DEPLOYMENT AND CHARACTERIZATION OF RECESSIVE RESISTANCE GENE *PVR1* IN THE SOLANACEAE

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Potyviruses are the largest group of plant viruses and can infect pepper, potato and tomato which were the focus of this dissertation. Pepper resistance to potyviruses has been attributed to point mutations at the *pvr1* locus which cause conformational shifts in encoded protein eukaryotic translation initiation factor eIF4E. In addition to susceptible allele $Pvr1^{+}$, three *pvr1* resistance alleles were the focus of this study: *pvr1*, $pvr1^{1}$, $pvr1^{2}$. Resistance conferred by these three *pvr1* alleles has been linked to the inability for eIF4E to interact with the viral encoded protein VPg, a protein covalently bound to the 5' end of the potyvirus genome. To determine if VPg was the virulence determinant for two strains of *Tobacco etch virus* (TEV), two VPg viral chimera were synthesized using a PCR based domain swapping method which we developed. The technique is a versatile and widely applicable alternative to conventional restriction enzyme digestion and ligation methods and is a valuable tool for determining which domains of a viral genome are essential for infectivity. Substituting VPg from an infectious TEV strain was found to determine the outcome of host infection for both *pvr1* and $pvr1^{2}$. In additional studies, we transformed widely cultivated potato variety Russet Burbank with one of the four pepper *pvr1* alleles to confer resistance to potyvirus *Potato virus Y* (PVY). Russet Burbank lines which overexpressed the $pvr1^{2}$ or the $pvr1^{1}$ allele from pepper were resistant to at least one of three PVY strains tested. The majority of $pvr1^{2}$ lines were resistant to all strains of PVY and plants grown from tubers of the inoculated resistant lines were virus free.

BIOGRAPHICAL SKETCH

Kari Perez was born and raised in Rhode Island. She received her B.S. from University of Wisconsin-Madison in Spanish while following the pre-medical curriculum and working in laboratory at the UW medical school. After graduation, Kari worked in several laboratory positions including a marine pathology laboratory at the University of Rhode Island, the molecular pathology division of a pharmaceutical company, for a small agricultural biotechnology company in rice and turfgrass transgenic development and as a technician in maize genetics laboratory at Yale University. Kari started the Plant Breeding and Genetics Ph.D. program at Cornell University in 2003 under the guidance of Dr. Molly Jahn.

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CHAPTER 1

INTRODUCTION

This dissertation contains three chapters in addition to this introduction, general conclusions and two appendices. Each chapter has been or will be submitted for publication as indicated on the title page of that chapter. The first chapter describes a PCR-based method which was used to replace a viral domain, the VPg domain, easily and efficiently among different viral strains. This method allowed for the identification of the virulence determinant in the *Capsicum-Tobacco etch virus* (TEV) pathosystem. The second chapter describes the resulting VPg viral chimeras and their infectivity in Capsicum, a host system that contains an allelic series of mutations at the *pvr1* locus (*poty*v*irus r*esistance gene *1*) that encode natural mutations in eIF4E which have been correlated with potyvirus resistance (Kang et al. 2005; Murphy et al. 1998). Mutations, now known to be clustered near the eIF4E cap binding pocket, can block potyvirus infection at the cellular level (replication/translation stage) in a protoplast assay (Murphy et al. 1998). Very few mutations in eIF4E are necessary to both confer resistance and eliminate interaction with VPg. In fact, a single mutation in the *pvr1* allele was sufficient to eliminate interaction with TEV VPg (Yeam et al. 2007). This study focuses on two alleles at the *pvr1* locus, pvr1 and *pvr1*^{2}, that differ with respect to susceptibility to two strains of TEV, TEV-Mex21 and TEV-N, respectively. Chapter 3 discusses a transgenic approach to confer PVY resistance in potato using alleles of the *pvr1* locus as transgenes. The potyvirus PVY is responsible for millions of dollars in loss each year to the potato industry (Brown 2009, personal communication). A pepper resistance screen using PVY strains currently devastating potato production was conducted and identified resistant pepper lines. *pvr1* alleles which conferred PVY resistance were subsequently used as transgenes to confer

resistance in potato. Given the wide array of resistance sources currently in the population which confer potyvirus resistance, it seems there is no limit to potyvirus control using this approach.

All the resistance genes described in this study are recessive resistance alleles. In fact, resistance to potyvirues is controlled primarily by recessive resistance genes (Robaglia and Caranta 2006). Recent reviews have tried to address why dominant susceptible alleles have been maintained in the environment. Robaglia and Caranta et al. hypothesized that since potyviruses are generally mild pathogens for wild plants, infection could therefore increase the plant's innate immunity against other more destructive pathogens. However, while potyviruses may be minor pathogens in wild plants, viruses such as PVY in potato can cause major crop losses. Recessive resistance alleles are much more difficult to maintain in breeding populations than dominant alleles, however, the approach described in this study demonstrates that a recessive allele can behave as a dominant gene when constitutively expressed. Alleles such as *pvr1*^{2} in pepper which have been used successfully to confer potyvirus resistance in pepper for over 50 years (Czaplewski 2009, personal communication), represent a tremendous resource for transgenic breeding approaches of the future.

Plant translation initiation factors play an important role in the initiation of host translation, but can also function in the plant virus life cycle and have been shown to be crucial for infection or resistance. In plants, most mRNA has a m^7G cap at the 5' end which is bound by one of two cap-binding proteins: eukaryotic translation initiation factor eIF4E or its isoform eIF(iso)4E (Shatkin 1976; Sonenberg et al. 1979). The protein, eIF4E or eIF(iso)4E, along with eIF4G or eIF(iso)4G make up one of two translation initiation complexes, eIF4F or eIF(iso)4F, respectively (Browning 1996). Both 4F complexes are involved in mRNA 5' cap recognition during translation initiation, and mRNA unwinding and recruitment to the ribosome (Browning 1996;

Gingras, Raught, and Sonenberg 1999; Kawaguchi and Bailey-Serres 2002; Nicaise et al. 2007). The mRNA cap binds eIF4E or eIF(iso)4E, while the mRNA poly-A tail binds the poly-A binding protein (PABP) (Le et al. 1997). Additionally, the PABP has been shown to bind eIF4G or eIF(iso)4G which was found to increase mRNA binding affinity, an interaction which occurs even in the absence of mRNA poly-A (Le et al. 1997). These translation factors, eIF4F or eIF(iso)4F and the PABP are just a few of the components which collectively initiate translation, a process which requires as many as 11 distinct proteins that serve as initiation factors (Cheng and Gallie 2007).

The existence of two isoforms of the 4F complex is unique to plants, and each appears to carry out distinct functions (Browning 1996). Recent data suggests that the 4F isoforms preferentially recognize different subsets of total mRNA. The eIF(iso)4F complex recognizes standard capped mRNA molecules, while the eIF4F complex recognizes abnormal mRNAs, e.g. mRNA without a cap, mRNA with secondary structure near the cap, or polycistronic mRNA (Gallie and Browning 2001). Not only do the two isoforms appear to preferentially recognize mRNA, but they also appear to preferentially recognize RNA viruses (Robaglia and Caranta 2006). *Brassica perviridis* plants infected with *Turnip mosaic virus* showed expression of eIF4E only in infected plants, while eIF(iso)4E was identified independent of virus infection (Leonard et al. 2004).

Given that viruses are obligate intracellular parasites, they encode very few proteins and rely heavily on their hosts to complete essential functions such as replication and translation. Any host protein that is essential for virus infection is a candidate gene for virus resistance. Several studies have shown that viral interaction with eIF4E or eIF(iso)4E is essential for virus infectivity. In fact, naturally occurring mutations in eIF4E or eIF(iso)4E have been identified with virus resistance in many systems. Resistance to *Tobacco etch virus, Turnip mosaic virus* and *Cucumber mosaic*

virus is observed in *Arabidopsis thaliana* eIF(iso)4E knock out mutants (Duprat et al. 2002; Lellis et al. 2002; Yoshii et al. 2004). To date, virus resistance conferred by naturally occurring eIF4E variants has been observed in five plant families including the Solanaceae (Kang et al. 2005; Ruffel et al. 2005; Ruffel et al. 2002), Asteraceae (Nicaise et al. 2003), Poaceae (Kanyuka et al. 2005; Stein et al. 2005), Fabaceae (Gao 2004), and the Cucurbitaceae (Nieto et al. 2006).

Based on genetic studies of host resistance, three genera of RNA plant viruses are known to require eIF4E or an isoform. Resistance in the Poaceae was observed against *Barley yellow mosaic virus*, a bymovirus, and Cucurbitaceae resistance was conferred against *Melon necrotic spot virus* (MNSV), a carmovirus. All other naturally occurring resistance alleles at the eIF4E locus involve potyviruses including *Potato virus Y* (PVY), *Tobacco etch virus* (TEV), *Lettuce mosaic virus* and *Pea seed-borne mosaic virus* (Kanyuka et al. 2005; Stein et al. 2005). Bymoviruses and potyviruses are both found within the Potyviridae family. The Potyviridae share structural similarity with host mRNA. These viruses do not encode a cap, however, they encode a viral protein known as VPg covalently bound by a tyrosine residue to the 5' terminus of the RNA genome (Murphy et al. 1991). Given the structural similarity and the observed correlation of the VPg-eIF4E interaction, it has been hypothesized that VPg acts as a cap mimic (Beauchemin, Boutet, and Laliberte 2007; Leonard et al. 2000; Roudet-Tavert et al. 2007; Schaad, Anderberg, and Carrington 2000; Wittmann et al. 1997). Several studies have shown that VPg must interact with eIF4E or isoform eIF(iso)4E for full susceptibility. In fact, several studies have identified VPg as a virulence determinant, or the pathogen factor essential for virus infection (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Rajamaki and Valkonen 1999; Rajamaki and Valkonen 2002).

