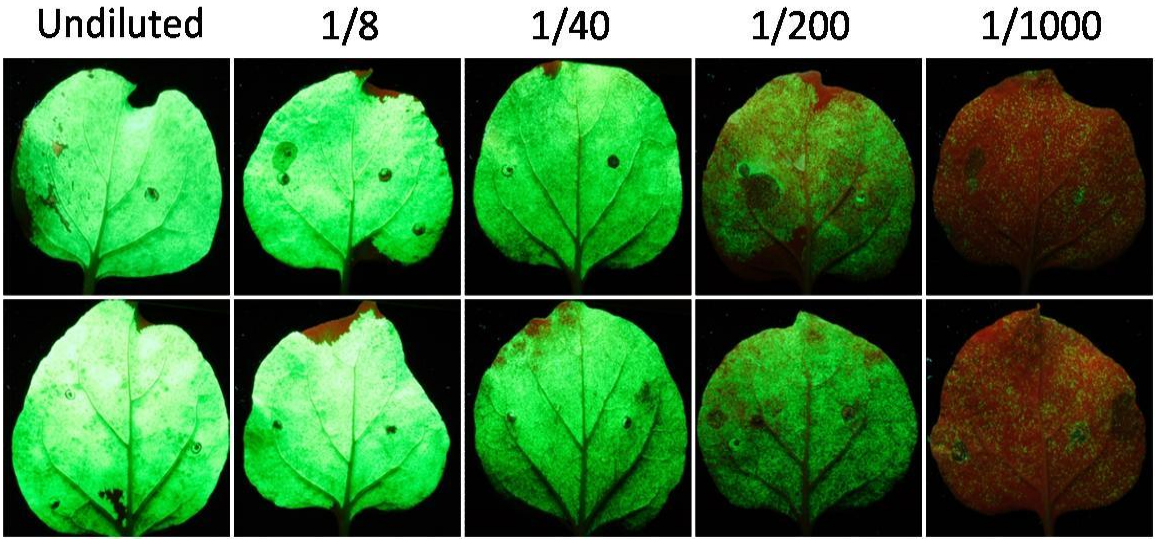


Figure 19. Quantification analysis of GFP expression. Quantification of GFP expression of FECT40/GFP + p19 and 35S/GFP + p19 was analyzed seven days postinfiltration in *N. benthamiana*. The GFP accumulation in infiltrated leaves was measured by spectrofluorometry. Values represent averages from three extracts ± SD.

Because the TGB and CP of FoMV is required for systemic and cell-to-cell movement (Bancroft *et al*., 1991, Cruz *et al*., 1998), the FECT viral vectors were not expected to move systemically and cell-to-cell in plants. To test this, *N. benthamiana* plants were inoculated with either JL24, a full length TMV vector which expresses GFP (Lindo, 2007), or FECT40/GFP by agroinfection mixed with p19. When leaves were infiltrated with higher concentration of JL24 or FECT/GFP containing *Agrobacterium*, almost all plant cells in the infiltrated area expressing GFP made it difficult to separate an individual focus. Serial dilution experiment of agroinfection was performed on *N. benthamiana* plants. Plants were observed under UV illumination to visualize GFP expression and, hence, viral movement. The vector JL24 expressed GFP and all of the genes of TMV, including the MP (corresponding for TGB in FoMV) and the CP (corresponding for CP in FoMV), and was observed to move cell-to-cell at 4 dpi and systemically at about 7 dpi. As TMV replicates and viral particles move cell-to-cell from inoculated cell to adjacent cells through the help of MP, individual GFP-expressing foci



20-Control

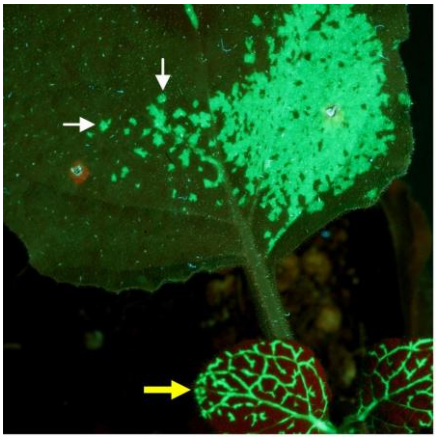


Figure 20. Dilution experiment of agroinfection. Dilution experiment of agroinfection with *A. tumefaciens* (*A.t.*) containing FECT40/GFP expression vector and p19 co-inoculated on *N. benthamiana* plants. Leaves of *N. benthamiana* plants were infiltrated with mixture of *A.t.* cell suspensions containing FECT40/GFP viral vector and p19 binary plasmids. *A.t.* cell suspensions were diluted, as noted in the figure, from an initial OD600 of 1.0. Pictures were taken under UV illumination at 4 dpi; all leaves shot at same exposure, color tone, etc. Control: Leaf infiltrated with 1:100 dilution of a mixture of A.t./JL24 and p19. Photos were taken at 8 dpi. Large green spots were indicated by white arrows; systemic green fluorescent leaf was indicated with yellow arrow.

enlarge it. The FECT replicon was never observed to move systemically in the agroinoculated plants in this or any other experiment, and the green fluorescent loci on the leaves never grew to larger spots by cell-to-cell movement (Fig 20). This effect was

especially clear in comparing the sizes of individual GFP-expressing cell foci in 4 dpi images of leaves infiltrated with 1:1000 dilutions of FECT/GFP and 1:100 JL24 (Fig 20).

FECT Expression Analysis in Monocots

We demonstrated agroinoculation of FECT vector together with a silencing-suppressor gene dramatically increased the production level of target protein in *N. benthamiana* plants. The host range in grasses, its natural hosts, was investigated in the same method. Switchgrass, foxtail millet, barley, wheat, oat and corn were co-agroinoculated with the mixture of two *Agrobacterium* cultures containing FECT40/GFP and p19, respectively. Fluorescing cells were observed in corn's leaves agroinoculated with FECT/GFP by fluorescence microscopy, but not in uninoculated control leaves (Fig 21). In barley a clump of about 20 fluorescing cells was seen, which was not found in controls (Fig 21). Single cell fluorescence was present in oats and other grasses, but there were also these kinds of fluorescing cells in noninfected leaves (data not shown). The low transformation efficiency in monocots via agroinoculation made it difficult to conclusively distinguish between autofluorescence in control leaves and GFP fluorescence in inoculated leaves. Successful reports of grass species agroinoculation were not found by the authors except for Vanderghenst *et al*. (2007).

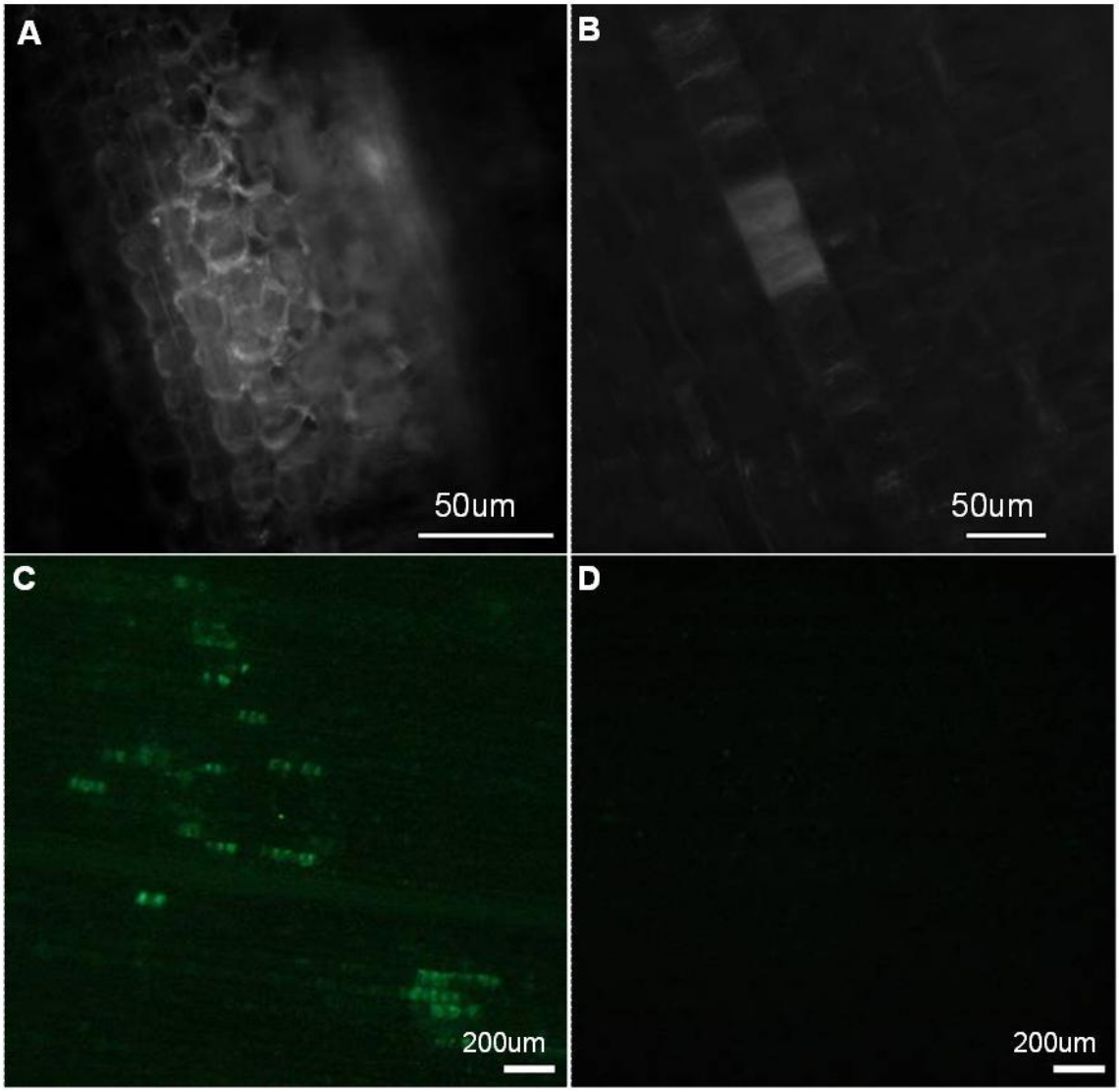


Figure 21. Fluorescence microscopy of monocots agroinfected with FECT40/GFP. Barley (A), oat (B) and corn (C) leaves were co-agroinfiltrated with mixture of FECT40/GFP and 35S/p19. Transient expression of GFP was visualized by fluorescence microscopy after 10 days post-inocualtion. Corn (D) leaves demonstrated the negative control without agroinfection.

Effect of Gene Silencing Suppressor in cis Construct

High yield protein production in *N. benthamiana* demonstrates that specific interaction between the suppressor and FECT viral vector is absolutely required for gene silencing suppression. However, only a small fraction of cells, at best, are expected to be

infected by *Agrobacterium* in the agroinoculation of grasses (VanderGheynst *et al*., 2007). As the trans construct, p19 has to be expressed in a separate binary vector and two *Agrobacterium* cultures have to be mixed before agroinoculation. Because of expected low rates of infection, co-agroinfection of the same cell with FECT and p19 was considered almost impossible in grasses. So, a combined FECT/p19 construct was built for the co-expression of p19 and FECT in the same cell. The p19 ORF was cloned into the FECT/GFP vector under the control of *nos* promoter and *nos* terminator. GFP expression was examined under a hand-hold UV lamp at 2 days post-inoculation in *N.benthamiana*. There is no significant difference between cis and trans p19 construct (Fig 22). At seven days post-inoculation, the trans treatment gained in fluorescence while the cis treatment remained at the same level (data not shown).

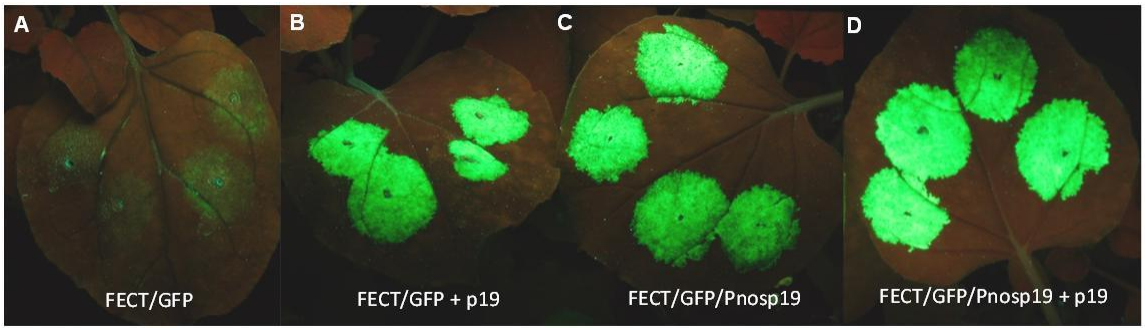


Figure 22. Effect of gene silencing suppressor p19 in cis and trans constructs. Effect of gene silencing suppressor p19 on GFP expression of FECT/GFP in cis and trans constructs at two days postinoculation in *N. benthamiana*. GFP fluorescence as seen under UV light. A: FECT/GFP alone; B: FECT/GFP + p19; C: FECT/GFP/p19 alone; D: FECT/GFP/p19 + p19.

This construct was inoculated to corn and foxtail millet, but, again, no overwhelming fluorescence greater than the controls was observed by fluorescence microscopy. However, we see no reason why FECT vectors will not express in grass

cells if they are able to be introduced to the cell. Protoplast studies are now being pursued.

Transient Antibody Expression with FECT Viral Vector

The FECT vector has been successfully used to express glucuronidase (GUS; data not shown), DsRed and GFP in *N. benthamiana*. To expand the repertoire of genes that could be expressed, we next chose a very large and multimeric protein. cDNA clones of anti-langerin mAb IgG4 HC and LC were prepared by PCR introducing restriction cloning sites for *Pac*I and *Avr*II at the 5' and 3' ends, respectively. The gene fragments encoding HC and LC were cloned into viral vector FECT40, under the control of the subgenomic promoter for FoMV TGB1 mRNA, obtaining FECT40/LC and FECT40/HC. *N. benthamiana* plants were co-agroinfiltrated with three *Agrobacterium* mixtures carrying the antibody expression constructs -FECT40/LC and FECT40/HC- and gene silencing suppressor p19 driven by 35S.