Consistent with this observation are several studies that demonstrate that mutations in eIF4E or eIF(iso)4E inhibiting interaction with VPg, both *in vitro* and *in planta*, correlate with virus resistance (Beauchemin, Boutet, and Laliberte 2007; Bruun-Rasmussen et al. 2007; Kang et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Leonard et al. 2004; Moury et al. 2004; Yeam et al. 2007). This form of resistance has been classified as "passive" virus resistance, defined as an alteration or a deletion in a host factor essential for the virus, but dispensable for the host (Fraser 1990). With respect to the mechanism by which this resistance is effected, there are two major possibilities. As in the host, VPg interaction with eIF4E or eIF(iso)4E could be essential for translation. In addition or alternatively, this interaction could also be involved in replication stabilization (Lellis et al. 2002; Robaglia and Caranta 2006). Interestingly, unlike potyviruses and bymoviruses, the virulence determinant for the carmovirus *Melon necrotic spot virus* has been mapped to the 3'-untranslated region (3'-UTR), rather than an expected 5' component of the viral genome. Carmoviruses do not encode a VPg protein or a cap. It is hypothesized that, like VPg, the carmovirus 3'UTR acts as a cap mimic since it has been shown that these viruses utilize structural elements in their 3'-UTR to carry out cap independent translation (Koh, Liu, and Wong 2002; Nieto et al. 2006).

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CHAPTER 2

MEGAPRIMER-MEDIATED DOMAIN SWAPPING FOR CONSTRUCTI0N OF CHIMERIC VIRUSES[1,2]

Abstract

Clones that encode viral genomes constructed from two viruses with contrasting biological properties have been widely used in studies of viral-host interactions, particularly when the objective is to determine the identity of the viral component recognized by the host in a resistant response, known as the avirulence factor. This paper presents an efficient method based on megaprimer-mediated domain swapping for the construction of clones encoding chimeric viral genomes as a versatile and widely applicable alternative to conventional restriction enzyme digestion and ligation methods. Potato Virus X (PVX)-derived vectors expressing genes encoding fluorescent proteins were used to demonstrate this concept. The cyan fluorescent protein (CFP) gene was cloned into a binary PVX vector and subsequently replaced with the yellow fluorescent protein (YFP) gene using the megaprimer amplification reaction. DNA fragments up to 1480 bp could be replaced efficiently and quickly. Most viral clones showed the expected change in phenotype without altered infectivity. Sequence analysis revealed mutations were not introduced into the four domain-swapped plasmids. This approach will provide a valuable tool for determining which domains of a viral genome are essential for infectivity, avirulence, or otherwise determine biologically significant properties of plant viruses.

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[2]Experiments included in this study were conceived by Byoung-Cheorl Kang. Kari Perez completed megaprimer PCR reactions, statistical analysis of the error rate, colony PCR and all the restriction digests. Byoung-Cheorl Kang performed all the other experiments including the primer design, virus inoculations, confocal microscopy and ELISA. This manuscript was written by Kari Perez and Byoung-Cheorl Kang with assistance from Inhwa Yeam and Molly M. Jahn.

Introduction

The component(s) of a plant virus responsible for triggering a resistance response is known as the viral avirulence determinant. In plant-pathogen interactions, pathogen avirulence genes have long been defined by the fact that the gene or its corresponding gene product are essential in eliciting the resistant response in hosts containing a disease resistance (R) gene (Flor 1971; Jordan et al. 2007). In plant-viral interactions, the avirulence determinant for a specific host/virus combination is typically identified by creating infectious chimeric viral clones derived from two viral genotypes that contrast with respect to virulence (Roger 2002). Once an avirulence domain is identified, subsequent mutational analysis via site-directed methods can be used to identify specific point mutations responsible for virulence (Roger 2002). Creating chimeric clones, however, can be problematic if the necessary endonuclease restriction sites are unavailable or unsuitable. Despite this constraint, very few studies have employed alternative methods that do not rely on endonuclease and/or ligase (Charlier et al. 2003; Dekker et al. 2000; Liang et al. 2004). Alternative methods published to date typically are very restricted in their application. An easy, efficient and widely applicable strategy to generate chimeric clones of viral genomes is therefore highly desirable.

The polymerase chain reaction (PCR)-based site-directed mutagenesis (SDM) technique is widely used to introduce desired mutations into target DNA sequences (Ishii et al. 1998). A variety of PCR-based SDM protocols have been established to achieve efficient mutagenesis. Among them, the 'megaprimer PCR' method is particularly attractive because it is simple and relatively inexpensive (Kammann et al. 1989; Landt, Grunert, and Hahn 1990; Sarkar and Sommer 1990). This approach relies on the fact that DNA synthesized *in vitro* is not methylated and therefore is resistant to digestion by the enzyme *Dpn*I. In the megaprimer method, *in vitro* DNA

synthesis takes place in two successive steps (Kirsch and Joly 1998; Sarkar and Sommer 1990). First, a target DNA fragment is amplified in a typical PCR reaction using two oligonucleotide primers. Then, the two strands of newly synthesized PCR fragments are used as megaprimers to synthesize the whole plasmid *in vitro* to incorporate mutagenic target DNA fragments. The plasmid DNA is synthesized by a high-fidelity thermostable DNA polymerase such as *Pfu* DNA polymerase (Cline, Braman, and Hogrefe 1996), and the original parental DNA is subsequently digested by *Dpn*I. The *Dpn*I-resistant mutated DNA is recovered directly by transformation into competent bacteria.

This paper demonstrates an alternative to conventional restriction enzyme digestion and ligation for construction of chimeric viral clones using the megaprimer approach. For simplicity, the *Potato virus X* (PVX) system was chosen because the PVX genome has been cloned into a binary vector suitable for *Agrobacterium tumefaciens*-mediated inoculation. For a visual assay to confirm domain-swapping, the cyan fluorescent protein (CFP) gene was cloned into a binary PVX vector and subsequently replaced the gene with a gene encoding yellow fluorescent protein (YFP) via the megaprimer PCR reaction.

Results

Cloning CFP and YFP into pgR106

To test the efficacy of the Megaprimer method to make specific exchanges of DNA sequences, a system based upon exchange of CFP and YFP was chosen because of the convenience of a fluorescence assay. CFP and YFP show 97% nucleotide sequence identity, a degree of divergence between nucleotide sequences that is approximately similar to the degree of divergence that may be observed between two different strains of the same plant virus in nature. These genes represent specific

functional units analogous in principle to viral domains that are very similar but not identical, and can be distinguished functionally.

CFP and YFP genes were cloned into a binary PVX vector pgR106 and designated pgR106::CFP and pgR106::YFP, respectively (Figure 2.1A). To test expression of CFP and YFP *in planta*, a transient assay was used. *N. benthamiana* plants were inoculated with *A. tumefaciens* carrying pgR106, pgR106::CFP and pgR106::YFP. Plants inoculated with *A. tumefaciens* carrying empty vector pgR106 showed typical systemic PVX mosaic symptoms at 6 - 9 dpi. Plants inoculated with pgR106::CFP and pgR106::YFP displayed similar symptoms to plants inoculated with empty vector. Introduction of CFP and YFP via PVX-derived vectors resulted in symptoms that developed 2 -3 days later than those observed in plants inoculated with *Agrobacterium* containing empty vector pgR106.

At 10 dpi, leaves displaying symptoms were harvested from each treatment and examined under a confocal microscope to detect fluorescent protein expressed by the PVX vectors. *N. benthamiana* leaves infected by the empty vector control did not show either CFP or YFP expression, whereas plant leaves infected with PVX carrying CFP and YFP showed the expected result, the obvious presence of fluorescent protein (Figure 2.1B).

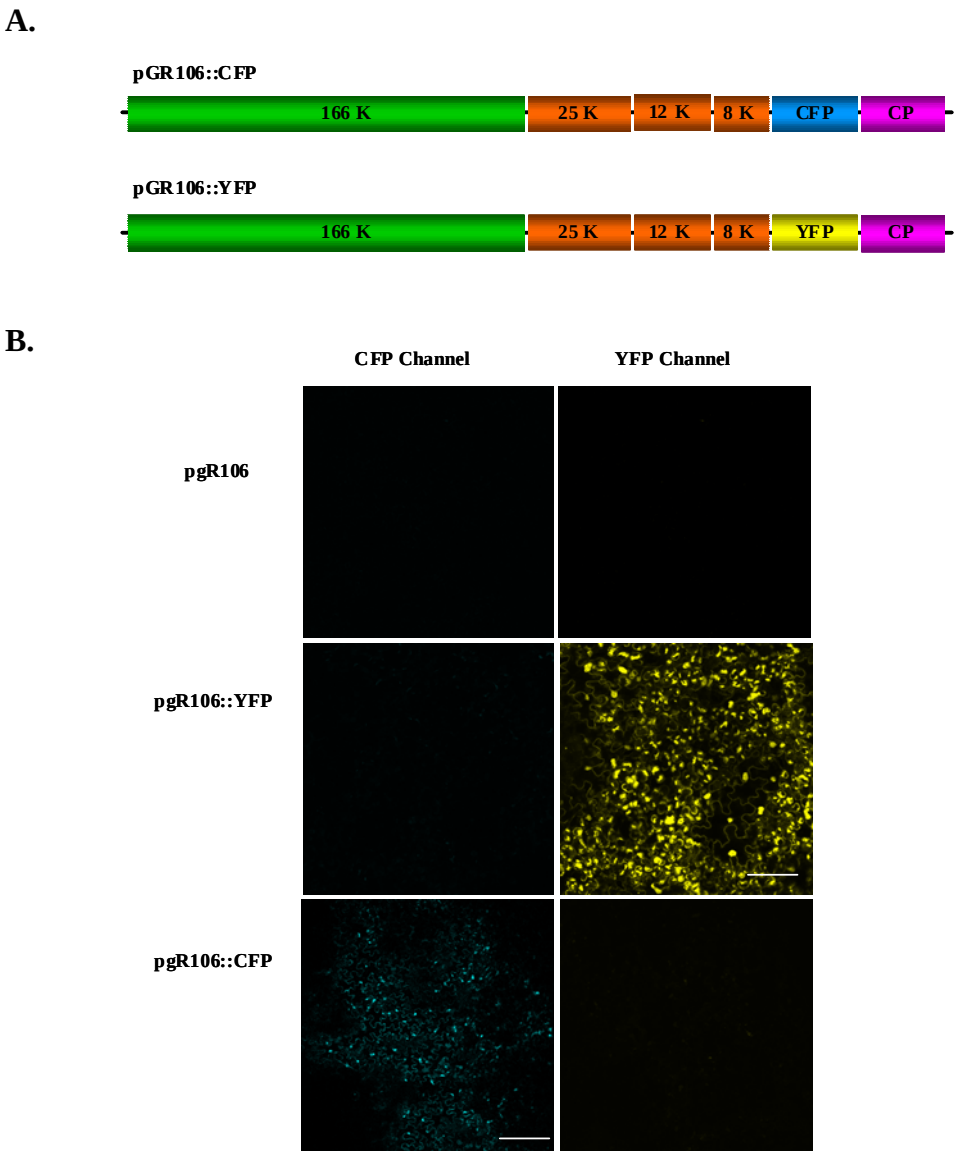

Figure 2.1. Cyan (CFP) and yellow fluorescent protein (YFP) expression in *N. benthamiana* cells infected with PVX-derived vectors.

A. PVX constructs used in this study. CFP and YFP genes were cloned into the binary PVX vector pgR106.

B. CFP and YFP expression observed by confocal laser microscopy 10 days after *Agrobacterium*-mediated inoculation. *Agrobacterium* containing empty vector pgR106 was used as a negative control. Scale bar = 100 μm.

Conversion of CFP to YFP by megaprimer-mediated amplification reaction

The megaprimer approach was applied to convert CFP cloned in pGR106 vector to YFP (Kirsch and Joly 1998). A schematic diagram of megaprimer primer amplifications is shown Fig. 2. A standard PCR reaction was performed to amplify 720 bp YFP DNA fragments (Figure 2.2, Figure 2.3A). To test megaprimers of different lengths, 1000, 1200, and 1500 bp DNA fragments were amplified using pgR106::YFP as a template; each fragment contained full length YFP (Figure 2.2 and Figure 2.3A). These four fragments served as megaprimers in the second amplification reaction to convert CFP in pgR106::CFP plasmids to YFP. Megaprimer amplification products were subjected to gel electrophoresis to confirm that bands of the expected size (12.3 kb) remained after *Dpn*I digestion (Figure 2.3B). A 1 μL aliquot of each reaction transformed into *E.coli* generated more than 100 colonies for each mutagenized plasmid (Table 2.1). The number of colonies obtained was dependent on primer concentration, rather than on primer size (Figure 2.3A and Table 2.1). Amplification products at higher concentrations (Figure 2.3B, lane 2 and 3) resulted in more colonies than products at lower concentration (Figure 2.3B, lane1 and 4).

To test if the colonies carried mutagenized plasmids, colony PCR and restriction enzyme digestion reactions were performed. CFP replacement could be screened easily using *Pst*I; an enzyme which cuts YFP genes into two fragments, 220 and 500 bp in length, but does not cleave CFP (Fig. 3C). For each mutagenized plasmid, 45 colonies were tested. Results are summarized in Table 2.1. When mutagenesis efficiency for megaprimers of various sizes was compared, all of the primers showed more than 90% efficiency. A slight trend towards increased efficiency was observed for longer primers (Table 2.1).

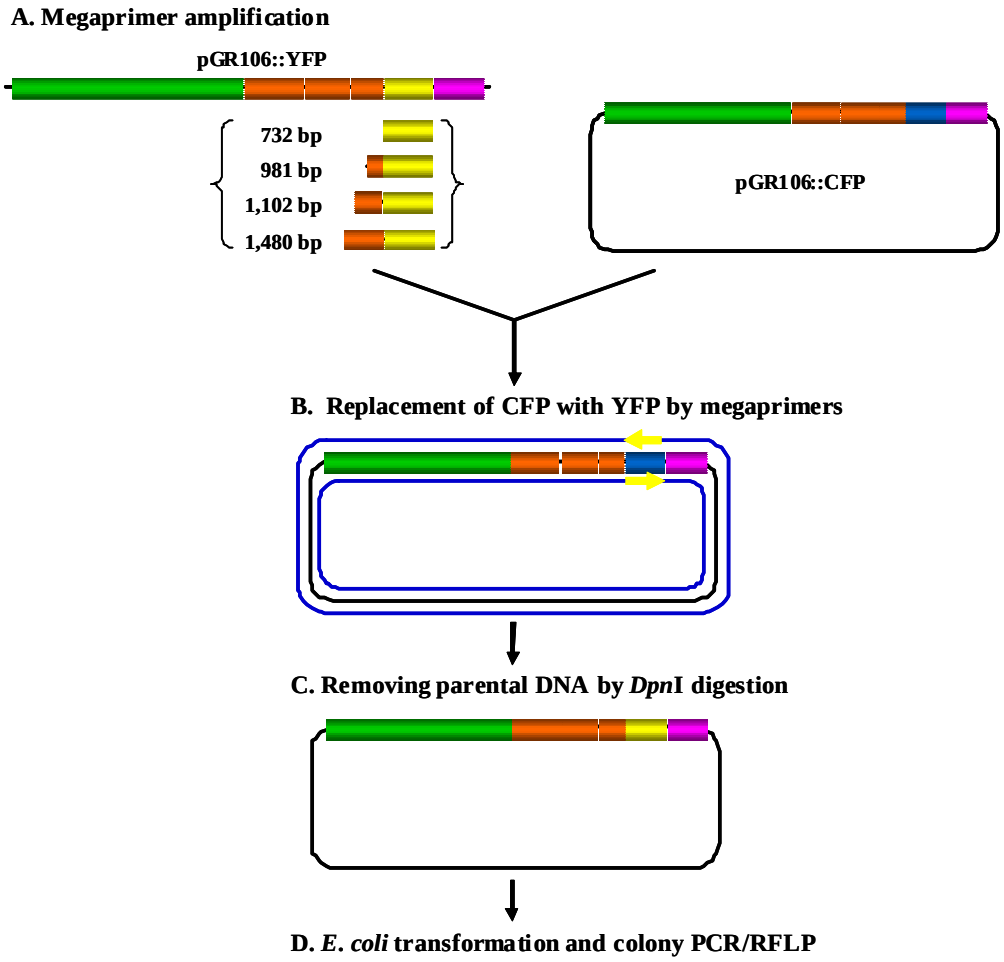

Figure 2.2. A schematic diagram of megaprimer-mediated domain swapping. Megaprimer amplification was performed in two steps. First megaprimers were generated containing YFP and different segments of the PVX genome.

A. Resulting amplification products were recovered and purified.

B. The target gene was amplified using the megaprimer.

C. Digested with *Dpn*I to remove parental template DNA.

D. The *in vitro* synthesized DNA was used to transform *E. coli*.

Figure 2.3. DNA gel analyses for megaprimer-mediated domain swapping.

A. PCR reaction to amplify megaprimers. Lane M, 1 kb ladder; lane 1, 732 bp megaprimer; lane 2, 981 bp megaprimer; lane 3, 1102 bp megaprimer; lane 4, 1480 megaprimer. Megaprimer 981, 1102, and 1480 bp contained PVX sequences of various lengths in addition to the 732 bp YFP gene sequence.

B. Domain swapping amplification reaction and removal of template plasmids. The DNAfragments resulting from the PCR reaction (A) served as megaprimers with pgR106::CFP as template. The amplification products were treated with *Dpn*I to remove template plasmids. Lane M, 1 kb ladder; lanes 1 - 4, DNA fragments amplified using megaprimers 732, 981, 1102 and1480 bp, respectively. Arrow indicates amplified and *Dpn*I digested DNA fragments.

C. Colony PCR and restriction enzyme digestion. *Dpn*I treated amplification (A) products were transformed into *E. coli*. Clones containing swapped plasmids were subject to PCR using primers YFP-F and YFP-R and the amplified DNA fragments were digested by *Pst*I. Lane M, 1 kb ladder; lane C, CFP; lane Y, YFP; other lanes, putative pgR106::CFP →YFP clones.

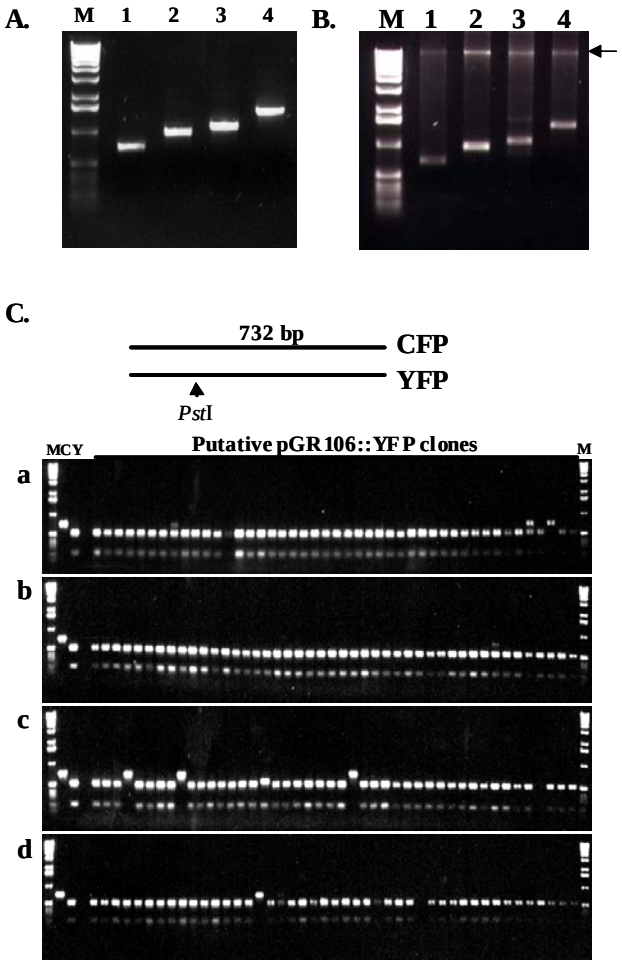
A.
M 1 2 3 4
B.
M 1 2 3 4
C.
732 bp
CFP
YFP
PstI
MCY
Putative pGR106::YFP clones
M
a
b
c
d

Table 2.1. Summary of mutagenesis efficiency using megaprimers of various sizes.