Infected leaves of *N. benthamiana* were homogenized in extraction buffer, centrifuged to clear the supernatant, and directly coated onto ELISA plates. Plant-based recombinant antibody bound to the antigen was detected using the Langerin alkaline phosphatase conjugate. Extracts from plants infected with FECT40/GFP were used as negative controls. The ELISA results demonstrate that the total soluble protein from agroinfiltrated leaves contained molecules which human heavy and light antibody chains where assembled. Western blot analysis of total soluble protein extracted by non-reducing conditions indicated that the protein material from transient expression contained a 170 kDa full-length antibody protein band of similar molecular size as the positive control antibody which is expressed by mammalian expression system (Fig 23).

The total soluble protein from leaves agroinfiltrated with *Agrobacterium tumefaciens* with FECT empty vector plasmid was negative by ELISA and western blot, as expected. The antibody yield was 10 ug/g fresh weight of agroinfiltrated leaves as calculated by ELISA using the standard antibody standard curve.

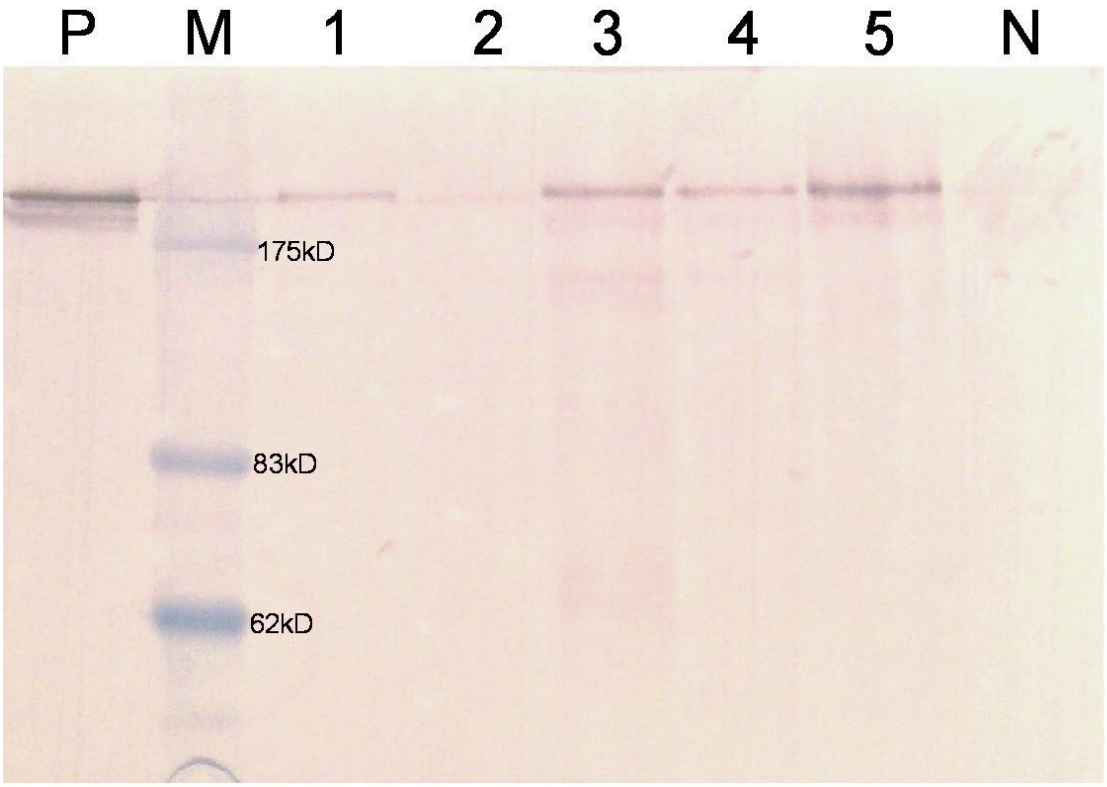


Figure 23. Western blot analysis of full-length recombinant antibody. Western blot analysis of full-length recombinant antibody expressed in *N. benthamiana* plants. Immunoblot after 7.5% SDS-PAGE in non-reducing condition. Lane M: Prestained protein marker with molecular weight labeled. Lane P: positive control of 100 ng antibody expressed by mammalian expression system. Lane 1-5. 10 ul of total soluble protein extracted from recombinant *Agrobacterium*-infiltrated leaves. Lane N: 10 ul of total soluble protein extracted from *Agrobacterium*-infiltrated leaves (negative control).

Discussion

We report a viral expression system based on a version of FoMV which is hyper-replicated in plants. This vector was efficiently delivered to cells by agroinfection with the co-expression of an RNA-silencing suppressor protein. By the removal of the TGB and CP genes to inhibit viral movement and greatly reduce the size of viral vector, the system allow the target protein to be produced to high levels similar to the best viral vectors ever reported before. The FECT constructs used in this study when p19 was coinfiltrated produced GFP levels in the same order of magnitude as the highest achieved with the TMV vector used in that study with 100% infection of plant cells (Lindbo, 2007; Marillonnet et al., 2005; Komarova et al., 2006).

In the process of agroinfection, the cDNA form of FoMV was introduced into the nucleus of plant cells as a part of T-DNA of Ti plasmid carried by *Agrobacterium* and the transcription of viral genome was driven by plant promoter 35S in plant nucleus. After FECT/GFP T-DNA is transcribed, the transcripts of viral RNA can be delivered into cytoplasm and initiate viral replication and gene expression in the cytoplasm. FECT/GFP expresses the RNA dependent RNA polymerase (RdRp) - replicase, which is the only viral open reading frame retained in the viral vector. Replicase directs the synthesis of negative strand viral RNA and subsequently the synthesis of positive strand viral genome and subgenomic RNA containing the gene of GFP. Because the FECT/GFP replicon cannot express the FoMV TGB and CP, it should not move cell-to-cell in the inoculated leaves and move long distance systemically.

FECT replicons without TGB and CP can be employed as a tool for expression of heterologous proteins in plants while ensuring the safety of the environment. For

example, one highly effective means of producing a plant-based vaccine is to infect the plants with recombinant viruses carrying the desired antigen that is driven by an additional subgenomic promoter or is fused to viral coat protein to display on the surface of virion. However, full length viral genomes will produce infectious virions, creating a potential biosafety issues. FECT replicons without CP and TGB genes cannot form infectious virions and cannot move systemically or even cell-to-cell. In fact, the poor GFP expression seen in inoculations lacking silencing suppressors indicates a very low rate of viral replication and an eventual elimination of the virus. Thus, on several levels, the FECT vector has much less liability in terms of environmental safety than do full length viral vectors.

As a deconstructed virus, another advantage of the FECT viral vector is a shorter period of evolution with each infection. This should allow for larger inserts to be stably expressed. Large inserts are deleted due to recombination, which results in smaller viral RNAs which are replicated more quickly and take over a viral population (Dawson *et al*, 1989). Evolution in full length vectors occurs over many replication periods interspersed with periods of spread from cell-to-cell or through the phloem. However, each FECT vector muliplies only in a single cell, allowing it, in theory, to carry larger heterologous genes more stably. For example, we were able to stably produce the 1.7 kb GUS construct, which is unstable in full length TMV vectors.

The control provided by an on/off switch, represented by the gene silencing suppressor and FECT under the control of an inducible promoter, may be useful to prevent leakiness in future inducible transgenic plants. For biotechnological applications, it is highly desirable to express genes in a controllable strategy. Under the control of an

inducible promoter, the target gene can be expressed at a specific duration of interest. The inducible system can be utilized to control the expression of a protein that is toxic to plant cells. To establish a reliable inducible expression system, it has to be tightly controlled and highly inducible and respond only to specific inducers. Several chemical-inducible viral expression systems have been developed. A chemically inducible brome mosaic virus (BMV)-based viral vector was controlled by the dexamethasone (DEX) glucocorticoid-inducible transcription system for production of human interferon in transgenic *N. benthamiana* (Mori *et al*., 2001). Bean yellow dwarf virus was used to transgenic express in cell culture up to 1.2% TSP without the use of p19 (Zhang and Mason, 2006). A cucumber mosaic virus inducible viral amplicon (CMViva) expression system has been developed that allows for tightly regulated chemically inducible expression of heterologous genes in *N. benthamiana* leaves (Sudarshana *et al*., 2006). These inducible systems have been successfully used in various studies, but they also show some drawbacks with with either background expression and/or low expression levels. The FECT system may provide both high expression and a tightly regulated system with minimal leakage in the absence of both inducer and gene silencing suppressor. Without the gene silencing suppressor, the protein expression of FECT was tightly controlled by gene silencing. If chemical inducible promoters could be added in front of both FECT and p19, they should be able to tightly control the timing of gene expression and resulting protein production even in non-transgenic plants. It will be interesting to determine how protein expression in non-transgenic plants utilizing the FECT system compares with that in other inducible virus systems.

Agrobacterium-mediated transient expression is a useful tool for assessing gene expression constructs in plants. It is rapid, giving fairly good results 3 to 4 days after inoculation, and has been demonstrated to work in whole leaf tissue with a range of plants (Levy *et al*., 2005; Marillonnet *et al*., 2005; Hoffmann *et al*., 2006), as well as in a range of cell culture systems (Collens *et al*., 2007; O'Neill *et al*., 2008). However, the method is limited in its applications because expression levels are low and transient in nature, because the protein production disappears after less than 5 days from inoculation. Post-transcriptional gene silencing (PTGS) is the limiting factor in *Agrobacterium*-mediated transient expression in tobacco. By mixing *Agrobacterium* cultures prior to agro-infiltration, one carrying a viral binary expression vector for the gene of interest and another carrying a binary expression vector for a suppressor of gene silencing, high level expression of the gene of interest can be achieved. Expression also persists for much longer, up to 12 days post inoculation and longer. Indeed the time period of expression may only be limited by senescence of the infiltrated plant tissue. As well as applications where rapid analysis of gene expression constructs in plants is required, the system could easily be scaled up to be a rapid and efficient protein production system.

Without the co-expression of the gene silencing suppressor, the slight level of transient expression of FECT/GFP usually peaks at three to four days post-inoculation and declines rapidly thereafter. We presume that post-transcriptional gene silencing (PTGS) limits the efficiency of *Agrobacterium*-mediated transient expression to cause this short transient expression period. Viral replicase-mediated plant virus resistance was definitely attributed to RNA silencing (Marano and Baulcombe, 1998). Replicase is the

only viral open reading frame reserved in the FECT viral vector, so the transcripts of viral replicase may be a potential gene silencing inducer.

Transient GUS expression in switchgrass leaves was accomplished by agroinfiltration (VanderGheynst *et al*., 2007), the assays showed low expression levels and only scattered individual cells. This is only case that was successful with agroinoculation in grasses. A host range study was attempted with FECT/GUS and FECT/GFP in monocots. However, GUS expression was detected in *Agrobacterium* cultures in absence of agroinoculation. Only limited cells were infected by FECT/GFP in grasses. Low transformation efficiency in monocots via agroinoculation is probably the reason for such low rates of infection. Co-agroinfection of the same cell with FECT and p19 is considered as the solution to solve the problem in grasses. An intron-containing GUS reporter gene has to be incorporated in the FECT/p19 viral vector and its application in grasses and also protoplasts will be investigated in the future.

In this study, we demonstrated the transient expression of antibody with FECT viral vector. The heavy chain and light chain were cloned separately and expressed together using the mixture of two viral vector constructs carried by *Agrobacterium*. The expected molecular size (170kDa) in SDS-PAGE and the recognition by anti-Fc IgG in western blot indicate that the full length antibody was correctly assembled. Furthermore, the recognition of specific antigen Langerin in the ELISA assay demonstrated that correctly assembled functional immunoglobin molecules were formed. The crude total soluble protein material from agroinfiltrated leaves recognized the antigenlangerin in a pattern similar to the mammalian system expressed antibody. Thus, presence of correctly

assembled full-length recombinant Ab chains and binding to the corresponding Ag-langerin has been demonstrated in ELISA and immunoblot.

Plant glycosylation in recombinant antibodies has been a concern in therapeutic applications, due to the possible induction of allergic reactions in humans (Gomord *et al.*, 2005). Humanizations of glycosylation in plant-based recombinant proteins were attempted in recent researches. Human immunoglobulins produced in tobacco plants with transgenic human β-1,4-galactosyltransferase had galactose residues and low levels of plant-specific xylose and fucose residues, but immunoglobulins produced in wild type tobacco plants had no galactose and contained high levels of xylose and fucose (Bakker *et al*., 2006). Meanwhile, gene silencing has been used to disable the plant-specific fucosyltransferase and xylosyltransferase enzymes, which incorporate xylose and fucose sugar residues to proteins. RNA silencing for lower expression of the plant enzymes was used to produce human antibodies with low amount of xylose and fucose incorporation in duckweed (Cox *et al*., 2006). The properties of the recombinant antibodies should be studied in more detail with a product coming from the mutated antibody without these plant-specific glycans.

In general, an effective heterologous expression platform for production of plant-derived biomedicals has been established, in which the deconstructed vector virus overcomes restrictions on the size of insert and potential problems of bio-contamination. High efficiency of agroinfection and high expression level make FECT transient expression system a useful approach to test candidate genes for their characteristics before generating stably transformed transgenic plants. Also, it is possible now to develop an on/off switch system under the control of a chemically-inducible promoter to

create inducible transgenic plants. The future application of this viral vector could be in edible vaccine overexpression and also for molecular biology and molecular virology studies.

CHAPTER FOUR

Plant-expressed Recombinant Mountain Cedar Allergen Jun a 1 is Immunogenic and has Pectate Lyase Activity

Abstract

Mountain cedar (*Juniperus ashei*) pollen causes severe allergies in Texas and the central USA. Jun a 1 is the dominant allergen protein of mountain cedar pollen. Recombinant Jun a 1 was expressed in *Nicotiana benthamiana* using an agroinfection-compatible tobacco mosaic virus vector and isolated in good quantity from the apoplast by vacuum infiltration (100 μg/g leaf material). The recombinant protein samples appeared authentic as characterized by SDS-PAGE and N-terminal amino acid sequence analysis. Monoclonal anti-Jun a 1 antibodies detected the recombinant Jun a 1 in an immunoblotting assay. IgE antibodies from the sera of cedar-hypersensitive allergic patients reacted with the recombinant protein in ELISA. Purified recombinant Jun a 1 induced the beta-hexosaminidase release from RBL SX-38 cells stimulated by sera from cedar pollen allergy patients. A severe necrotic reaction occurred in plants in the later stages of infection with the viral vector carrying Jun a 1 or a control pectate lyase gene from banana. Pectate lyase activity was detected from plant extracts, suggesting the cause of the plant necrosis. Our inexpensive and rapid expression system should prove useful for structural studies based on molecular engineering and for diagnostic and therapeutic purposes.