Megaprimer size (bp)	No. colonies obtained[a]	No. plasmids analyzed[b]	No. plasmid swapped[c]	Mutagenesis efficiency[d] (%)
732	100	44	41	93.0
981	301	45	43	95.5
1102	347	44	43	97.7
1480	177	45	45	100.0

[a] Number of colonies obtained from transformation of 1 µl out of 25 µl reaction.
[b] Number of colonies that amplified 732 bp inserts.
[c] Number of PCR products showing the same *Pst*I digestion pattern as the YFP gene.
[d] Mutagenesis efficiency was calculated as the number of mutants confirmed by restriction enzyme digestion of PCR products/total number of plasmids analyzed.

Verification of fluorescent phenotype change by confocal microscopy

To demonstrate that the mutagenized plasmids yielded infectious transcripts in addition to producing fluorescence, *Agrobacterium*-mediated PVX inoculation was performed as described previously (Jones et al. 1999). Sixteen randomly selected plasmids (pgR106::CFP→YFP) confirmed by PCR/RFLP were isolated from *E. coli* and re-transformed into *A. tumefaciens* GV3101. *Agrobacterium* cells carrying the plasmids were grown and inoculated on *N. benthamiana* leaves; four plants were inoculated for each plasmid. All inoculated *N. benthamiana* plants displayed systemic mosaic symptoms typical of PVX infection at 8 - 10 dpi comparable to results obtained with pgR106::CFP and pgR106::YFP. Symptom development observed in plants inoculated with pgR106::CFP→YFP plasmids was identical to control plants inoculated with pgR106::YFP. In order to test for differences in virus accumulation among treatments, indirect ELISA analysis was performed using anti-PVX antibody.

In plants inoculated with the swapped PVX mutants, PVX proteins apparently accumulated to slightly higher levels, but were very similar to control plants (Figure 2.4). This observation suggests that there were no detectible mutations in these plasmids that affected viral infection and pathogenesis.

N. benthamiana plants were inoculated with *Agrobacterium* carrying plasmids resulting from the CFP/YFP swap. Leaves showing PVX symptoms were observed using a confocal microscope to confirm a shift from cyan to yellow fluorescence. Except for one case, leaves of all plants tested showed strong yellow fluorescent protein expression comparable to control plants inoculated with pgR106::YFP plasmid. This result demonstrated that the CFP gene was successfully replaced by YFP (Figure 2.5).

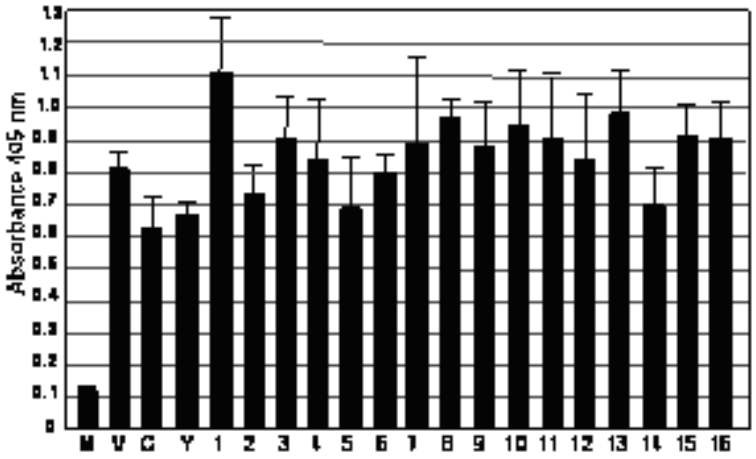

Figure 2.4. ELISA analysis of *N. benthamiana* following inoculation with test constructs and controls. Accumulation of PVX coat protein as determined by ELISA of systemically infected leaves of mock-inoculated (M) and vector-inoculated (V) *N. benthamiana* plants and plants inoculated with pgR106::CFP, pgR106::YFP, or the domain-swapped pgR106::CFP→YFP (1 - 16). Virus accumulation in upper uninoculated leaves was determined at 21 dpi.

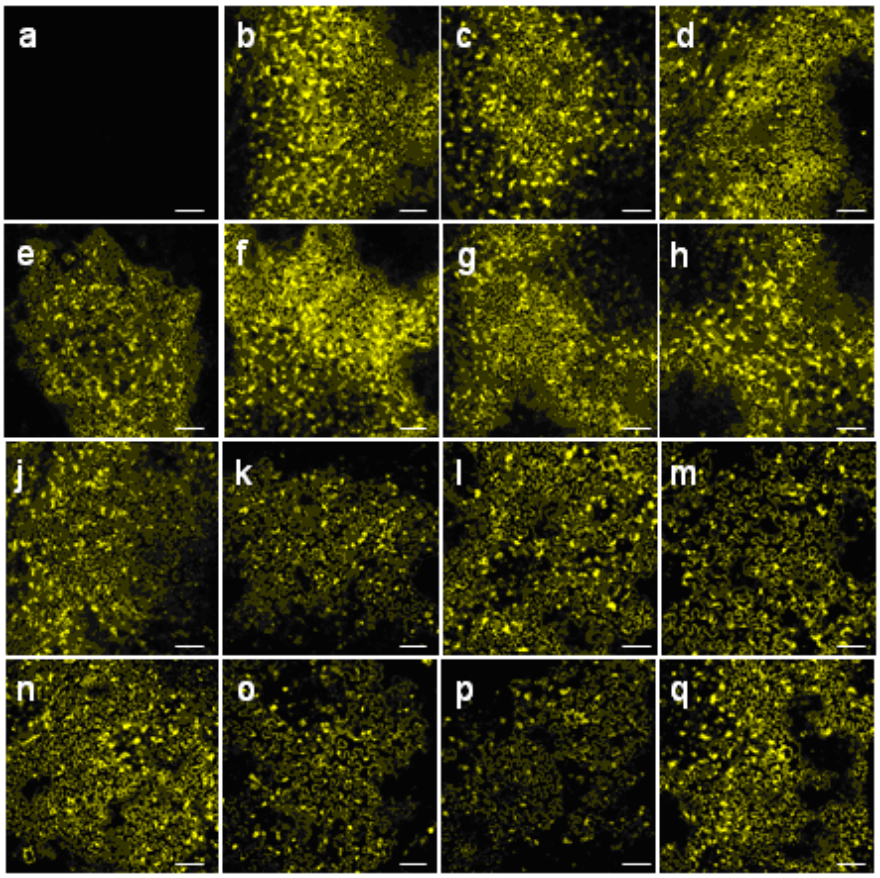

Figure 2.5. Yellow fluorescent protein expression in *N. benthamiana* leaves inoculated with *Agrobacterium* cultures containing *in vitro* mutagenized pgR106::YFP. *In vitro* mutagenized pgR106::YFP plasmids were transformed into *A. tumefaciens* and inoculated in *N. benthamiana* plants. Systemically infected leaves showing PVX symptoms were observed under the confocal microscope and photographed at 10 dpi. a - q: *N. benthamiana* leaves infected by pgR106::CFP→YFP clones. Scale bar = 100 μm**.**

Frequency of unintended mutations in chimeric clones

One potential disadvantage of this method might be the introduction of undesired mutations during *in vitro* plasmid synthesis. The mutation frequency for the megaprimer amplification reaction was determined according to Lundberg *et al.,* (1991) as described below, and confirmed by plasmid sequencing. The mutation frequency (mf) of any PCR reaction using *PfuUltra* can be calculated using the *PfuUltra* error rate (ER) in the formula ER = mf/ (bp × d) (Lundberg et al. 1991) where "bp" reflects the number of base pairs in the sequence of interest. The term, d, number of cycles of template duplications, is determined by the equation 2^d = (amount of PCR product) / (amount of starting target) because the amount of product increases exponentially. However, in this reaction, the mutagenized product contains staggered nicks which make it incapable of serving as template for the next round of PCR (Strategene 2004). Template doublings can be calculated for this 18 cycle reaction using the formula 2^d = 36 copies (2 ×18 cycles), d = 5.14. According to this formula, just 3.4% of the mutagenized pGR106 PCR product, a 12.3 kb plasmid with approximately 5 template doublings, would contain a mutation introduced in error.

Using the binomial proportion, a 95% confidence level could be assigned to the proportion of non-mutated plasmids that would be greater than a defined cutoff of 25% (calculated using S-plus; power 0.8, $\alpha = 0.05$). This mutation frequency accounts for both silent and detectable single nucleotide mutations in the PCR product. According to this calculation, 25%, or at least one in four plasmids screened per reaction should not contain a single mutation. To test this prediction, four randomly selected plasmids were sequenced. Forward and reverse primers were synthesized at 600 bp intervals covering the entire plasmid sequence (Table 2.1). One plasmid was entirely mutation free, while 4 point mutations were detected in the other three plasmids (Table 2.2). However, all 4 mutations were located in the YFP gene

indicating the mutations were introduced during megaprimer synthesis. High fidelity *Taq* DNA polymerase was not used for this PCR reaction. Excluding the 4 mutations in the megaprimer, sequencing analysis revealed unintentional mutations were not generated during the domain-swapping reaction.

Table 2.2. Summary of sequence analysis to determine rate of unintentional mutation.

Plasmid sequenced	Mutations	Gene affected by mutation
pgR106::CFP→YFP #3	G→A (2716), A→G (2917)	YFP
pgR106::CFP→YFP #4	G→A (2816)	YFP
pgR106::CFP→YFP #5	-	-
pgR106::CFP→YFP #6	G→A (2831)	YFP

Discussion

This study demonstrates an efficient and reliable domain-swapping method that allows for quick construction of clones encoding chimeric viral genomes. In place of restriction enzymes and ligases, a two-step site-directed mutagenesis method allowed for the replacement of the CFP gene in a binary PVX-derived vector with the YFP gene. Fragments up to 1480 bp could be exchanged efficiently. Although previous studies showed that larger megaprimers were exchanged less efficiently than shorter megaprimers (Barik and Galinski 1991; Upender, Raj, and Weir 1995), these results have demonstrated efficient exchange of an extremely large fragment (1480 bp), equivalent in size to some of the larger genes encoded by plant viruses.