Introduction

Cedar pollen from the mountain cedar *Juniperus asheii* is a major cause of seasonal hypersensitivity in the central United States (Goetz *et al*., 1995). It is one of the most common sources of IgE-mediated allergic disease in early springtime. Jun a 1, the major allergen protein of this pollen, was isolated and characterized by Midoro *et al.* in 1999 (Midoro *et al.*, 1999-1). The cDNA of Jun a 1 was cloned and sequenced by the same group in 1999 (Midoro *et al*., 1999-2). The sequence shows Jun a 1 possesses a high level of amino acid sequence homology with other grouop 1 allergens of cedar pollen. Linear IgE epitopes of Jun a 1 were identified using synthetic overlap peptides and serum IgE from mountain cedar hypersensitivity (Midoro *et al.*, 2003). Crystallization of Jun a 1 extracted from pollen was achieved using the hanging-drop vapor diffusion method in 2003 (Liu *et al*., 2003) and its crystal structure was determined in 2005 (Czerwinski *et al.*, 2003).

Group 1 allergens are structurally similar to bacterial pectate and pectin lyases (Czerwinski *et al.*, 2005). Pectate lyase is an enzyme secreted by microorganisms and is important in plant pathogenesis (Keen *et al*., 1984). These pectolytic enzymes attack the plant cell wall, randomly cleaving the α-1,4 glycosidic bond of pectate, the major component of plant cell walls. Pectate lyases also promote germination by pollen grains and the ripening (softening) of fruits (Carpita *et al*., 2000, Marin-Rodriguez *et al.*, 2003). Although the highly homologous Japanese cedar allergen Cry j 1 was found to have pectate lyase activity (Taniguchi *et al.*, 1995), Jun a 1 has not yet demonstrated any enzymatic activity (Czerwinski *et al.*, 2005). The putative active-site residues of pectate lyase are conserved in the Jun a 1 and Cry j 1 sequences (Midoro-Horiuti *et al.*, 1999).

Investigating the enzymatic activity of Jun a 1 would help explain the amino acid sequence and structure homology of group 1 allergens with pectate lyase.

There are several structural features of Jun a 1 which make it very similar to pectate/pectin lyases. The core of the Jun a 1 structure consists primarily of a parallel beta-helix, which is nearly identical to that found in the pectin/pectate lyases from several plant pathogenic microorganisms. Though Jun a 1 has only 20-50% sequence identity with Pel and Pnl proteins of microorganisms, the structure is conserved (Czerwinski *et al*., 2005). Pnl and Pel proteins contain two highly conserved sequences, vWiDH and RxPxxR (uppercase letters indicate identity residues between enzymes, the v and i indicate conserved residues, and x any residue) along with a characteristic beta helical core (Herron *et al*., 2000). The residue sequences vWiDH and RxPxxR, and Ca^{++} binding sites are also present in Jun a 1. According to the hypothesis (Czerwinski *et al*., 2005), the inactivity of Jun a 1 is due to steric hindrance of the enzyme's active site. The first 30 residues of the N terminus cover the conserved site vWiDH by making a complex loop. vWiDH site has been found to play an important role in the export and proper functioning of enzyme (Kita *et al*., 1996). In addition, the salt bridge between Asp177 and His203 traverses the presumed substrate-binding groove in the vicinity of the putative active site at Arg229 (Czerswinski *et al*., 2005). Mutations were proposed which should expose the pectolytic site and result in the activity of Jun a 1. Truncation of N terminus of Jun a 1 should eliminate the complex loop covering the vWiDH site. A His203 mutant will remove the histidine-aspartate bridge covering the substrate binding groove at this site (Czerswinski *et al*., 2005).

Currently, treatment of allergic disease is to suppress the immune reaction to the allergen by specific immunotherapy. Desensitization therapy with crude extracts from cedar has met with limited success, with only 30% of patients responding after 2 years of weekly injections (Platts-Mills *et al*., 1998). The allergen extracts are hard to standardize as they contain mixtures varying from extract to extract yielding little consistency. There is a rising need for recombinant protein for causing IgE – mediated diseases. Successful expression of recombinant allergen will aid in the development of more effective allergy diagnosis and vaccines.

Plant-based expression systems are considered ideal for the recombinant production of allergen protein of plant origin (Breiteneder and Wagner, 2002). Plant expression systems have advantages compared to other methods of recombinant protein production - the comparatively low cost of large-scale production; much cheaper and easier cultivation without sterile condition of cell culture; the eukaryotic protein modification machinery, allowing sub-cellular targeting, proper folding, and post-translational modifications; the absence of human or animal pathogens (Streatfield, 2005).

Plant viral vectors can be applied for the rapid, inexpensive, high level, transient expression of allergen proteins. A number of different plant viruses have been developed as protein production vector, including tobacco mosaic virus (TMV), potato virus X (PVX) (Pogue *et al*., 2002; Richard *et al*., 2003; Yusibov *et al*., 2006).

Heterologous recombinant allergens have been produced in plants via a tobacco mosaic virus (TMV) vector (Krebitz *et al*., 2000, 2003; Gadermaier *et al*., 2003). However, previous TMV vectors have to be in vitro transcribed to generate capped

infectious RNA using RNA polymerase, which is a time-consuming procedure with expensive materials. Agroinfection provides a high efficient viral infection platform without in vitro transcription. Agroinfection is a quick and highly efficient delivery strategy for transient expression using viral replicons. *Agrobacterium tumbfaciens* suspension harboring T-DNA containing the viral genome is syringe-infiltrated into a plant leaves. Almost 100% of cells take up a copy of the viral vector into their chromosomes and from this transgene the virus launches an infection (Gleba *et al*., 2005; Linbo *et al*., 2007).

Moreover, extraction from ground leaves can make purification difficult. This can be overcome by trafficking the allergen protein to the apoplast (interstital area), where there are few native proteins, using the allergen's own signal peptide. Expressing these allergens as secreted proteins allows the protein to be extracted from the apoplast by vacuum infiltration of the leaf with buffer. The leaf is then centrifuged, apoplastic fluid (dew) is found at the bottom of the tube while the leaves remain intact in the top basket. This approach achieves a high purity of initial purification step by eliminating most plant cellular proteins (McCormick, *et al*., 1999; Moehnke *et al*., 2008).

RBL SX-38 cells are rat basophilic leukemia cells expressing human FcεRI, which is the human high-affinity receptor for IgE and including α, β, and γ chains (Wiegand *et al*, 1996). These cells can bind IgE from the patient sera of allergic individuals and be activated (Wiegand *et al*, 1996). After sensitization with IgE from allergic human patients, RBL SX-38 cells can deregulated upon exposure to specific allergen (Dibbern *et al*., 2003). This is a useful model system to explore IgE-allergen interactions and to analyze the biological activity of recombinant allergens.

Jun a 1 protein has not been successfully expressed in a heterologous system, though repeated attempts have been made. The production of Jun a 1 proteins in bacteria, insect cells or other non-plant systems has been hampered by several problems such as toxicity, incorrect processing, incorrect folding, and insolubility (R. Goldblum, personal communication). Here we describe the expression of recombinant Jun a 1 (rJun a 1) in *Nicotiana benthamiana* using an agroinfection-compatible tobacco mosaic virus vector. It is the first report of the expression of immunogenic recombinant Jun a 1 and the first report of pectate lyase activity for this allergen.

Materials and Methods

Construction of the TMV-Jun a 1 Recombinant Expression Vector

We utilized the agroinfection-based TMV expression vector JL36 (Lindbo *et al*., 2007). pJL36 contains the full components of TMV genes as well as an additional subgenomic promoter with a multiple cloning site (*Pac*I, *Avr*II and *Not*I). The Jun a 1 ORF and the point mutation (his^{203} to ala) , with its native signal peptide, were amplified to add *Pac* I and *Avr* II sites to a previously made Jun a 1 containing constructs (Midoro-Horiuti *et al*., 1999). The point mutation (his^{203} to ala) was generated by site directed mutagenesis of the Jun a 1 ORF (NIEHS Center Molecular Genetics Core, UTMB). The oligonucleotide primers used for 5' and 3' ends were: Jun a 1 Up (5' CCATCC TTAATTAA ATGGCTTCCCCATGCTTA - 3') and Jun a 1 Down (5' CCATCC CCTAGG TTAGGTTACAACTCCAGC - 3') (*Pac*I and *Avr*II sites underlined). The 38-base N-terminal truncation and 38-base N-terminal truncated his^{203} to ala mutant of Jun a 1 were created using appropriately designed 5'end primer (5' CGG TTAATTAA

ATGGCTTCCCCATGCTTAATAGCAGTCCTTGTTTTCCTTTGTGCAATTGTATCT TGTTACTCT GATTTTTACACCGTCACAAG 3') by UTMB NIEHS Center Molecular Genetics Core.The amplified fragments were then cloned into *Pac*I-*Avr*II digested JL36 to create Jun a 1/JL36 (Fig 22). Phusion DNA polymerase (NEB, Beverly, MA) was used according to company protocols. Recombinant clones were screened by PCR using Taq polymerase (NEB, Beverly, MA) or restriction digest and then sequenced using a CEQ capillary sequencer (Beckman Coulter, Fullerton, CA).

Agro-inoculation of N. benthamiana *Plants*

Agrobacterium tumefaciens LBA4404 was used for the agroinoculation of *N. benthamiana*. Plasmids were transformed into *A. tumefaciens* LBA4404 by electroporation at 1.44 kV and 129 Ω for 5 ms using a BTX 600 Electro Cell Manipulator (BTX Inc., San Diego, CA, USA). *A. tumefaciens* transformants were selected on LB plates containing 10 μg/ml rifampicin, 30 μg/ml streptomycin and 50 μg/ml kanamycin. Individual colonies of *A. tumefaciens* transformed with a binary plasmid were grown to an OD600 of 1.0 in liquid LB media supplemented with 10 mM MES pH 5.7, the same antibiotics, and 20 μM acetosyringone. Cells were collected by centrifugation and resuspended in 10 mM MES, pH 5.7, 10 mM $MgCl_2$, and 200 μM acetosyringone (Johansen *et al*., 2001).

After 2–8 hours incubation, *N. benthamiana* leaves were infiltrated into the abaxial surface using a 3 ml syringe with no needle.

Detection of Viral RNA and Extraction of Jun a 1 Protein

Symptoms of infection were visible after 5–6 days as mild leaf deformation, with some variable leaf mottling and growth retardation. Seven to 12 days after inoculation, as necrosis commenced, systemic leaves (~500 mg) from each plant were used for total RNA extraction with TRI Reagent (Sigma, St Louis, MI). The RNA served as a template for RT-PCR using the screening primers: (5' J1sigScr) 5'ATGGCTTCACCATGCTTAAT3' and (3'J1Scr) 5'TTAGGTTACAACTCC AGCAT3'. Eleven to 14 days after inoculation, leaf and stem material was harvested, weighed, and then subjected to a 700 mmHg (93 kPa) vacuum for 2 min in infiltration buffer (100 mM Tris.Cl, pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA). Secreted proteins were then recovered from infiltrated leaves by mild centrifugation at 2,000 × g (Beckman JA-14) on supported nylon mesh discs (McCormick *et al*., 1999). The apoplastic fluid containing recombinant protein was collected at the bottom of the tube while the leaves remain intact in the top basket.

Detection of Pectate Lyase Activity by Mass Spectrometry

A mixture of galacturonic acid oligomers was produced by autoclave hydrolysis of pectic acid as described by Robertsen (Robersen, 1986). $GalA_9$ was purified from the mixture by chromatography on a 22 x 250 mm PA1 anion exchange column (Dionex Corp., Sunnyvale, CA) as described previously (Zheng and Mort, 2008). The GalA9 oligomer was labeled with aminopyrene trisulfonic acid and purified away from labeling reagents following procedures of Naran et al (Naran *et al*., 2007).

Five hundred ug of galacturonic acid oligomers, GalA9, was dissolved in 25 ul 0.1 M tris buffer, pH 8.5 and mixed with 0.5 ul 5mM $CaCl_2$ and 25 ul of recombinant Jun

a 1. Two ul of toluene was added to prevent microbial growth. The progress of the digestion was followed by taking 1 ul aliquots, labeling the oligomers with aminonaphthalene trisulphonic acid and analysis by capillary zone electrophoresis. After 4 days incubation at room temperature the oligomers were desalted by passing through a Shodex protein KW-802.5 gel permeation column (Shodex, Inc., New York, NY) in 200 mM ammonium acetate buffer with detection via a refractive index monitor. Oligosaccharide fractions were analyzed using a Perseptive Biosystems (www.appliedbiosystems.com/) ABI Voyager De-Pro MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) in the negative ion reflector mode with a matrix of 100 mg/ml trihydroxyacetophenone in methanol. These pectate lyase determinations were carried out by collaborator Andrew Mort of Oklahoma State University.

Purification of rJun a 1

Recombinant Jun a 1 was purified with HiTrap NHS-HP anti-Jun a 1 affinity column chromatography. The column was prepared by washing with 3 ml binding buffer (PBS, pH 7.4, 0.01% NaN_3). Recombinant Jun a 1 sample was centrifuged at full speed for 5 minutes and filtered through a 0.45 um filter before it was applied to the column. The column was equilibrated with 10 ml binding buffer; sample flow rate was then 0.2 ml/min. Following a wash with binding buffer, the first 1 ml effluent was collected, followed by a second 10 ml wash. Elution was carried out with elution buffer (0.2 M Glycine-HCl, pH 2.6) and 1 ml purified fractions were collected. The column was re-equilibrated by washing with 10 ml of binding buffer. Purified rJun a 1 was then analyzed by SDS polyacrylamide gel (4-20%, Biorad, Hercultes, CA) electrophoresis and subsequently stained with Coomassie brilliant blue R-250 (Sigma, St Louis, MO). The

concentrations of purified proteins were determined by serial diluted BSA (NEB, Beverly, MA) protein as standard.

SDS-PAGE and Immunoblotting of Recombinant Jun a 1

Total plant extracts of Jun a 1 and purified rJun a 1 were separated on 4-20% Tris-HCl PAGE gels (Biorad, Hercultes, CA) and subsequently stained with Coomassie Blue R-250. For immunoblotting experiments, recombinant Jun a 1 was electroblotted from SDS-PAGE gels onto a PVDF membrane (Amersham) by semi-dry transfer cells (Biorad, Hercules, CA). The blot was blocked with TBST buffer (100 mM tris-HCl, 0.9% NaCl, 0.1% tween 20, pH 7.5) containing 10% BSA at room temperature for 1h and was then incubated with anti-Jun a 1 monoclonal antibodies mixture: s91, L1b-18, L86-10, L27b-21, L20-15 (0.5 ug/ml each) at 4°C overnight. Bound mouse IgG was detected by biotinylated goat antimouse IgG and horseradish peroxidase–streptavidin (Zymed, San Francisco, CA) (Midoro-Horiuti *et al*., 1999). Visualization was performed via ECL detection reagents (Amersham) followed by immunofluorescence detection.

IgE Binding to Native and Recombinant Jun a 1 Allergens

Serum samples were obtained from *J. ashei* allergic patients from Austin, Texas, USA. IgE binding to Jun a 1 allergens was measured by ELISA. ELISA plates were coated with 3 ug/ml native or recombinant Jun a 1 in borate buffer (0.125 M boric acid, pH 8.8, 0.225 M NaCl, 5 mM EDTA) at 37^{o}C overnight. Patients' sera were serially diluted into 1:4; 1:16; 1:64 for the detection of specific IgE. Coated plates were incubated with sera (IgE) at room temperature for two hours. Bound IgE was detected by adding biotin conjugated goat anti-human IgE (1:300) (Sigma) and horseradish-

peroxidase (HRP) -coupled streptavidin (1:1000) (Sigma, St. Louis, MO) with shaking at room temperature. Colorimetric substrate, FAST OPD (Sigma, St. Louis, MO), was added and incubated for 20 min at room temperature. The reaction was stopped by adding 5 N H_2SO_4 and the OD value at 492 nm was measured.

RBL SX-38 Cell Culture and Beta-hexosaminidase Release Assay

RBL SX-38 cells were cultured in DMEM with 10% FCS, 1% penicillin/streptomycin and 1.2 mg/ml G418 (Invitrogen, Carlsbad, CA) (Wiegand *et al*., 1996). Cells were subcultured in 96 wells plate at 1×10^5 cells/well in DMEM without G418, at 37°C, 5% CO_2 over night. Cells were sensitized with Jun a 1 patient serum (1:10 dilution), 100 ul/well, at 37°C, 5% CO_2 for over night. Cells were washed with warmed HEPES-Tyrode's buffer (137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES and 0.1% BSA, pH 7.3) three times, 100 ul/well. Native or recombinant Jun a 1 allergen and A23187 were added and incubated at 37°C, 5% CO_2 for 30 min. Twenty five ul supernatants were transferred to new 96 well plates. Five ul of 1.2% Triton X100 and 30 ul NAG (p-nitrophenyl-N-acetyl-beta-D-glucopyranoside) (Sigma) buffer were added in the well at the same time. The NAG buffer was made with 4 volumes 0.01 M NAG and 1 volume 0.4 M pH 4.5 citrate buffer. The 96 well plates were shaken at 37°C for 2 hr. To stop the reaction, 20 ul 1 N NaOH was added per well and the plate was read at 410 nm (Dastych *et al*., 1999; Zhu *et al*., 1998).

Results

Expression and Identification of rJun a 1 in N. benthamiana *Plants*

The Jun a 1 and mutant coding sequences, including its signal peptide, were successfully amplified and inserted into the pJL36 vector under the control of the TMV-U1 coat protein subgenomic promoter (Fig 24). Insertions of the gene, as well as correct orientation, were verified with PCR and DNA sequencing. RT-PCR products confirmed that the Jun a 1 sequence was stably maintained in the TMV vector.

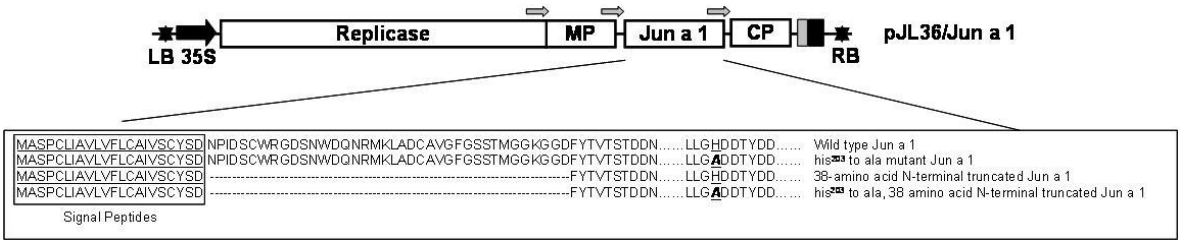
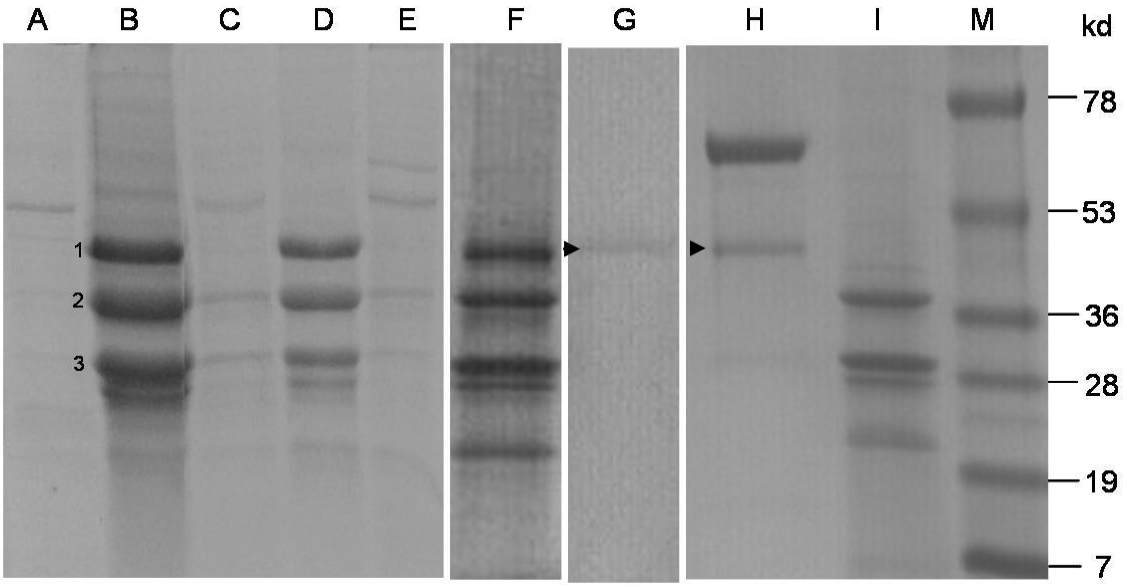


Figure 24. Diagram of binary plasmid pJL36/Jun a 1 and mutant variants. Diagram of the T-DNA regions of binary plasmid pJL36/Jun a 1 and schematic representation of mutated Jun a 1 variants. Black stars: left border and right border of T-DNA. Black arrow, CaMV duplicated 35S promoter. Black box, CaMV polyA signal sequence/ terminator. Gray box, Ribozyme. Gray arrows, Subgenomic promoters. ORFs are represented by white boxes. Identities of ORFs are labeled in white boxes. Replicase, TMV 126K/183K ORF; MP, movement protein; Jun a 1, mountain cedar allergen protein; CP, coat protein.

Recombinant wild type and his^{203} to ala mutant Jun a 1 protein were expressed in good quantity in *N. benthamiana* plants. SDS-PAGE revealed a Coomasie-Blue-stained band migrating at 44 kDa which was not present in the empty vector or unifected control plants (Fig 25-IB.). However, no protein was detected for the truncation mutants of Jun a 1. Recombinant Jun a 1 was expressed at 100 ug from the vacuum infiltrate of 1g of intact leaves, as estimated from a seriallt diluted BSA standard. Also, rJun a 1 was

purified from infected *N. benthamiana* leaves by using HiTrap NHS-HP affinity column (Fig 25-I-H.) yielding 6 ug rJun a 1 per gram of leaf tissues. The rJun a 1 protein was

I.



II.

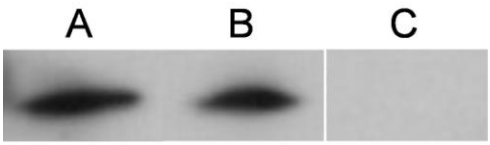


Figure 25. Protein analysis of rJun a 1 expression and purification.
I. SDS-PAGE analysis of rJun a 1 expression and purification. Protein samples were separated by gradient SDS-PAGE (4-20%) and subsequently stained by Coomassie brilliant blue; Arrow: Purified Jun a 1. Lane A. *N. Bentamiana* negative control; Lane B, F. plant extract of wild type Jun a 1; Lane C. 38 amino acid N-terminal truncated Jun a 1; Lane D: his^{203} to ala mutant Jun a 1; Lane E: his^{203} to ala, N-terminal truncated Jun a 1; Lane G: Purified recombinant Jun a 1; Lane H. Purified recombinant Jun a 1 with protective BSA; Lane I. Monitoring of the flow-through (FT) of affinity purification; Lane M. Protein Marker, Marker band sizes (in kDa) are listed.

MALDI-TOF analysis of the three bands from TMV/Jun a 1 (Lane B, F) shown band 1 as Jun a 1, band 2 as β-1,3-glucanase and band 3 as chitinase, which are apoplast-residing PR-proteins responding to the necrosis.

II. Western blot analyses of wild type Jun a 1 and his^{203} to ala mutant Jun a 1. A. Recombinant wild type Jun a 1; B. his^{203} to ala mutant Jun a 1; C. uninoculated *N. Bentamiana* as negative control.

totally removed from the Flow Through of purification procedure (Fig 25-I.). These results were confirmed by western blotting with the anti-Jun a 1 antibodies (Fig 25-II.).

N-terminal Amino Acid Sequence

MALDI-TOF analysis on the bands shown determined one band as Jun a 1; the other two bands as β-1,3-glucanase and chitinase, which are apoplast-residing pathogenesis response proteins which are commonly induced by tissue necrosis (Fig. 25-I.). N-Terminal sequencing of this band revealed that the signal sequence of the rJun a 1 was correctly eliminated by the plant.

Symptoms of Jun a 1 Expressing Plants

Symptoms of viral infection were visible after 5–6 days inoculation as mild leaf deformation, with some variable leaf mottling and growth retardation. Ten days post-inoculation, signs of TMV infection, such as mosaic and deformed leaves, were apparent not only in locally inoculated leaves but in systemic leaves as well.

Jun a 1 protein expression was always associated with a necrotic phenotypic response in plants. Twelve days post-inoculation, Jun a 1 caused a necrotic response in *N. benthamiana*, starting veinally then spreading through the plant. Protein was collected from seven to eleven days after inoculation before the severe necrosis occurred. Complete plant death culminated at 20-30 days after inoculation (Fig 26). This necrosis and death phenomenon was perfectly duplicated by the expression of banana pectate lyase (Marín-Rodríguez *et al.*, 2003) in the JL36 TMV expression vector on *N. Bentamiana* (data not shown). A his^{203} to ala mutant was created in order to remove the histidine-aspartate bridge which is thought to cover the putative catalytic site. Plants

inoculated with wild type and his^{203} to ala mutant of Jun a 1 have veinal necrosis and sudden death of infected plants. None of truncation mutants infected plants had necrosis or sudden death (Fig 26), but the viral RNA associated with these was found to have deleted the Jun a 1 sequence (data not shown).

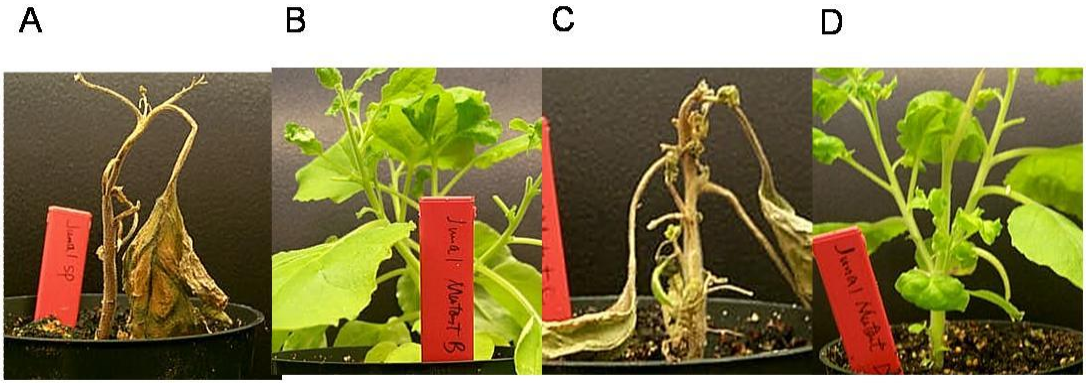


Figure 26. Symptoms of Jun a 1 expressing plants. Agroinfected *Nicotiana benthamiana* plant was expressing Jun a 1 protein (30 days after infiltration). Severe necrosis, leading to plant death, resulted from infection with TMV carrying Jun a 1. A: wild type Jun a 1, B: 38 amino acid N-terminal truncated Jun a 1, C: his203 to ala mutant, full length Jun a 1, and D: his203 to ala, N-terminal truncated Jun a 1. Severe necrosis, leading to plant death, resulted from infection with TMV carrying wild type and his203 to ala mutant Jun a 1. Truncation mutants were able to infect plants but none of the infected plants had necrosis or sudden death.