Because plasmids were mutagenized *in vitro* using thermostable DNA polymerase, there was a possibility the mutagenized plasmids could contain unwanted mutations.

However, in contrast to standard PCR, the predicted mutation rate for this method is extremely low for several reasons. First, the newly synthesized product of each round of PCR is not used as a template for the following round of amplification because it contains nicks at the initiation site of each mutagenic primer (Strategene 2004). Second, this method requires fewer reaction cycles (less than 20 thermal cycles) than a typical PCR, further reducing the potential mutation rate. Finally, *PfuUltra* has exonuclease-dependent proofreading activity resulting in 18-fold higher fidelity than regular *Taq* DNA polymerase (Flaman *et al.*, 1994, Cline *et al.*, 1996). The error rate of *Taq* polymerase has been reported as approximately 10-fold higher than *Pfu*, and 30-fold higher than *PfuUltra* (Cline *et al.*, 1996, Stratagene personal communication). The *Pfu* error rate has been determined to fall between 1.3×10^{-6} and 1.6×10^{-6} (Cline *et al.,* 1996; Lundberg *et al.,* 1991). The error rate of *PfuUltra* could therefore be estimated to fall between 4.3×10^{-7} and 5.33×10^{-7}.

The mutation frequency for the megaprimer amplification reaction was estimated as previously described (Lundberg *et al.,* 1991). According to this calculation, at least one out of four plasmids isolated from each reaction should be free of any mutations. In fact, sequence analysis revealed the megaprimer PCR reaction using *PfuUltra* did not generate a single mutation in the four plasmids screened. Plasmid mutations were all located within the megaprimer sequence which was synthesized using standard *Taq* DNA polymerase. These mutations could likely have been prevented with the use of high fidelity *Taq* DNA polymerase.

This study tested whether the mutagenized plasmids were capable of producing an infectious viral genome. While slight variation in YFP accumulation was observed among the clones as expected due to subtle differences that typically occur during viral infections as a consequence of environmental or unwanted YFP mutations (see Figure 2.5), all 16 plasmids tested showed no noticeable differences in infectivity and

symptom development, indicating this method is generally reliable for creating chimeric virus mutants. This technique has been critical to identify precisely the avirulence determinant of *Tobacco etch virus* (TEV) that is involved in overcoming *pvr1*-mediated resistance in *Capsicum* (Kang et al. 2005; Murphy et al. 1998). Using megaprimer PCR to swap domains of avirulent TEV strains with domains of virulent TEV strains circumvented the limitation posed by absence of restriction sites surrounding the domains of interest. Rather than adding additional steps to generate unique restriction sites necessary for conventional cloning and domain exchange, this technique provided a particularly simple and easy method to swap TEV domains implicated in host resistance. Megaprimer domain swapping will likely find wide applicability in any study where clones encoding precisely defined regions of plant viral genomes are required.

Materials and methods

Cloning CFP and YFP into a binary PVX vector

CFP and YFP genes were purchased from BD biosciences (Palo Alto, CA) and cloned into pgR106 provided by D. Baulcombe (Jones et al. 1999). The 720 bp coding region of each gene was amplified via PCR from the plasmids using the primers YFP-ClaI and YFP-SalI. Amplified fragments were cloned into pGEM^{T} (Promega, Madison, WI) and confirmed by sequencing. Sequence confirmed plasmids were digested with *Cla*I and *Sal*I and subcloned into corresponding sites in the pgR106 vector. The resulting pgR106 derivatives were designated pgR106::CFP and pgR106::YFP.

Megaprimer-mediated domain swapping to replace CFP with YFP

Megaprimer amplication was performed in two steps. The first step was to generate the YFP megaprimer, a 732 bp DNA fragment, amplified using the YFP-F and YFP-R primers (Table 2.3). Additional megaprimer reactions were carried out with three different forward primers, PVX_{6421}, PVX_{6300}, and $PVX_{5922,}$ each combined with YFP-R. These reactions generated megaprimers of 981, 1102, and 1480 bp, respectively.

The 50 µL PCR reaction contained 10 ng YFP plasmid, 200 nM of each primer, 200 µM dNTPs and 1.0 M Advatage2 DNA polymerase (Invitrogen, Carlsbad, CA) in 1× Advantage2 reaction buffer. PCR amplification was performed in a MJR PCT-100 thermal cycler (MJ Research Inc., Watertown, MA). After initial denaturation at 94^{o}C for 2 min, DNA fragments were amplified through 30 cycles (94^{o}C, 60 sec; 55^{o}C, 60 sec; 72^{o}C, 90 sec) followed by elongation at 72^{o}C for 5 min. Amplification products were electrophoresed on 1% agarose gels; amplified DNA fragments were recovered and purified using Qiagen gel purification kit (Valencia, CA).

Table 2. 3. Primer sequences used in this study.

Primer name[a]	Orientation	Primer sequence[b]
YFP-ClaI	Forward	5`-CCATCGATATGGTGAGCAAGGGCGAGG-3`
YFP-SalI	Reverse	5`-CGGGTCGACTTACTTGTACAGCTCGTCCATG-3`
YFP-F	Forward	5`-ATCGATATGGTGAGCAAGGGCGAGG-3`
YFP-R	Reverse	5`-TTACTTGTACAGCTCGTCCATG-3`
PVX_{5922}	Forward	5`-GGAAGAAGGGCACTTAGAG-3`
PVX_{6300}	Forward	5`-ACAGCTTGCCACACGGAGGAG-3`
PVX_{6421}	Forward	5`-GCTGATCTATGGAAGTAAATAC-3`
pgRPVXF1	Forward	5`-CCCGGAAGCCTGTGGATAG-3`
pGRPVXR1	Reverse	5`-CTATCCACAGGCTTCCGGG-3`
pGRPVXF2	Forward	5`-GGTCAACATGGTGGAGCACG-3`
pGRPVXR2	Reverse	5`-CGTGCTCCACCATGTTGACC-3`
pGRPVXF3	Forward	5`-GGAAAAACACAAACTAGCT-3`
pGRPVXR3	Reverse	5`-AGCTAGTTTGTGTTTTTCC-3`
pGRPVXF4	Forward	5`-CCAAGGATAGCTTTCTC-3`
pGRPVXR4	Reverse	5`-GAGAAAGCTATCCTTGG-3`
pGRPVXf5	Forward	5`-GCAAGCGTGGAAAGCCTTCCGA-3`
pGRPVXR5	Reverse	5`-TCGGAAGGCTTTCCACGCTTGC-3`
pGRPVXF6	Forward	5`-CAAGGCGCTGGAAATTCAGAGG-3`
pGRPVXR6	Reverse	5`-CCTCTGAATTTCCAGCGCCTTG-3`
pGRPVXF7	Forward	5`-GACAAAGCGTCTACCATGAAACT-3`
pGRPVXR7	Reverse	5`-AGTTTCATGGTAGACGCTTTGTC-3`
pGRPVXF8	Forward	5`-GGAAAAGTTTGACAGAGAGATC-3`
pGRPVXR8	Reverse	5`-GATCTCTCTGTCAAACTTTTCC-3`
pGRPVXF9	Forward	5`-GGAATTTCAGCAGACCTAGC-3`
pGRPVXR9	Reverse	5`-GCTAGGTCTGCTGAAATTCC-3`
pGRPVXF10	Forward	5`-CACACACTCACTTGCAGAACA-3`
pGRPVXR10	Reverse	5`-TGTTCTGCAAGTGAGTGTGTG-3`
pGRPVXF11	Forward	5`-GGAGTCTGAGACAACACTG-3`
pGRPVXR11	Reverse	5`-CAGTGTTGTCTCAGACTCC-3`
pGRPVXF12	Forward	5`-GGAAAGAACGCAGCATTTGCT-3`
pGRPVXR12	Reverse	5`-AGCAAATGCTGCGTTCTTTCC-3`
pGRPVXF13	Forward	5`-ATGGAAGTAAATACATATC-3`
pGRPVXR13	Reverse	5`-GATATGTATTTACTTCCAT-3`
pGRPVXF14	Forward	5`-GATACAGGTCCCTATTCCAAC-3`
pGRPVXR14	Reverse	5`-GTTGGAATAGGGACCTGTATC-3`
pGRPVXF15	Forward	5`-CGTTCAAACATTTGGCAAT-3`
pGRPVXR15	Reverse	5`-ATTGCCAAATGTTTGAACG-3`
pGRPVXF16	Forward	5`-GCGCTCACTGCCCGCTTT-3`
pGRPVXR16	Reverse	5`-AAAGCGGGCAGTGAGCGC-3`
pGRPVXF17	Forward	5`-GGGCTGTGTGCACGAACC-3`
pGRPVXR17	Reverse	5`-GGTTCGTGCACACAGCCC-3`

Table 2. 3. Continued

pGRPVXF18	Forward	5`-CCAACATCAATACAACCTATT-3`
pGRPVXR18	Reverse	5`-AATAGGTTGTATTGATGTTGG-3`
pGRPVXF19	Forward	5`-GGCGCATCGGGCTTCCC-3`
pGRPVXR19	Reverse	5`-GGGAAGCCCGATGCGCC-3`
pGRPVXF20	Forward	5`-GCCCGCCGTCGAGCGGGC-3`
pGRPVXR20	Reverse	5`-GCCCGCTCGACGGCGGGC-3`

[a] pgRPVXOO primers were used for plasmid sequencing.
[b] Underlined sequences indicate introduced restriction enzyme sites.