Detection of Pectate Lyase Activity by Mass Spectrometry

Recombinant Jun a 1 protein was submitted to collaborator Andrew Mort (Oklahoma State University) for pectate lyase activity analysis. One microliter of native Jun a 1 solution was incubated with a small amount of a fluorescently labeled nonamer of galacturonic acid (GalA9). Pure fluorescent oligomers incubated with Jun a 1 were converted into shorter oligomers in a time-dependent manner. After only 30 min about half of the oligomer had been converted to smaller products by native pollen Jun a 1; the same products were produced by our recombinant Jun a 1 from *N. benthamiana*. There was only a slight amount of pectate lyase activity in the uninoculated *N. benthamiana*

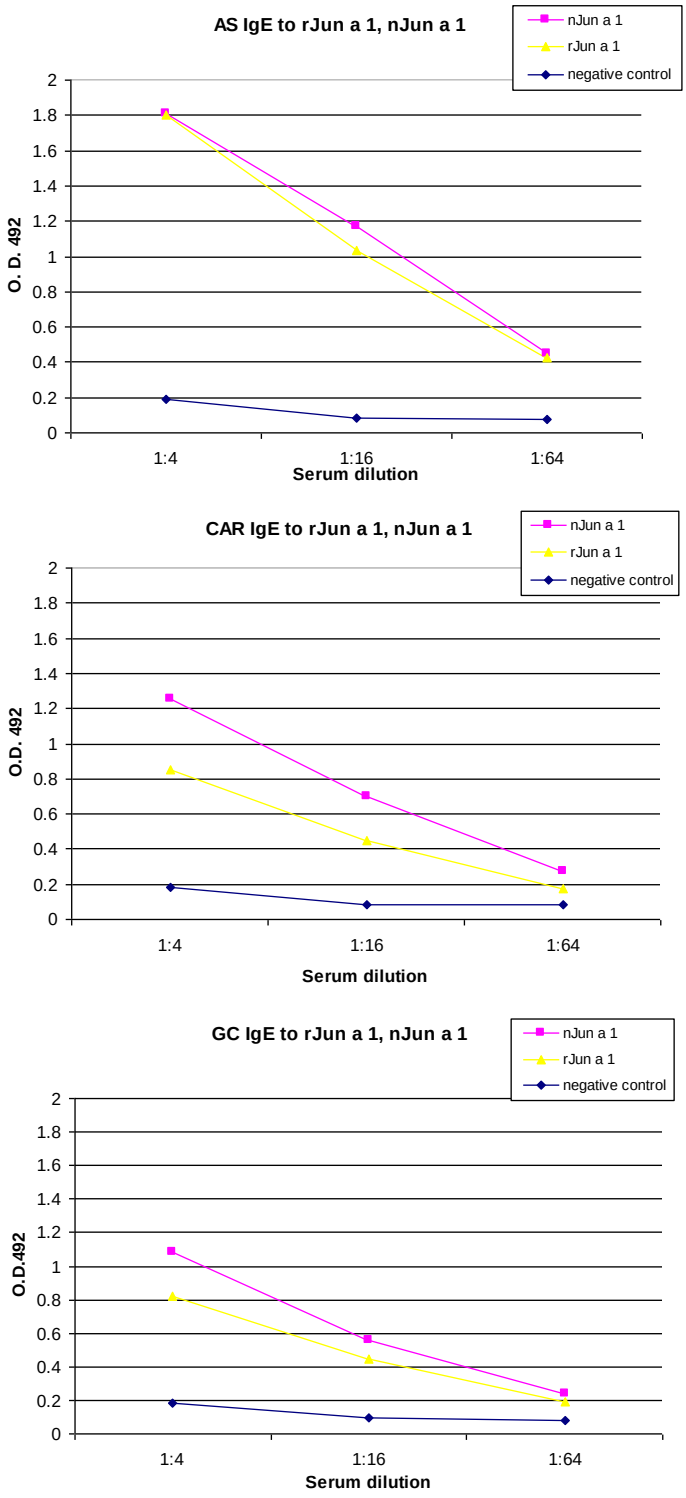


Figure 27. Human IgE ELISA assay of native and recombinant Jun a 1. ELISA showing reactivity of IgE from three allergic patient sera (AS, CAR and GC) with native and recombinant wild type Jun a 1 expressed in *N. benthamiana*, and with uninoculated *N. benthamiana* as negative control.

apoplast control sample. Since pectate lyase activity was detected from both native and recombinant Jun a 1, this suggests the cause of the plant necrosis and perhaps the cause of the allergenicity

IgE Binding Capacity of Recombinant Jun a 1 Allergens

To test the binding capacity to IgE in the patient sera, serial dilutions of patient sera were tested in ELISA experiments. For each antibody concentration, three determinations were performed and mean values are given (Fig 27.). ELISA experiments demonstrated that IgE antibodies from sera of subjects with *J. ashei* pollinosis bound to the recombinant Jun a 1 extracted by vacuum infiltration, and the IgE bindings were significantly increased relative to the negative control of uninoculated *N. benthamiana* plants.

Purified Recombinant Jun a 1 Induces Degranulation of RBL SX-38 Cells

To demonstrate specific beta-hexosaminidase release capacity of recombinant Jun a 1, RBL SX-38 cells were cultured and stimulated with patient sera of cedar pollen allergy. Activated basophils were incubated with purified recombinant Jun a 1, and with native Jun a 1 as positive control and the flow-through (FT) sample of affinity purification (refer to Fig 25-I) as negative control. Fig 28 shows that purified recombinant allergens induced specific and dose-dependent beta-hexosaminidase release in cultured basophils. Comparing the IgE reactivity of the patients displayed in the ELISA in Fig 27 with beta-hexosaminidase release capacity of recombinant allergens, all patients sera with IgE antibodies specific for the recombinant mountain cedar pollen allergens also had positive beta-hexosaminidase release results (Fig 28.).

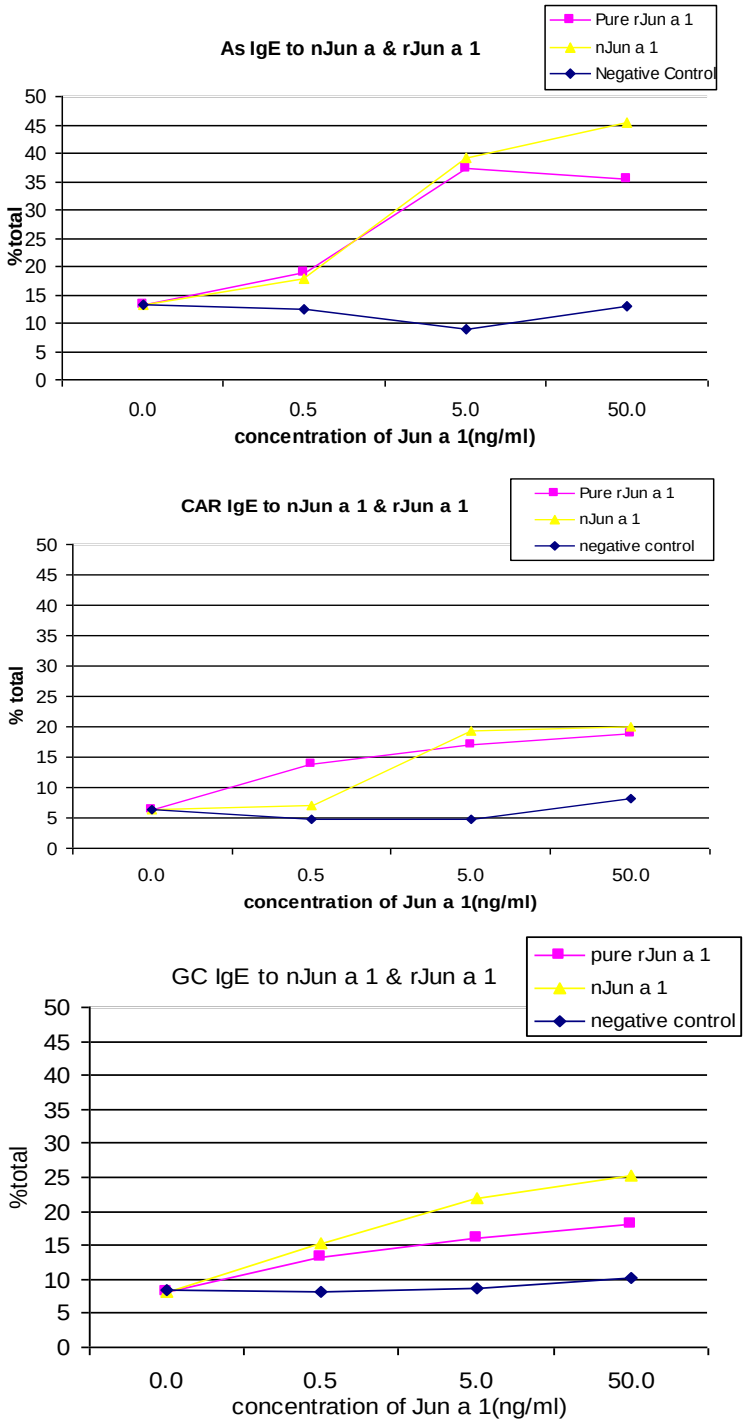


Figure 28: Biological activity of wild type and recombinant Jun a 1. Induction of beta-hexosaminidase release from RBL SX-38 cells sensitized with sera from mountain cedar allergic patients was stimulated with both purified native and recombinant allergen Jun a 1, and with the flow-through (FT) sample of affinity purification (refer to Fig 25-I) as negative control. Humanized rat basophilic leukemia cells, RBL SX-38 cells, were incubated with three mountain cedar pollen-allergic patients sera (AS, CAR and GC) and then were incubated with various concentrations (ng/ml) of allergen samples (x-axis) and the percentage of total beta-hexosaminidase release is expressed on the y-axis.

Discustion

Jun a 1 is the dominant allergen of mountain cedar pollen and has a high degree of cross reactivity with other group 1 allergens. The three-dimensional structures of Jun a 1 consists primarily of a parallel β-helix, which is nearly identical to that found in the pectin/pectate lyases from several plant pathogenic microorganisms (Czerwinski *et al*., 2005). The residue sequences vWiDH and RxPxxR, and Ca^{++} binding sites present in both pectin/pectate lyases and Jun a 1. The present work represents the first demonstration that the mountain cedar allergen, Jun a 1, possesses the pectate lyase activity, which explains the amino acid sequence and structure homology of group 1 allergens with pectate lyase. The allergenicity of Jun a 1 was expected to be due to pectate lyase activity which would facilitate penetration through the cellular glycocalyx (Midoro-Horiuti *et al*., 1999). The epithelium forms a barrier that allergens must cross before they can cause sensitization. Jun a 1 is a pollen specific protein and there is a crucial function for pectate lyases in pollen germination (Taylor and Hepler, 1997). There may be similarities between glycosaminoglycans in the human extracellular matrix and pectin in the plant apoplast. For example, *Treponema pectinovorum*, which is the oral spirochete causing periodontal disease, produces a pectate lyase and is dependent on pectin, pectic acid, glucuronate or galacturonate for in vitro growth (Weber *et al*., 1984). As an analogy, the allergenicity of Der p 1, a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, is dependent on its cysteine protease activity which causes disruption of intercellular tight junctions (TJs), which are the principal components of the epithelial paracellular permeability barrier (Schulz *et al*., 1998). The movement of Jun a 1 through epithelium to dendritic antigen-presenting cells via the

paracellular pathway may be promoted by the allergen's own glycanase activity. Moreover, enzyme active allergen may inadvertently function to induce allergies by releasing oligosaccharides when hydrolyzing mammalian structures, such as the polysaccharide compounds on the luminal surface of epithelial cells (Midoro-Horiuti *et al*., 1999). It is known that when hyaluronan (HA), which is the extracellular matrix component, is cleaved at sites of inflammation, small HA fragments contact and activate dendritic cells (DC) to induce a primary immune responses (Termeer *et al*., 2000). As another example, heparan sulfate is an acidic, biological active polysaccharide in the cell membrane and extracellular matrices. Soluble heparin sulfate, as a product of the degradation of heparin sulfate proteoglycan in inflammation and tissue damage, induces maturation of dendritic cells, and contributes to the primary immune responses (Kodaira *et al*., 2000). Also, heparin sulfate glycosaminoglycans can active macrophages and alternate T cell responses; and the glycosaminoglycans help to regulate the behavior of antigen-presenting cells (APCs) in inflammatory processes (Wrenshall *et al*., 1999).

To determine the structural requirements of pectolytic activity and the possible role of enzyme activity in allergenicity, mutations were created in order to test a structural hypothesis for the enzymatic activity of Jun a 1 (Czerwinski *et al*., 2005, Dissertation by Dr.Shikha Varshney, Baylor University). A his^{203} to ala mutant was created in order to remove the histidine-aspartate bridge which is thought to cover the putative catalytic site. Truncation mutants were made by eliminating the first 38 amino acids which are also posited to inhibit enzymatic activity via the covering on the vWiDH sequence as a complex loop. Recombinant wild type and his^{203} to ala mutant of Jun a 1 were successfully expressed in *N. benthamiana*, but with veinal necrosis and sudden

death of infected plants. Venial necrosis may be due either to pectolytic activity or to some hypersensitive reaction of the plant to the protein. The expressed Jun a 1 protein with his^{203} to ala mutant has the same enzyme activity as the wild type recombinant Jun a 1, which indicates that the histidine-aspartate bridge does not preclude enzyme activity. The truncated versions were unable to be expressed in plants due to insert deletion. The lack of expression on truncations may be due to an excessively high pectolytic activity that destroys the initial plant cells in which the virus replicates. Eliminating the first 38 amino acids from Jun a 1 help to expose the vWiDH site, which play an important role in the export and proper functioning of enzyme (Kita *et al*., 1996) and highly increase the enzyme activity.

Plant viral expression systems, which provides the homologous system to produce plant allergen protein with correct glycosylation, are the only platform that can produce this enzyme active recombinant allergen Jun a 1 before necrosis occurs. Jun a 1 is a glycoprotein of 43kDa with two putative glycosylation sites at positions Asn^{127}ThrSer and Asn^{157}ValThr both of which are located on the surface of the protein (Midoro-Horiuti *et al*., 2003). In bacteria, Jun a 1 has been difficult to express in its soluble, correctly folded state due to the lack of glycosylation necessary for proper folding (R. Goldblum, personal communication). The expression of Jun a 1 in insect cells with baculovirus virus infection has been attempted, but resulted in the death of insect cells by necrosis (R. Goldblum, personal communication). It was observed that in the case of Jun a 1 sequences carried by the TMV vector, protein expression was also always associated with a necrotic response. In addition to the mosaic and leaf deformation that distinguishes TMV-infected from uninfected *N. benthamiana* plants, all plants expressing Jun a 1 also

died of a sudden necrosis at 40 days after inoculation. All of the above demonstrates that the allergen protein Jun a 1 has a highly enzymatic activity which is toxic to the host cells and the plant expression system is the unique system to produce allergen protein Jun a 1 with glycosylation modification before the necrosis occurs.

One of the goals of allergen research is to develop more effective immunotherapy strategies. Current immunotherapy utilizes an allergen vaccine from crude extracts of a specific source plant. It takes months to years to become effective and also carries a high risk for adverse immune responses. New specific immunotherapy focuses on reducing the allergic inflammation by shifting the Th2 response to Th1 response or inducing the blocking antibodies. The molecular mutagenesis of allergens can be used to explore new allergen vaccines, which have reduced IgE binding capacity while still retaining their ability to T cell induction. Recombinant allergens allow developing vaccines for allergy therapy to produce the native allergens and further create the innovative mimic. Plant-based expression systems can provide the platform to produce high quantities of soluble, immunogenic, and, most importantly, biologically active recombinant allergen proteins. The method I describe allows for the convenient and inexpensive expression of the recombinant allergen, Jun a 1, which will allow for further structural studies and may prove useful in diagnostic and immunotherapeutic strategies for cedar allergy.

APPENDIX

APPENDIX

Bench Protocol

Formaldehyde-Agarose Gel Electrophoresis

Reference: Qiagen RNeasy MiniHandbook.

Formaldehyde-agarose gels are used for RNA gel electrophoresis. The presence of formaldehyde in the gel and running buffer, as well as a sample heating step, prevent the RNA from forming secondary structures, thus allowing RNA to run properly on the gel.

1. Prepare a 1.2% gel by combining 360 mg of agarose, 3 ml of 10×FA gel buffer, and 27 ml of DEPC-treated water.
2. Microwave at 50% power to melt agarose. Allow solution to cool to about 65°C.
3. Add 545 µl of 37% formaldehyde and 3µl of ethidium bromide (1 mg/ml).
4. Mix and pour into gel casting tray with the appropriate comb for well formation.
5. Allow gel to polymerize for 30 minutes.
6. Place gel in the electrophoresis chamber and add 1×FA running buffer. Allow gel to equilibrate in buffer for 30 minutes.
7. Prepare samples in 5×RNA loading buffer and incubate 5 minutes at 65°C.
8. Load samples onto gel and run at 60 volts.
9. When gel is finished running, wash in DEPC-water and visualize under UV light.

10x FA gel buffer:

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA, pH to 7.0 with NaOH.

1x FA gel running buffer:

100 ml 10x FA gel buffer, 20 ml 37% (12.3 M) formaldehyde, 880 ml DEPC water.

5x RNA loading buffer:

16 µl saturated aqueous bromophenol blue solution, 80 µl 500 mM EDTA, pH 8.0, 720 µl 37% (12.3 M) formaldehyde, 2 ml 100% glycerol, 3.084 ml formamide, 4 ml 10 x FA gel buffer, RNase-free water to 10 ml, Stability: approximately three months at 4°C.

Quantification of RNA Using Spectrophotometry

Reference: Pharmacia Biotech GeneQuant pro

1. Wash the Quartz cuvette (path length 5 mm) with 1 ml of 0.1 M NaOH, 1 ml of 1mM EDTA, 1 ml of water, and 1 ml of ethanol. Remove any remaining ethanol and allow drying.
2. Turn on the spectrophotometer and wait for the display to say "Instrument Ready".
3. Press "set up" to enter the desired parameters: Select "Base Type-RNA". Set path length to 5 mm. Enter the dilution factor (1 if running an undiluted sample.).
4. Fill the cuvette with 10 µl of the buffer used to dissolve RNA. Place cuvette in the spec and press "set ref". Remove the cuvette when the display reads " Instrument Ready".
5. Remove buffer from the cuvette and fill with 10 µl of sample. Insert cuvette and press "RNA" to read. Record absorbance values. (Pure RNA has A260/A280 = 2.0. A good range is 1.7-2.1)
6. Remove the sample and wash the cuvette with 1 ml of water and 1 ml of ethanol. Remove any remaining ethanol and allow drying.

RNA Extraction from Plant Tissue

Reference: Qiagen RNeasy Plant Mini Kit

1. Determine the amount of plant material. Do not use more than 100 mg.
2. Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.
3. Add 450 µl Buffer RLT to a maximum of 100 mg tissue powder. Vortex vigorously.
4. Transfer the lysate to a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube.
5. Add 225 µl of 100% ethanol to the cleared lysate, and mix immediately by pipetting.
6. Transfer the sample including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Centrifuge for 15 s at maximum speed.
7. Add 700 µl Buffer RW1 to the RNeasy spin column. Centrifuge for 15 s at maximum speed to wash the spin column membrane.
8. Add 500 µl Buffer RPE to the RNeasy spin column. Centrifuge for 15 s at maximum speed to wash the spin column membrane.

9. Add 500 µl Buffer RPE to the RNeasy spin column. Centrifuge for 2 min at maximum speed to wash the spin column membrane.
10. Place the RNeasy spin column in a new 1.5 ml microcentrifuge tube. Add 50 µl RNase-free water directly to the spin column membrane. Centrifuge for 1 min at maximum speed to elute the RNA.
11. If the expected RNA yield is >30 µg, repeat step 10 using another 50 µl RNasefree water.

RNA Extraction from Plant Tissue with Tri Reagent

1. Homogenize plant leaves sample in liquid nitrogen with a mortar and pestle and then add 1 ml Tri Reagent.
2. After homogenization, centrifuge the homogenate at 12,000×g for 10 minutes at 4^{o}C to remove the insoluble material. The supernatant contains RNA and protein. Transfer the clear supernatant to a fresh tube.
3. Add 0.2 ml of chloroform per ml of Tri Reagent used. Cover the sample tightly, shake vigorously for 15 seconds and allow standing for 2-15 minutes at room temperature. Centrifuge the resulting mixture at 12,000×g for 15 minutes at 4^{o}C.
4. Transfer the aqueous phase to a fresh tube and add 0.5 ml of isopropanol per ml of Tri Reagent used in sample preparation. Allow the sample to stand for 5-10 minutes at room temperature. Centrifuge at 12,000×g for 10 minutes at 4^{o}C.
5. Remove the supernatant and wash the RNA pellet by adding 1 ml of 75% ethanol per 1 ml of Tri Reagent. Vortex the sample and then centrifuge at 7,500×g for 5 minutes at 4^{o}C. Dry the RNA pellet for 5-10 mins by air drying or under a vacuum. Resuspend RNA in 50 ul formamide water.

First Strand Synthesis of cDNA

1. Add 2 µg RNA in a sterile RNase-free microcentrifuge tube.
2. Add 0.5 µg of the primer per µg of the RNA sample to a total volume of water no greater than 15 µL.
3. Heat the tubes to 70°C for 5 minutes to denature secondary structures within the template.
4. Cool the tubes immediately on ice to allow DNA to remain in its linear form.
5. Spin the samples briefly to collect the solution at the bottom of the tubes.
6. Add the following components to the annealed primer/template in the order shown:

M-MLV 5X Reaction Buffer	5 µL
dATP, 10 mM	1.25 µL
dCTP, 10 mM	1.25 µL
dGTP, 10 mM	1.25 µL
dTTP, 10 mM	1.25 µL
rRNasin Ribonuclease Inhibitor	25 units
M-MLV RT	200 units
add sterile dH_2O to final volume	25 µL

7. Mix the solution gently by flicking the tube and incubate for 60 minutes at 37°C for random primers or 42°C for other primers or primer-adaptors.

Alkaline Lysis Miniprep: Small Scale Isolation of Plasmid DNA

1. Incubate cultures for 14 to 18 hours at 37^{o}C in Terrific Broth (TB), with ampicillin at a concentration 100 ug/ml.
2. Pellet 1.5 ml aliquots of culture for 1 minute at high speed in a microcentrifuge. Repeat until culture completely pelleted – this allows you to increase the DNA yield. Remove any remaining supernatant with a micropipette.
3. Resuspend the bacterial pellet in 200 ul of GTE buffer by vortexing or pipetting up and down at room temperature.
4. Add 300 ul of freshly prepared 0.2 N NaOH/1%SDS and then mix the contents of the tube by inversion until the solution clears. Do not vortex as this will shear the contaminating chromosomal DNA present. This step should not last longer than 5 minutes as it can damage the DNA at room temperature.
5. Neutralize the solution by adding 300 ul of 3.0 M potassium acetate, pH 4.8, (4^{o}C) mix by inverting the tube, and incubate on ice for 5 min. Sodium acetate can also be used but results are better with the potassium acetate.
6. Remove cellular debris by centrifuging for 10 minutes at room temperature, and then transfer the supernatant to a clean tube.
2. Add 2 ul of RNase A (10 mg/ml) [final concentration 20 ug/ml] and incubate the tube at 37^{o}C for 20 minutes.
3. After the RNase A treatment, extract the supernatant twice with 400 ul of phenol:chloroform:isamyl alcohol (25:24:1) (4^{o}C). Mix the layers by vortexing for 15-30 sec. Centrifuge the tube for 1 min to separate the phases and remove the aqueous (top) phase to a clean tube.

4. Precipitate the total DNA by adding an equal volume of 100% isopropanol (hood) and immediately centrifuging the tube for 15 min at room temperature.
5. Wash the DNA pellet with 500 ul of 70% ethanol by pipetting up and down. Spin for 5 minutes. Remove the supernatant by decanting, spin again briefly for 1 minute and remove any remaining ethanol with micropipette.
6. Dry under vacuum for 10 minutes.
7. Dissolve pellet in 30-40 ul of deionized sterile H_2O.

DNA Plasmid Maxiprep Protocol

1. Pick single colony and inoculate 250 ml of LB broth containing 100 mg/l ampicillin or appropriate antibiotic. Shake at 250 RPM overnight.
2. Centrifuge cells at 5 k × g for 10 minutes.
3. Resuspend cell pellet in 5 ml of GTE buffer (50 mM Glucose, 25 mM Tris-Cl, and 10 mM EDTA, pH 8.0) by pipetting up and down with a 10 ml pipette. Optional: add a spatula tip of lysozyme powder. A good suspension is consistent without clumps of cell pellet.
4. Add 10 ml of NaOH/SDS lysis solution (0.2 M NaOH, 1% SDS). Use a spatula to stir and dissolve the cells until the solution becomes clear, yellow. Alternatively, you can shake the bottle for 5 seconds.
5. Quickly add 7.5 ml 5 M potassium acetate solution (pH 4.8). This solution neutralizes NaOH in the previous lysis step while precipitating the genomic DNA and SDS in an insoluble white, rubbery precipitate. Shake bottle thoroughly. Centrifuge at 10 k × g for 10 minutes.
6. Pour the supernatant into a clean SS-34 tube through a small two-ply square of cheesecloth placed in the center of a funnel. The cheese cloth catches any fragments of SDS/genomic DNA pellet floating on the surface.
7. Precipitate the nucleic acids by adding 20ml of isopropanol and ice for 10 minutes. Centrifuge at 10k × g for 10 minutes.
8. Aspirate off all the isopropanol supernatant. Dissolve the pellet in 5 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). Add 5 ml of 5 M LiCl solution to

precipitate RNA. Leave on ice for 10 minutes and centrifuge at 10k × g for 10 minutes.

9. Pour off the supernatant containing plasmid DNA into a clean tube. Add an equal volume of isopropanol (10 ml) and precipitate the nucleic acids on ice for 10 minutes. Centrifuge at 10 k × g for 10 minutes.
10. Aspirate off all the isopropanol supernatant. Dissolve the pellet in 1 ml of TE buffer. Transfer TE solution into a 1.5 ml centrifuge tube. Add 15 ul of RNAse A solution (20 mg/ml stock stored at -20°C), vortex and incubate at 37°C for 20 to 30 minutes to digest remaining RNA.
11. Precipitate the plasmid DNA with PEG solution (30% polyethylene glycol, 1.6 M NaCl) by adding 0.4 ml and incubating one hour to overnight on ice. This step discriminates very large plasmid DNA from small nucleic acid fragments as only the larger plasmid DNA precipitate.
12. Spin the PEG solution in the centrifuge at full speed for one minute. Aspirate off the supernatant PEG buffer and dissolve the PEG pellet in 0.4 ml of TE buffer. If it is difficult to resuspend with pipetteman, let it sit at room temperature for 10 minutes and try again.
13. Extract proteins from the plasmid DNA using PCIA (phenol/chloroform/isoamyl alcohol) by adding about 0.3 ml. Vortex vigorously for 30 seconds. Centrifuge at full speed for 5 minutes at room temperature. Note organic PCIA layer will be at the bottom of the tube.
14. Remove upper aqueous layer containing the plasmid DNA carefully avoiding the white precipitated protein layer above the PCIA layer, transferring to a clean 1.5 ml

centrifuge tube. If you catch any precipitate when removing aqueous layer, add fresh PCIA and repeat.

15. Add 100 ul of 7.5 M ammonium acetate solution and 1 ml of absolute ethanol to precipitate the plasmid DNA, usually on ice for 10 minutes. Centrifuge at full speed for 5 minutes at room temperature.
16. Aspirate off ethanol solution and resuspend or dissolve DNA pellet in 0.3 to 0.5 ml of TE buffer. This is the final stock of PEG pure plasmid DNA which is suitable for DNA sequencing and long term storage.
17. Measure the concentration of the plasmid DNA by diluting stock into water at 1:200 or 5 ml of DNA per 1 ml of water (blank spectrophotometer to water). The absorbance at 260 nm multiplied by ten is the concentration of the DNA in units of mg/ml (i.e. 50 mg/ml/OD 260nm). A 250 ml flask should yield about 0.5mg of DNA.

LB (Luria-Bertani) broth recipe:

10 grams tryptone

5 grams of yeast extract

10 grams of NaCl

Dissolve in 1 L of water (tap or distilled water work fine).

Portion into flasks and cover will aluminum foil. Fill the flask to half its volume.

Autoclave 20 to 30 minutes.

GTE Solution:

Stock solution	Volume	Final concentration
40% sterile glucose	2.27 ml	50 mM
0.5 M EDTA, pH 8.0	2.0 ml	10 mM
1 M Tris-HCl, pH 8.0	2.5 ml	25 mM
sterile ddH_2O	93.23 ml	
Total:	100.0 ml	

Use all sterile stock solutions. Store at 4^oC.

NaOH/SDS lysis solution:

Stock solution	Volume	Final concentration
1 N NaOH	2.0 ml	0.2 N
10 % SDS	1.0 ml	1%
sterile ddH_2O	7.0 ml	
Total:	10.0 ml	

5 M potassium acetate solution

Stock solution	Volume
5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
ddH_2O	28.5 ml
total:	100 ml

Filter sterilize. The resulting solution is 3 M potassium and 5 M acetate and has a pH of about 4.8.

OR use the following recipe when you don't have KOAc

Stock solution	Volume
glacial acetic acid	29.5 ml
KOH	several pellets
ddH2O	70.5 ml
Total	100 ml

Add the KOH pellets until the pH is 4.8.

TE Buffer

10 mM Tris-Cl, pH 7.5

1 mM EDTA

Make from 1 M stock of Tris-Cl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).

PEG solution

30% PEG 8000 (polyethylene glycol)

1.6 M NaCl

Store at 4°C, but you can get away with storing at room temperature.