The second step involved amplifying the target gene using the megaprimer. For the reaction, the Quikchange XL kit manufacturer's protocol was followed exactly (Stratagene, La Jolla, CA). The 50 µL amplification reactions contained 100 ng of megaprimers, 10 ng of pgr106::CFP plasmid, 2.5 U *Pfu* DNA polymerase, 50 µM dNTPs. The thermal cycle program used was: 95 ^{o}C for 90 sec, 18 cycles of 94^{o}C, 60 sec; 55^{o}C, 60 sec; 68^{o}C, 12 min, 72 ^{o}C for 15 min. Following this PCR reaction, a digestion with 10 U *Dpn*I was performed at 37 ^{o}C for 1 hr. After digestion, 1 – 2 µL of the reaction was transformed into *E. coli*. Resulting clones were evaluated by PCR, restriction enzyme digestion, and sequenced to verify that the selected clones contained the desired mutation.

***Agrobacterium*-mediated PVX inoculation**

Mutated plasmids (pgR106::CFP → YFP) were electro-transformed into *Agrobacterium* strain GV3101. Cells were allowed to grow for 2 days at 28 ^{o}C on LB plates containing tetracycline (12.5 µg/mL) and kanamycin (50 µg/mL). Selected colonies were inoculated with a toothpick near main veins of lower leaves of *N. benthamiana* plants at the 4 to 6 leaf stage of development. Four plants were inoculated per plasmid construct including empty vector pgR106 as a control.

Monitoring YFP fluorescence using a confocal microscope

Ten days after inoculation, *Nicotiana benthamiana* leaves were monitored for YFP and CFP fluorescence using a Leica TCS SP2 scanning confocal microscope (Leica, Wetzlar, Germany). YFP was excited by a 4-line argon laser at 514 nm and detected between 525 nm and 600 nm.

Enzyme-linked immunosorbent assay

Plants were monitored daily for the appearance and severity of symptoms. Leaf tissue was evaluated for the presence of virus using antigen plate-coating indirect enzyme-linked immunosorbent assay (ELISA) as previously described (Kang et al. 2005). Anti-PVX antibodies were obtained from Agdia, Inc. (Elkhart, IN) and were used according to the manufacturer's instructions. Virus accumulation was tested 10 dpi for inoculated leaves or at 21 dpi for un-inoculated leaves.

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CHAPTER 3

A NARROW RANGE OF VARIATION IN VPG DETERMINES *TOBACCO ETCH VIRUS* INFECTIVITY SPECTRUM IN *CAPSICUM*[1,2]

Abstract

Potyvirus resistance in *Capsicum* has been attributed to point mutations at the *pvr1* locus which cause conformational shifts in eukaryotic translation initiation factor eIF4E. Two recessive *pvr1* alleles were the focus of this study, *pvr1* and *pvr1*^{2}. Resistance conferred by these alleles has been linked to the protein's inability to interact with the viral encoded protein VPg, covalently bound to the 5' end of the potyvirus genome. In this study, the VPg domains within three *Tobacco etch virus* (TEV) strains: TEV HAT, TEV Mex21 and TEV N, which differentially infect *Capsicum* lines encoding *pvr1* and *pvr1*^{2} were compared. To determine if VPg was the virulence determinant for these virus strains two VPg viral chimeras were synthesized. Substituting VPg from an infectious TEV strain was found to determine the outcome of host infection for both *pvr1* and *pvr1*^{2}. Additionally, TEV-HAT VPg interaction with susceptible allele *Pvr1*$^+$ was analyzed in a yeast two hybrid assay. The interaction between the VPg and eIF4E was not detected when 80 or more amino acids were deleted from the 5' end of the VPg. 3D models constructed of VPg and eIF4E to analyze protein-protein interaction between the two proteins correlate with susceptibility.

[1]In final Preparation, Molecular Plant-Microbe Interactions
Kari Perez, Byoung Cheorl Kang, Inhwa Yeam, John F. Murphy, Daniel R. Ripoll and Molly M. Jahn
[2]Byoung-Cheorl Kang and Molly Jahn conceived of the chimeric viruses including in this study. Kari Perez completed all the experiments except for the protein modeling work which was completed by Daniel Ripoll. Kari wrote this document with assistance from Inhwa Yeam and John Murphy.

Introduction

Potyviruses make up the largest genus of plant viruses and are found within the *Potyviridae* family. Examples such as *Tobacco etch virus* (TEV) can infect agronomically important solanaceous crops such as pepper, tomato, potato and tobacco. Based on structural similarity, potyviruses reside within the same class or supergroup as members of the *Picornavirdae* family which includes polioviruses and rhinoviruses (Koonin, Dolja, and Morris 1993). They are single-stranded positive sense RNA molecules approximately 10 kb in length that encode a polyprotein that is subsequently cleaved into functional proteins by three virus encoded proteinases: P1, the helper component proteinase (HC-Pro) and the nuclear inclusion a (NIa) (Dougherty and Carrington 1988; Dougherty and Semler 1993). The NIa, which is also referred to as VPg-Pro, encodes a proteinase domain in its C terminus, and viral genome linked protein (VPg) in its N-terminus (Carrington and Dougherty 1987; Dougherty and Dawn Parks 1991; Murphy et al. 1990). After proteinase processing, the cleaved VPg protein is then covalently bound to the 5' end of the virus genome by a tyrosine residue (Murphy et al. 1991). Mutation of this tyrosine residue in potyvirus *Tobacco vein mottling virus* rendered the virus noninfectious (Murphy et al. 1996).

Virulence determinants, which are encoded by the virus and critical for infection, provide clues into host-virus interactions. VPg has been implicated as the virulence determinant in many resistance systems controlled by recessively inherited genes. VPg has been identified as the pathogenicity determinant for recessive resistance in nine pathosystems, although the virus is controlled at different stages of infection in these studies (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Rajamaki and Valkonen 1999; Rajamaki and Valkonen 2002). Functions of VPg in the virus infection cycle have been proposed previously (Lellis et al. 2002; Robaglia and

Caranta 2006) and includes potential roles in replication, translation, host gene inactivation and potyviral genome stabilization. In poliovirus replication, the uridylylated form of VPg is required for viral replication (Murray and Barton 2003; Paul et al. 1998). In vitro uridylylation has also been observed in the potyviruses, *Potato virus A* (PVA) and *Pepper vein banding virus* (Anindya, Chittori, and Savithri 2005; Puustinen and Makinen 2004). In these studies, uridylylation was carried out by nuclear inclusion protein b (NIb), which has RNA-dependent RNA polymerase activity (Hong and Hunt 1996). These findings suggest that VPg uridylyation is a requirement for replication; however, this has yet to be definitively demonstrated. In addition to a role in replication, studies have also implicated VPg has a role in viral translation. This role is in part contradicted by the fact that several VPg-encoding viruses, including TEV, have an internal ribosome entry site (IRES). It has been shown in several IRES encoding viruses, including TEV, that translation can still proceed without a VPg (Carrington and Freed 1990; Gallie 2001; Niepel and Gallie 1999). However, we cannot rule out the potential role of VPg as a co-initiator or an enhancer of viral translation along with the IRES. This is supported by evidence that VPg interacts with host eukaryotic translation initiation factor eIF4E (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Graner and Bauer 1993; Kanyuka et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Rajamaki and Valkonen 1999; Rajamaki and Valkonen 2002; Roudet-Tavert et al. 2007; Wittmann et al. 1997; Yambao et al. 2003). eIF4E is part of the translation initiation complex eIF4F, which consists of eIF4A, eIF4E and eIF4G (Browning 2004; Gingras, Raught, and Sonenberg 1999). This complex is responsible for translation initiation which includes mRNA 5' cap recognition, mRNA unwinding and recruitment of the 40S ribosomal subunit (Browning 2004; Browning 1996; Gingras, Raught, and Sonenberg 1999; Kawaguchi and Bailey-Serres 2002; Nicaise et al. 2007).

Naturally occurring mutations encoded in eIF4E have been correlated with virus resistance in five plant families and include resistance to bymoviruses, carmoviruses and potyviruses (Gao 2004; Kang et al. 2005; Kanyuka et al. 2005; Nicaise et al. 2003; Nieto et al. 2006; Ruffel et al. 2005; Ruffel et al. 2002; Stein et al. 2005). Several studies which have identified eIF4E as the recessive resistance gene have identified VPg as a virulence determinant (Borgstrom and Johansen 2001; Kuhne et al. 2003; Moury et al. 2004). Since potyviral RNA is structurally similar to host mRNA, it has been hypothesized that VPg serves as a cap mimic, suggesting VPg interaction with eIF4E is essential for the viral translation. Mutations in eIF4E have been found to inhibit its interaction with VPg *in planta*, as well as in yeast, and have been correlated with potyvirus resistance (Bruun-Rasmussen et al. 2007; Kang et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Yeam et al. 2007). Furthermore, the central region of VPg has been identified as essential both for its interaction with eIF4E and for virus infectivity (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Graner and Bauer 1993; Kanyuka et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Rajamaki and Valkonen 1999; Rajamaki and Valkonen 2002; Roudet-Tavert et al. 2007; Wittmann et al. 1997; Yambao et al. 2003). The central region of PVA VPg was found to be essential for *Solanum commersonii* resistance, and mapped to a single amino acid change His118Tyr (Rajamaki and Valkonen 2002). *Nicandra physaloides* susceptibility to PVA was also found to be determined by a single amino acid change in the same region of the VPg, Leu185Ser (Rajamaki and Valkonen 2002). Resistance to *Potato virus Y* (PVY) in *Lycopersicum hirsutum*, conferred by resistance gene *pot1*, was no longer effective if there was a single amino acid substitution in the central region of PVY VPg, Arg119His (Moury et al. 2004). In *Pisum sativum, Pea seedborne mosaicvirus* (PSbMV) resistance conferred by the mutated eIF4E gene, *sbm1*[1], was rendered

ineffective by mutations encoded in the central region (amino acids 105-107) of PSbMV VPg (Borgstrom and Johansen 2001). *P. sativum* resistance to *Bean yellow mosaic virus* (BYMV) conferred by the *wlv* gene, which also encodes eIF4E, did not confer resistance to a BYMV isolate encoding a single mutation in the central region of VPg, Arg116His (Bruun-Rasmussen et al. 2007). All these studies concerned potyviruses or bymoviruses which both reside in the *Potyviridae* family. In fact, all studies used potyviruses, except for a one study which found the central region of VPg determined pathogenicity to the bymovirus, *Barley yellow mosaic virus* in barley plants containing the eIF4E resistance gene *rym4* (Graner and Bauer 1993; Kanyuka et al. 2005; Kuhne et al. 2003).

In addition to the role eIF4E-VPg interaction could play in the viral infection cycle, this interaction may prohibit or alter expression of eIF4E from participating in normal cell functions and subsequently adversely affect the host. This has been shown in plants infected with potyvirus *Pea seedborne mosaic virus* where host mRNA and protein levels were suppressed in infected leaves (Wang and Maule 1995). In addition to cytoplasmic host translation, eIF4E has been detected in the nucleus where it was believed to function in nascent nuclear translation and mRNA export to the cytoplasm (Iborra, Jackson, and Cook 2001; Lejbkowicz et al. 1992). Interaction with VPg could inhibit these crucial cellular functions and potentially make the plant more vulnerable to viral infection.

TEV VPg has been the focus of several studies (Dougherty and Dawn Parks 1991; Murphy et al. 1990; Schaad and Carrington 1996; Schaad, Lellis, and Carrington 1997; Schaad et al. 1996). eIF4E mediated resistance conferred by the *pvr1* locus correlated with a lack of interaction with TEV VPg (Kang et al. 2005; Murphy et al. 1998). In our study, we focused on two alleles at the *pvr1* locus demonstrating differential infectivity for two strains of TEV. Pepper plants encoding

the eIF4E resistance allele *pvr1* are susceptible to TEV-Mex21 (Murphy et al. 1998), and pepper plants encoding the *pvr1*^{2} allele are susceptible to TEV-N (Jahn lab, unpublished results), whereas both of these alleles confer resistance to TEV-HAT (Deom, Murphy, and Paguio 1997; Murphy et al. 1998). This study identifies naturally occurring amino acid substitutions in TEV VPg, primarily occurring in the central domain, which are sufficient to establish infection in a previously resistant host. This domain was additionally confirmed to be critical for eIF4E-VPg interaction in a yeast two hybrid assay which showed interaction was eliminated after 80 amino acids (aa) or more of the 188 aa TEV-VPg protein were deleted.

Results

Chimeric TEV strains containing VPg from different TEV strains are generated to determine if the VPg is the virulence determinant of TEV.

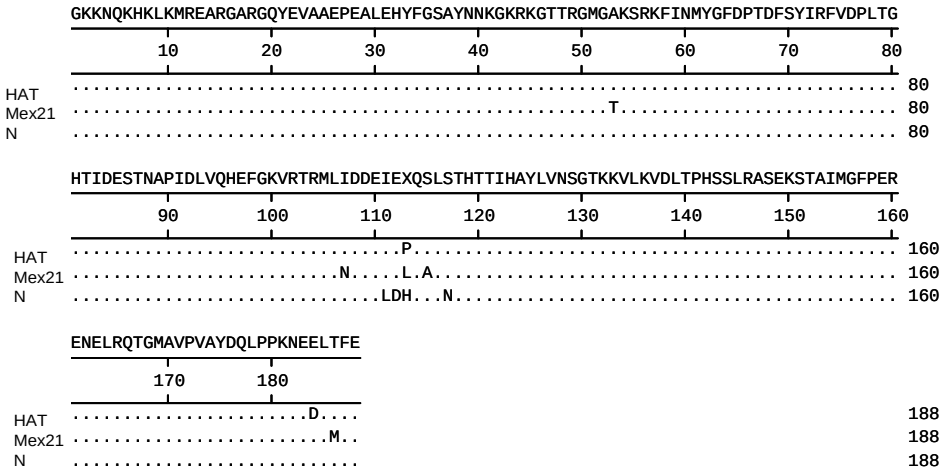
The *pvr1* resistance alleles *pvr1* and *pvr1*^{2} differ from susceptible allele *Pvr1*$^+$ by four and three distinct amino acid substitutions respectively; however they vary in their TEV resistance spectra (Figure 3.1A) (Kang et al. 2005). Pepper plants containing the eIF4E resistance allele *pvr1* were resistant to all TEV strains tested except TEV-Mex21 (Murphy et al. 1998), and pepper plants containing the *pvr1*^{2} allele were also resistant to all TEV strains tested, except they were susceptible to TEV-N (Jahn lab, unpublished results), whereas both of these alleles confer resistance to TEV-HAT (Deom, Murphy, and Paguio 1997; Murphy et al. 1998). Sequence analysis of the 188 amino acids encoding the TEV VPg revealed 97 % amino acid similarity between both pairs: TEV-Mex21 and TEV-HAT and TEV-N and TEV-HAT (Figure 3.1B). TEV-Mex21, TEV-N and TEV-HAT, referred to hereafter as Mex21, N and HAT (Figure 3.1B).

Figure 3.1. Amino acid changes of eIF4E and VPg proteins.

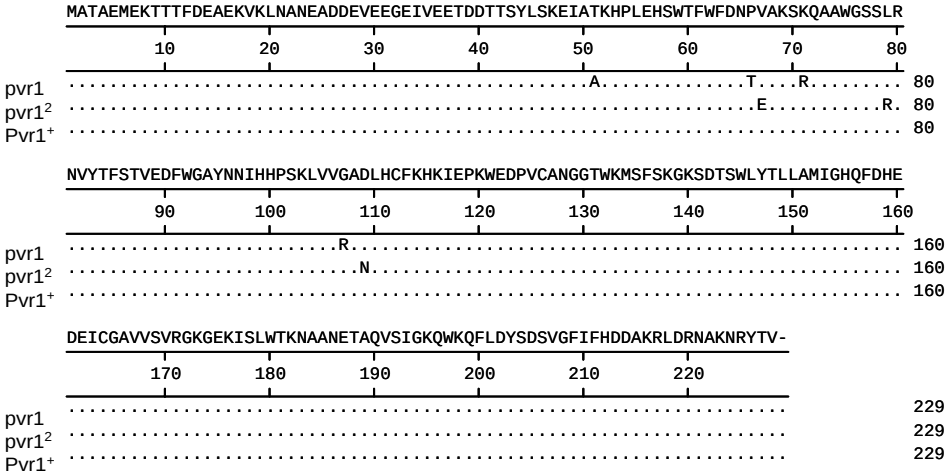
A. **pvr1 amino acid alignment**. Comparison of eIF4E proteins encoded by pvr1 resistance alleles, *pvr1*, *pvr1*^{1}, *pvr1*^{2} as well as the susceptibility allele, *Pvr1*$^{+}$. eIF4E proteins are 98% similar to susceptibility allele Pvr1^{+}. pvr1 variation includes: T51A, P66T and G107R. pvr1^{2} variation includes: VG7E, L79R and D109N.

B. **VPg amino acid alignment**. Amino acid sequence alignment of TEV-HAT, TEV-Mex21 and TEV-N VPg proteins. Alignment shows TEV-Mex21 and TEV-N are 97% similar to TEV-HAT. Amino acid Changes are concentrated in the central and C-terminal region of the protein. The VPg amino acid variations between TEV-HAT and TEV-N included A53T, I111L, E112D, P113H, S117N and D184E . Those differing between TEV-HAT and TEV-Mex21 include I107N, P113L, S115A, D184E andT186M.

A.


GKKNQKHKLKMREARGARGQYEVAAEPEALEHYFGSAYNNKGKRKGTTRGMGAKSRKFINMYGFDPTDFSYIRFVDPLTG
10 20 30 40 50 60 70 80
HAT 80
Mex21 .....T..... 80
N 80
HTIDESTNAPIDLVQHEFGKVRTRMLIDDEIEXQSLSTHTTIHAYLVNSGTKKVLKVDLTPHSSLRASEKSTAIMGFPER
90 100 110 120 130 140 150 160
HAT .....P..... 160
Mex21 .....N.....L.A..... 160
N .....LDH...N..... 160
ENELRQTGMAVPVAYDQLPPKNEELTFE
170 180
HAT .....D.... 188
Mex21 .....M.. 188
N 188

B.


MATAEMEKTTTFDEAEKVKLNANEADDEVEEGEIVEETDDTTSYLSKEIATKHPLEHSWTFWFDNPVAKSKQAAWGSSLR
10 20 30 40 50 60 70 80
pvr1 .....A.....T....R..... 80
pvr1² .....E.....R. 80
Pvr1⁺ 80
NVYTFSTVEDFWGAYNNIHHPSKLVVGADLHCFKHKIEPKWEDPVCANGGTWKMSFSKGKSDTSWLYTLLAMIGHQFDHE
90 100 110 120 130 140 150 160
pvr1 .....R..... 160
pvr1² .....N..... 160
Pvr1⁺ 160
DEICGAVVSVRGKGEKISLWTKNAANETAQVSIGKQWKQFLDYSDSVGFIFHDDAKRLDRNAKNRYTV-
170 180 190 200 210 220
pvr1 229
pvr1² 229
Pvr1⁺ 229