Preparing E. coli Electrocompentent Cells
(Kearney Lab, rev. 6/06)

Day 1:

1. Autoclave LB broth in flasks for shaking. Rinse flasks free of any soap residual (tap then DI water) before using. To end with 400 ul of competent cells (10 shots), you'll start with 100 ml of broth. Use a 500 ml flask to shake 100 ml of broth; a liter flask for 200 ml.
2. Autoclave De-Ionized water and 10% glycerol for overnight chilling if not already available. You'll need 200 ml of water and 8.2 ml of 10% glycerol per 100 ml of broth processed.
3. Reserve centrifuge and swinging bucket rotor (in refrigerator overnight) for next day. Use black buckets with three 50 ml spaces each.
4. Verify that you'll have sufficient liquid nitrogen for tomorrow.
5. Autoclave medium and pour plates if you're going to use the cells immediately. Autoclave any centrifuge tubes if you're not going to be using disposables.
6. Late in the day (or at night), put 5 ml of sterile broth into a sterile 40 ml green-cap culture tube and inoculate with an *E. coli* colony growing on LB plate (alternative: from frozen stock). Shake overnight at 37 C.

Day 2:

1. Transfer 1 to 2 ml of culture to 100 ml of broth and shake. Grow about 4 hrs to OD600 = 0.5 to 0.9 (0.5-0.6 optimal). Set reference with 3 ml of LB broth, replace with 3 ml of culture, and press "cell culture" button to get OD600 reading.
2. Transfer water and 10% glycerol from refrigerator to an ice bucket. Put a freezer box in -80 freezer to pre-chill it for the last step in this protocol.

3. Pour culture into 50 ml conical bottom tubes and set in ice. Balance to within 0.1 g (50 mg preferred) using sterile pipets/filter pipet tips to transfer culture from tube to tube to get balance right. Be sure ice and water on outside of tubes aren't affecting balancing. Let cool on ice 15 min as you balance tubes.
4. Centrifuge 12 min at 1000 x g at 4 C. Immediately transfer tubes to ice bucket and, and sterilely as you can, pour off supernatant into flask while at centrifuge. Super will be cloudy; pellet is very loose.
5. Add 5 ml of ice-cold water to pellets. Should be able to resuspend with just a little vortexing. Keep vortexing to minimum. Never take tubes off ice except for an instant at a time. Bring ice-cold water up to 45 ml per tube and mix by inversion.
6. Balance, centrifuge and pour off super as before.
7. Resuspend each tube in 45 ml ice-cold water as before.
8. Balance, centrifuge and pour off super as before.
9. Resuspend each tube in 4 ml of ice-cold 10% glycerol.
10. Balance, centrifuge and pour off super as before.
11. Add 100ul of ice-cold 10% glycerol to each tube. There will be 50 ul of cells and 150 ul of residual glycerol that didn't pour off, bringing the total to 300 ul per tube. Resuspend by flicking tube. The cells will be happy on ice in the glycerol for up to an hour at least, so no need to hurry.
12. Label microcentrifuge tubes (if desired) and place on ice. Transfer 40 ul of cells to each tube. Keep the cells cold throughout the transfer process.

13. Flash freeze in liquid nitrogen: Dispense nitrogen to thermos, pour into cup that comes with thermos, and toss some tubes of cells in and "stir-freeze" with long forceps. Transfer to prechilled box in -80^{o}C freezer.

SOC (100 ml):

2 g Bacto tryptone

0.5 g Bacto yeast extract

58 mg NaCl (brings conc. to 10 mM)

19 mg KCl (brings conc. to 2.5 mM)

-- autoclave—

Then add these filter-sterilized solutions:

1 ml of 1 M $MgSO_4$/$MgCl_2$ stock (brings conc. to 10 mM each)

1 ml of 2 M glucose stock

Bacterial Transformation by Electroporation

1. Gently thaw cells on ice. Place clean (sterile) cuvettes on ice. Place the white chamber slide on ice. Get 1 ml of S.O.C media into a 15 ml Falcon tube. Do this under sterile conditions.
2. In a cold 1.5 ml polypropylene tube, mix 80 μl of the cells with 1-2 μl of the ligation.
3. Set the *E. Coli* Pulser for 1.8 kV if using 0.1 cm cuvettes, 2.5 kV if using 0.2 cm cuvettes. Try only to use the 0.1 cm cuvettes. They give the best results.
4. Transfer the mixture of cells and ligation to the cold cuvette with a pipette. Do not create any bubbles. Tap the cuvette gently to make sure the level of cell suspension is even when looking at both sides of the cuvette. Place the cuvette in the safety chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber.
5. Pulse by holding onto and not letting go of the twin pulse buttons.
6. Immediately, add 1 ml of S.O.C media to the cuvette directly. Mix it with the cells quickly, but gently. This step is very important.
7. Transfer the cell suspension to a Falcon 2059 tube and incubate at 37°C. Shaking is not compulsory but better.
8. After 1 hour of shaking, plate out a certain volume.

Bacterial Transformation by Heat-shock

1. Thaw competent cells on wet ice. Place required number of Falcon 15 ml round bottom tubes on ice.
2. Chill approximately 5 ng (2 µl) of the ligation mixture in a 1.5 ml microcentrifuge tube.
3. Add 50 µl of competent cells to the DNA and mix gently by pipetting up and down.
4. Incubate on ice for 30 min.
5. Heat shock for 45 seconds at 42°C, chill on ice for 2 min.
6. Add 950 µl of room temperature LB or SOC media and incubate at 37°C for 1 hour.
7. Shake at 225 rpm (37˚C) for 1 hour.
8. Spread 50-250 µl onto the appropriate number of LB/agarose/ampicillin plates.
9. Incubate overnight at 37°C.

Preparation of electroporation competent Agrobacterium *cells*

1. Inoculate 500 ml of LB (with appropriate antibiotics) with 200-400 ul of a fresh overnight culture of the *Agrobacterium* strain. (Note: *Agrobacterium* strain GV3101 with 25 ug/ml gentamycin and 10 ug/ml rifampicin; LBA4404 with 30ug/ml streptomycin and 10 ug/ml rifampicin; EHA105 with 10 ug/ml rifampicin.)
2. Grow cells at 28°C to an ABS_{600} of about 0.5 (the cells should be at early- to mid-log phase, it usually takes over night).
3. To harvest, chill the flask on ice for 15-30 minutes, transfer to centrifuge bottles and centrifuge at 4000×g for 15 minutes at 4°C. From this stage the cells should be kept cold throughout the preparation.
4. Remove as much of the supernatant as possible and resuspend the cells in 500 ml of 1 mM hepes pH 7.4. Centrifuge as above.
5. Resuspend in 250 ml 1 mM hepes, centrifuge as above.
6. Resuspend in 10 ml 1 mM hepes, transfer to 50 ml conical bottom tubes and centrifuge at 4000×g at 4°C for 15 minutes.
7. Resuspend in 2 ml ice-cold 10% glycerol, make aliquots in eppendorf tubes (45 ul/tube) and freeze in liquid nitrogen. Store at -80 °C.

Transformation of Agrobacterium *Cells by Electroporation*

1. Thaw the *Agrobacterium* competent cells in room temperature and immediately place on ice. Transfer 40 ul of the competent cells to a chilled 1mm gap electroporation cuvette on ice.
2. Add 1-2 ul of plasmid in the cuvette, mix and keep on ice.
3. Set the electroporation machine BTX 600 Electro Cell Manipulator (BTX Inc., San Diego, CA, USA) at 1.44 kV and 129 Ω.
4. Place the cuvettes in the electroporation chamber and activate the pulse. The pulse length should be approximately 5 ms.
5. Immediately add 600 ul of SOC broth, transfer to an eppendorf tube and incubate at room temperature for 1 hour without agitation.
6. Plate the cells on appropriate antibiotics selection and grow over two nights at 28°C.

Agroinfiltration

1. Pick individual *Agrobacterium* colonies from plates with toothpick. Inoculate into 3 mls L broth with appropriate antibiotic.
2. Grow in shaker at room temp 24 to 30 hours until culture is very dense (late log or stationary phase).
3. Inoculate 5 mls L-MESA media with 250 ul of agro culture. Grow in shaker at room temp about 6- 8 hours.
4. Record OD after 6-8 hours of culturing. Target OD 600 is between 0.5 and 1.0, or more preferably between 0.8 and 1.0.
5. Harvest cells by centrifuging for 10 minutes at 3500 x g, or 15 minutes at 2000 x g.
6. Pour off liquid from cell pellet. Remove as much liquid as possible without disturbing pellet.
7. Resuspend cell pellet in appropriate volume of Agroinduction media so that a final OD 600 of about 1.0 to 1.1 is obtained.
8. Let resuspended cells sit in induction media for at least 2-3 hours to overnight, if needed, at room temp and on bench top.
9. Combine cultures (if desired). Label leaves to be infiltrated with sharpie marker (wide). Poke small hole in each leaf half with toothpick or yellow tip. Infiltrate culture from underside of leaf using 1 or 3 ml syringe with no needle, at site of small hole, blocking hole from other side with a gloved finger.
10. Should see GFP reporter activity in infiltrated zones by 30 hours post infiltration. If no silencing suppressor is present, GFP will generally be at peak expression levels at about 2-3 days post infiltration and fades (disappears) after that.

Materials:

LB media (5 g NaCl, 5 g yeast extract, 10 g bacto-tryptone per L).

0.5 M MES pH 5.7

0.1 M Acetosyringone (in DMSO)

1 M $MgCl_2$

L-MESA Media:

100 ml LB broth

2 ml 0.5 M MES (pH 5.7)

20 ul 0.1 M Acetosyringone

(Note: if growing *Agrobacterium* strain GV3101 with pCB based vector, add Gentamycin to 25 ug/ml, Kanamycin to 50 ug/ml and Rifampicin to 10 ug/ml. If growing LBA4404 with pCB based vector, add Streptomycin to 30 ug/ml, Kanamycin to 50 ug/ml and Rifampicin to 10 ug/ml. If growing EHA105 with pCB based vector, add Kanamycin to 50 ug/ml and Rifampicin to 10 ug/ml.)

Agroinduction Media:

50 mls dH20

0.5 mls 1 M $MgCl_2$

1 ml 0.5 M MES (pH 5.7)

50 ul 0.1 M acetosyringone

Histochemical GUS Assay

1. Dissolve 50 mg X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 1 mM) in 100 ul dimethylformamide in a chemical hood.
2. Add to 100 ml of 100 mM sodium phosphate buffer, pH 7.0. Wrap the tube in aluminium foil to protect from light.
3. Dissolve potassium ferricyanide and potassium ferrocyanide in water at final concentration of 50 mM. Add 1 ml of each stock solution to 100 ml X-Gluc solution for a final concentration of 0.5 mM.
4. Place fresh plant tissue in X-Gluc solution.
5. Leaves are infiltrated with the 700 mmHg (93 kPa) vacuum for 5 mins to improve the penetration of the substrate.
6. Incubate the samples in the dark at 37°C overnight.
7. Clear the tissue with 70% ethanol to improve contrast by removing photosynthetic pigments.
8. View GUS-stained leaves under a light microscope.

Spectrophotometric GUS Assay.

Transient GUS activity is determined using the spectrophotometric GUS assay (Jefferson *et al*., 1987).

1. Harvest and grind the inoculated leaves as frozen tissue in liquid nitrogen.
2. Add freshly prepared 1 ml of GUS lysis buffer (50 mM NaPO4 pH7.0, 10 mM beta-mercaptoethanol, 0.1% Triron X-100) in grinded tissue powder.
3. Centrifuge the crude extracted homogenate in a microcentrifuge at 12,000 g for 5 mins and collect the extract supernatant. Add 100 ul of 10 mM p-nitrophenyl beta-D-glucuronide (PNPG, Sigma) in lysis buffer and incubate the reaction at 37^{o}C for 15 h.
4. Add 0.4 ml 2.5 M 2-amino-2-methyl propanediol (Sigma) to terminate the reaction.
5. Measure the absorbance at 415 nm against a substrate blank.
6. Under these conditions the molar extinction coefficient of *p*-nitrophenol is assumed to be 14,000; thus in 1.4 ml final volume, an absorbance of 0.010 represents 1 nmol of product produced. One unit is defined as the amount of enzyme that produces 1 nmol of product/min at 37^{o}C. This represents about 5 ng of pure β–glucuronidase.

Protein Extraction by Grinding

1. Extract proteins by grinding in liquid nitrogen or chilled mortar-pestle (200 mg leaves/500 µl extraction buffer). Extraction buffer is 50 mM Tris HCl pH 8.0, 50 mM NaCl, 50µM AEBSF/PMSF). AEBSF/PMSF (4-(2-aminoethyl)-benzenesulfonyl fluoride/Phenylmethyl sulfonyl fluoride) needs to be added fresh to the buffer. In general, it's better to make fresh buffer from stocks and store no more than few days in the refrigerator.
2. Centrifuge at 4ºC for 15 min, remove supernatant and save in the refrigerator.

Protein Extraction by Vacuum Infiltration

1. Take 50 ml infiltration buffer (100 mM Tris.Cl, pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA) in a sterile beaker. Soak leaves in it. Cover leaves with a big leaf from the uninoculated plant (to keep the other leaves in the liquid while the vacuum is applied).
2. Put in the vacuum assembly; turn the vacuum on for 2 min. Should see bubbles coming out of leaves. Release the vacuum.
3. Leaves start turning darker when the buffer infiltrates the leaves.
4. Take out leaves gently with blunt forceps, put them on filter paper and tap dry.
5. Put leaves (without injuring them) in one of the drain cups. Put the drain cup in an autoclaved centrifuge bottle (250 ml).
6. Centrifuge at 2000 g for 5 min.
7. Collect the protein drops at the bottom of the centrifuge bottle and save in the refrigerator.

SDS Polyacrylamide Gel Electrophoresis

BioRad Mini-PROTEAN 3 Cell gel apparatus and electrophoresis equipment

30% acrylamide/0.8% bis-acrylamide stock solution

2.5x separating gel buffer

5x stacking gel buffer

TEMED (N,N,N',N'-tetramethylethylenediamine)

10% (w/v) ammonium persulfate

Compositions and separation properties of SDS-PAGE gels.

Gel acrylamide concentration (%)	Linear range of separation (kDa)	30 % acrylamide /0.8% bis-acrylamide stock solution (ml)	2.5x separating gel buffer (ml)	Distilled water (ml)
15.0	12–43	2.75	2.2	0.55
10.0	16–68	1.83	2.2	1.47
7.5	36–94	1.38	2.2	1.92
5.0	57–212	0.92	2.2	2.38

Volumes given are used for an 8 x 8 or 8 x 10 cm, 1 mm thick minigel, final volume 5.5 ml.

1. Assemble gel plates with spacers according to the BioRad Mini-PROTEAN 3 Cell instructions. The plates should be thoroughly cleaned and dried before use.
2. Mark the level to which the separating gel should be poured a few millimeters below the level where the wells will be formed by the comb.
3. Mix the suitable amount solutions in a beaker as the table above. According to the size of protein to be separated, the volumes of acrylamide/bis-acrylamide solution and water should be adjusted according to the percentage acrylamide required.
4. Just before pouring, add 5 µl TEMED and 50 µl 10% ammonium persulfate, and mix well. Pour the gel between the assembled gel plates to the level marked in step 2. Overlay with water.
5. After polymerization is complete (around 20 min), pour off water and dry.

6. For the stacking gel, mix the following:

   0.28 ml 30% acrylamide/0.8% bis-acrylamide stock solution

   0.33 ml 5x stacking gel buffer

   1 ml distilled water

   2 µl TEMED

   15 µl 10% ammonium persulfate

7. Pour on top of the separating gel. Insert comb, avoiding introduction of air bubbles.
8. After the stacking gel polymerizes (around 10 min), the gel can be placed in the electrophoresis chamber. Fill the chamber with electrophoresis buffer and remove the comb.
9. Before loading, add 1 volume 3x SDS-PAGE sample buffer (BioRad) to 2 volumes of protein sample (i.e., add 5 µl sample buffer to 10 µl sample giving a final volume of 15 µl). Vortex briefly and heat at 95°C for 5 min.
10. Before loading the samples, rinse out wells with 1x electrophoresis buffer using a suitable syringe and needle.
11. Load immediately onto gel and run at 200 volts for 45-60 minures.
12. Running the gel until the bromophenol blue dye reaches the bottom edge usually gives a satisfactory spread of protein bands.
13. Incubate the gel in Coomassie staining solution for between 30 min and 2 h with gentle shaking. Coomassie Brilliant Blue reacts nonspecifically with proteins.
14. Gently agitate the stained gel in destaining solution until the background becomes clear (1–2 h). A folded paper towel placed in the destaining bath will soak up excess stain and allow the re-use of destaining solution.

Transfer of Protein from Gel to PVDF Membrane
(Biorad Semi-Dry Electrophoretic Transfer Cell)

1. Equilibrate the gels in transfer buffer 15 min for a 0.75 mm SDS-PAGE gel.
2. Cut the membrane to the dimensions of the gel. Wet the membrane by slowly sliding it at a 45 degree angle into transfer buffer an allow it to soak for 15-30 min. Complete wetting of the membrane is important to ensure protein binding.
3. Cut filter paper to the dimensions of the gel. Two pieces of extra thick filter paper or four pieces of thick filter paper. Completely saturate the filter paper by soaking in transfer buffer.
4. Place a pre-soaked sheet of extra thick filter paper onto the platinum anode. Roll a pipette or test tube over the surface of the filter paper to exclude all air bubbles.
5. Place the pre-wetted membrane on top of the filter paper. Roll out all air bubbles.
6. Carefully place the equilibrated gel on top of the transfer membrane, aligning the gel on the center of the membrane. Roll out all air bubbles.
7. Place the other sheet of pre-soaked filter paper on top of the gel, carefully removing air bubbles from between the gel and filter paper.
8. Carefully place the cathode onto the stack. Press to engage the latches with the guide posts without disturbing the filter paper stack.
9. Place the safety cover on the unit. Plug the unit into the power supply. Normal transfer polarity is cathode to anode. Don't reverse polarity.
10. Turn on the power supply. Transfer mini gels for 15-30 min at 10-15V. Following transfer, turn the power supply off, and disconnect the unit from the power supply. Remove the safety cover and the cathode assembly. Discard filter papers.

Ponceau Staining (Sensitive Staining of Proteins)

1. Dilute 10 x Ponceau solution to 1 x and incubate membrane for 5-20 minutes.
2. Remove stain and destain excess with water (optional: buffers such as PBS and TBS remove the stain much faster). Protein positions should be obvious within 5 minutes of destaining.
3. Mark any positions with an appropriate marker and continue destaning until all the stain has been removed.

Western Blot for the Detection of Jun a 1

1. After transferring proteins to PVDF membrane, and checking the presence of proteins on the membrane by Ponceau staining, block the membrane for 30 min/1hr/overnight in 10% milk in TTBS.
2. Incubate in primary antibody: 1:10000 dilution. Seal in a small Ziploc bag or put in a new 50 ml Falcon tube. Shake at least 2-3 hrs or O/N. Make sure that the membrane stays wet during this time.
3. Wash 3 x, 10 min each with TTBS.
4. Incubate 1-2 hrs in secondary antibody, 1:10000 dilution.
5. Wash 3 x with TTBS, 10 min each.
6. Develop with ECL or DAB according to manufacturer's instructions and take pictures.

Alkaline Phosphatase ELISA Protocol

1. Coat a Costar 96 wells flat bottom EIA/RIA plate (Corning Inc., Corning, NY) with the capture antibody (50ul per well of Goat anti-human IgG from Jackson 109-005-088 diluted 1:1,000 to 1ug/ml in carbonate buffer.
2. Incubate at 4^{o}C overnight or 37^{o}C for one hour.
3. Wash 2× with PBS 0.05% Tween.
4. Add 35 ul supernatant (diluted in blocking buffer: Starting Block T20 (TBS) Blocking Buffer, from Pierce #37543).
5. Incubate at 37^{o}C for one to one and half hours.
6. Wash 3× with PBS 0.05% Tween.
7. Add 45 ul of Langrin alkaline phophatase supernatant diluted in blocking buffer 1:5. And incubate at 37^{o}C for one and half hours.
8. Wash 3× with PBS 0.05% Tween.
9. Add 100 ul pNPP substrate (Sigma). And incubate at 37^{o}C from a few hours to overnight to develop.
10. Read at 405nm.

Carbonate Buffer:

5.8 g $NaHCO_3$

3.2 g Na_2CO_3

Bring up to 2 L, pH 9.6.

Purification of Jun a 1

All buffers for columns should be 0.2 uM filtered.

1. Weigh 50 g Cedar pollen. Note the lot no. and the batch no. of pollen.
2. Extract with 500 ml 0.125 M NH_4HCO_3 (4.94 g/500 ml), 0.02% NaN3 (0.1 g/500 ml), 50uM 4-(2-Aminoethyl)-Benzenesulfonyl fluoride (6 mg/500 ml, 50 mM stock solution is in -20ºC), pH 8.0, at 4ºC for 48 hrs.
3. Centrifuge 10,000 rpm (JA-14) at 4ºC for 10 min or 3,000 g for 30min, and collect supernatant. Repeat extraction from above precipitate for 24 hrs with 250 ml/50g pollen, collect supernatant.
4. $(NH_4)_2SO_4$ 80% precipitation (561 g/L).

   Slowly add 56.8 g/L ammonium sulfate, add salt more slowly on cooling bath in 5-10 min stirring, and continue string over night at 4 º C.

   Centrifuge 10,000 g for 10 min or 3,000 g for 30 mins.

   Add 80% $(NH_4)SO_4$ in 0.025 M Tris, pH7.8, 0.1% NaN_3 to wash the pellet.

   Centrifuge 10,000 g for 10 mins or 3,000 g for 30 mins.

   Repeat washing total of three times.

   Resuspend pellet in 1-2 pellet volumes of 0.05 M Tris, pH 7.8, 0.1% NaN_3

   (Tris-HCl=157.6 g, 31.52 g/4 L and NaN_3 4 g/4 L)

   Centrifuge 10,000 g for 30mins to remove small particles
5. Dialyze against DEAE column buffer – 1 M NaCl with activated charcoal at 4ºC for 48 hrs.

Dialyze against DEAE column buffer (0.05 M Tris, pH 7.8, 1 M NaCl, 0.1% NaN_3) at 4ºC over night (Tris-HCl 31.52 g/4 L, NaCl 233.76 g/4 L, NaN_3 4 g and about 20 pellets of NaOH/4 L).

Apply 15 ml DEAE –Sepharose on the funnel.

Wash with 45 ml column buffer (3 column volume).

Apply sample to DEAE and collect flow through.

Add 15 ml column buffer (1 column volume) and combine with the flow through.

6. Dialyze against Con A Sepharose column buffer (0.02 M Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3) at 4ºC over night. (Tris-HCl 12.61 g, NaCl 116.88 g with 8 ml stock solution for $CaCl_2$, $MgCl_2$ and NaN_3 (500 mM $CaCl_2$, 500 mM $MgCl_2$ and 10% NaN_3) and about 3 pellets for NaOH/4 L, pH 7.4).

   Wash Sepharose column (5 ml) with 3 column volumns of Con A buffer, apply sample and collect flow through and one column with 6 ml (3 column volumes) of column buffer.

   Apply sample by gravity.

   Wash with Con A column buffer until O.D.280<0.05.

   Elute with 0.5 M mannoside in column buffer, 2 ml/tube. (Methyl α-D-Mannopyranoside=194.2, Sigma M-6882, 0.97 g/10 ml Con A buffer).

   Collect fractions O.D.280>0.05, wash Con A column with 5 column volume buffer and store in 4ºC.

7. Check the purity (5 ul) by 4-20% gel, stain with Coomassie stain, and determine the concentration on the gel.

Purification of rJun a 1

Recombinant Jun a 1 was purified with HiTrap NHS-HP anti-Jun a 1 affinity Chromatography column.

1. The column was prepared by wash with 3ml binding buffer (PBS, pH 7.4, 0.01% NaN_3).
2. Recombinant Jun a 1 sample was centrifuged at full speed for 5 minutes and filtered through a 0.45 um filter before it was applied to the column.
3. The column is equilibrated with 10 ml binding buffer. Apply the sample by pumping it onto the column. Flow rate is 0.2 ml/min.
4. Recirculate the sample solution by connecting a peristaltic pump and gently pump back and forth for 30 mins.
5. Wash with binding buffer. Collect the first 1ml effluent as the Flow Through of purification. Wash with 10 ml binding buffer.
6. Elute with elution buffer (0.2 M Glycine-HCl, pH 2.6). Collect 1ml purified fractions in 1 M Borate Buffer (pH 8.2, 100 ul). Continue 2 ml elution buffer.
7. Re-equilibrate the column by washing with 10 ml binding buffer.
8. Purified rJun a 1 was electrophoresesed on 4-20% SDS-PAGE, subsequently Coomassie stained.

Enzyme-Linked Immunoassays ELISAs

1. Coat 100 µl of 3 µg/ml purified Jun a1 (Midoro-Horiuti *et al* 1999) to a 96 (8×12) well plate with the use of 0.125 M borate buffer.
2. Leave one 12-well row uncoated as a negative control and instead filled with 100 µl of borate buffer.
3. Incubate the plate overnight in a 37°C environment.
4. Wash the plate four times in Tween-Saline solution.
5. Add the primary antibody, mouse serum in this case in 1:50 dilutions to the first row and then serially diluted five times.
6. Set another negative control to add 1× TTBS in the place of any primary antibody.
7. Incubate the primary antibody solutions for two hours while shaking at room temperature.
8. Wash four times with Tween-saline solution.
9. Prepare the secondary antibody solution. Dilute the Peridase labeled Goat anti-mouse Ig(G+M+A) antibody to 1:2000 in 1×TTBS. Add 100 µl of this secondary antibody solution to each well and sit for one hour while shaking at room temperature.
10. Wash six times with Tween-saline solution.
11. Prepare a developing solution of 10ml citrate buffer, 10 µl 30% hydrogen peroxide, and one 10 mg OPD (O-Phenylenediamine dihydrochloride) tablet.
12. Add 30 µl of the developing solution to each well and cover the plate for three minutes to allow the reaction to occur.

13. Add 10 µl of 5 N sulfuric acid to stop the reaction. Read the absorbance of each well at 492nm.

Freezing Cells

1) Freezing medium (2x):

84% Fetal Calf Serum	25.2 ml (Hyclone #SH30072.02)	from -20°C
16% DMSO	4.8 ml /30 ml	from Core shelf

2) Cell stock:

1. Count cells.
2. Spin cell down 800 rpm for 5-8 min.
3. Resuspend cells in culture medium.
4. Place on ice.
5. Prep. vials, label (cell name, cell #, date).
6. Slowly add, drop by drop, freezing medium, mix while add media.
7. Pipetting immediately place 1 ml of cells in vials.
8. Place -80^{o}C for 24 hrs.
9. Transfer to liquid N_2

Cell density: 1~10 x 10^6/tube.

Mix rate: 1:1 = freezing medium : culture medium.

Beta-hexosaminidase Release Assay

I. RBL SX-38 cell culture:

1. Culture cells in DMEM, with 10% FCS, Pen/Str, 1.2 mg/ml G418.
2. Subculture in 96 wells plate at 2×10^5 cells/well, 37°C, 5% CO_2 over night.
3. Sensitize with human IgE myeloma protein or patient serum, at 37°C, 5% CO_2 for 2 hours or over night.

II. Release Assay:

4. Wash with warm (room temperature) Tyrode's buffer three times, 100 ul/well.
5. Add anti human IgE or allergen, A23187 or compound 48/80, incubate at 37°C, 5% CO_2 for 30 mins.
6. Centrifuge at 100×g for 3 mins. Transfer 25 ul supernatant to well of 96 well plate (plus 5 ul of 1.2% Triton ×100).
7. Add 30 ul NAG in pH 4.5 citrate buffer (4 vol. 0.01 M NAG + 1 vol. 0.4 M pH 4.5 Citrate buffer). Shake at 37°C for 2 hours.

III. Reading:

8. Add 30 ul 1 N NaOH, read O.D. value at 410 nm.

HEPES-Tyrode's buffer pH7.3, 0.2 μm filtered, once you opened the bottle, keeps it in 4 °C.

137 mM Na	FW=58.44	8.01 g/L
5.6 mM glucose	FW=180.2	1.01 g/L
2.7 mM KCl	FW=74.55	0.20 g/L
0.5 mM NaH_2PO_4	FW=120	0.06 g/L
1 mM $CaCl_2$	FW ($CaCl_2.2H_2O$)=147.02	0.15 g/L
10 mM HEPES	FW=238.3	2.38 g/L
0.1% BSA		1 g/L

Thawing Cells

1. Remove cells immediately place in 37°C water bath.
2. When most of the suspension has melted (there is still a little check of ice left), remove the tube allow finishing melting at room temperature.
3. Transfer cells to 15 ml centrifuge tube.
4. Slowly add ice cold media (5 ml) = BCM for solve DMSO.
5. Spin cells at 800-1000 rpm for 5-8 min.
6. Remove supernatant.
7. Resuspend cells with 10 ml at the appropriate media.
8. Transfer to flask / dish.
9. Place in incubator.

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