Mex21 VPg differed from HAT VPg by six amino acids, while N VPg differed from HAT VPg by five amino acids. We created viral chimera which differed from HAT only in the VPg protein domain, encoded in the N terminus of NIa or VPg-Pro, to determine if the N-VPg or the Mex21-VPg protein would make HAT infectious in pepper plants containing the *pvr1* or *pvr1*^{2} genotypes. A full-length infectious HAT-GFP clone, harboring the *green fluorescent protein* (*GFP*) gene fused to HC-Pro, was used as the primary template (Figure 3.2) (Dolja, McBride, and Carrington 1992; Schaad, Lellis, and Carrington 1997). The two TEV-based chimera were synthesized by replacement of the HAT-GFP VPg with the VPg of Mex21 or N. The resulting chimeric viruses were designated HAT $^{Mex21\text{-}VPg}$-GFP and HAT $^{N\text{-}VPg}$-GFP (Figure 3.2). The chimera were synthesized using the megaprimer method (Perez et al. 2006) and transcribed *in vitro* as described previously (Perez et al. 2006; Schaad, Lellis, and Carrington 1997). *C. Chinense* 'CA4' (*pvr1*/*pvr1*), *C. annuum* 'Dempsey' (*pvr1*^{2}/*pvr1*^{2}) , *C. annuum* 'Rnaky' (*Pvr1*$^+$/*Pvr1*$^+$) and *C. chinense* 'Habanero' (*Pvr1*$^+$/*Pvr1*$^+$) were used for the infectivity study and will be subsequently referred to by the *pvr1* allele each encodes: *pvr1*, *pvr1*^{2} and *Pvr1*$^+$. A different *Pvr1*$^+$ line was infected depending on species classification of the *pvr1* and *pvr1*^{2} lines included in the study. For the Mex21 and HAT $^{Mex21\text{-}VPg}$-GFP infectivity test, which was conducted to test *pvr1* susceptibility to the HAT $^{Mex21\text{-}VPg}$-GFP, *Pvr1*$^+$ line *C. chinense* 'Habanero' was included for comparison to *pvr1 C. chinense* line 'CA4'. For the N and HAT$^{N\text{-}VPg}$-GFP test, *C. annuum Pvr1*$^+$ line 'RNaky' was included for comparison to the *C. annuum pvr1*^{2} line 'Dempsey'. Both RNaky and Habanero were used in the HAT-GFP screen. Inoculated plants were routinely monitored for visible symptom development from seven days post inoculation (dpi) to approximately 35 dpi. Symptoms were recorded at 10 dpi because they were most visible at this time point and remained consistent for the remainder of the screen. HAT-GFP induced systemic mosaic

symptoms and mild deformation in uninoculated leaves of the susceptible genotype *Pvr1*$^{+}$ by 10 dpi. In contrast, no symptoms were observed in *pvr1* or *pvr1*^{2} genotypes inoculated with HAT-GFP, and plants developed similar to similar to mock inoculated controls. *pvr1* plants inoculated with Mex21 or HAT$^{Mex21\text{-}VPg}$-GFP displayed systemic, mild mosaic symptoms in uninoculated leaves by 10 dpi. Mex21 caused systemic mosaic symptoms and mild distortion in uninoculated *Pvr1*$^{+}$ leaves by 10 dpi, whereas HAT$^{Mex21\text{-}VPg}$-GFP symptoms were less severe consisting of systemic mosaic without leaf distortion. No visible symptoms developed in *pvr1*^{2} plants inoculated with Mex21 or HAT$^{Mex21\text{-}VPg}$-GFP. By 10 dpi, N infection of *pvr1*^{2} plants resulted in systemic mosaic symptoms with chlorosis and leaf deformation, whereas HAT$^{N\text{-}VPg}$-GFP induced a mild systemic mosaic symptom that was visible. Even though the severity of visible symptoms induced by HAT$^{N\text{-}VPg}$-GFP in *pvr1*^{2} was less than that induced by N, symptoms were clearly visible when compared to mock inoculated and HAT-GFP inoculated *pvr1*^{2} plants which were symptom-free (Table 3.1). *Pvr1*$^{+}$ plants inoculated with N resulted in severe systemic mosaic symptoms and leaf distortion at 10 dpi. Symptoms of reduced severity were observed for *Pvr1*$^{+}$ plants inoculated with HAT$^{N\text{-}VPg}$-GFP infection by 10 dpi. *pvr1* plants inoculated with N or HAT$^{N\text{-}VPg}$-GFP did not show any visible symptoms.

Visible symptoms can be summarized as follows: HAT$^{N\text{-}VPg}$-GFP induced symptoms in *Pvr1*$^{+}$ of lesser severity than N, but equivalent to symptoms induced by TEV-HAT. *Pvr1*$^{+}$ plants inoculated with HAT$^{Mex21\text{-}VPg}$-GFP displayed the least severe symptoms of the five virus strains tested. A systemic infection with similar types and severity of symptoms occurred in *pvr1* plants inoculated with Mex21 and HAT$^{Mex21\text{-}VPg}$-GFP. *pvr1*^{2} plants were systemically infected by both N and HAT$^{N\text{-}VPg}$-GFP; however, N induced much more severe symptoms than those induced by HAT$^{N\text{-}VPg}$-GFP.

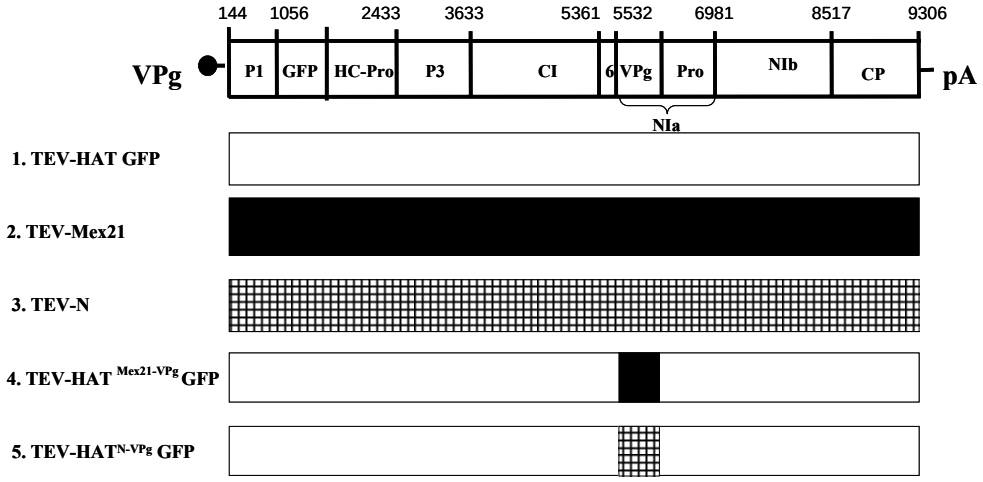


Figure 3.2. Diagrammatic representation of the TEV-green fluorescent protein (TEV-GFP) chimeric strains used in this study. TEV-GFP consists of the TEV-HAT (HAT – highly aphid transmissible) genome with GFP inserted between P1 and HC-Pro. All chimera were derived from the TEV-HAT GFP where GFP is fused to the HC-Pro domain of the virus (Dolja, McBride, and Carrington 1992). Chimeric viruses were entirely TEV-HAT GFP, except for VPg (encoded in the NIa 5' end) which was replaced by TEV-Mex21 VPg or TEV-N VPg. Above are 1: TEV-HAT GFP, 2: TEV-Mex21, 3: TEV-N, 4: TEV-HAT$^{Mex21\text{-}VPg}$ GFP and 5: TEV-HAT $^{N\text{-}VPg}$ GFP.

Plants of each genotype were tested by enzyme-linked imunosorbant assay (ELISA) to assess virus accumulation in uninoculated leaves. All viruses were inoculated on the same day, however, sampling times differed. Plants inoculated with N or HAT$^{N\text{-}VPg}$-GFP were sampled at 15dpi. Plants inoculated with HAT-GFP were sampled at 15 and at 30dpi. HAT-GFP was detected in *Pvr1*$^{+}$ leaves, but was not detected in *pvr1* or *pvr1*^{2} leaf tissue (Figure 3.3). N and HAT$^{N\text{-}VPg}$-GFP were detected in *pvr1*^{2} and *Pvr1*$^{+}$ plants. Neither N nor HAT$^{N\text{-}VPg}$-GFP was detected in *pvr1* samples. HAT-GFP and HAT$^{N\text{-}VPg}$-GFP accumulated to similar titers in *Pvr1*$^{+}$ plants.

Plants inoculated with Mex21 and HAT$^{Mex21\text{-}VPg}$-GFP were sampled at 30dpi along with the second HAT-GFP sample. Mex21 and HAT$^{Mex21\text{-}VPg}$-GFP were detected in uninoculated tissue of both *Pvr1*$^{+}$ and *pvr1* genotypes (Figure 3.3).

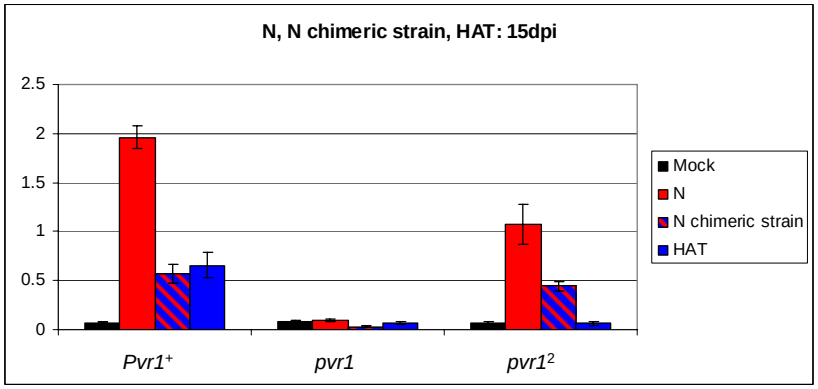
Consistent with the 15dpi sample, HAT-GFP was detected in uninoculated *Pvr1*$^{+}$ leaves at 30dpi, but was not detected at either time point in *pvr1* or *pvr1*^{2} plants. Neither Mex21 nor HAT$^{Mex21\text{-}VPg}$-GFP was detected in uninoculated leaf tissues from *pvr1*^{2} plants at 30dpi. In conclusion, systemic infection developed in *pvr1* plants inoculated with both Mex21 and the chimeric HAT$^{Mex21\text{-}VPg}$-GFP, but no systemic infection was detected for HAT-GFP. *pvr1*^{2} plants were systemically infected with both N and chimeric HAT$^{N\text{-}VPg}$-GFP, but not HAT-GFP. The VPg protein was the only difference between the chimera and the template HAT-GFP. ELISA absorbance values for *Pvr1*$^{+}$ samples infected with HAT-GFP the chimera were very similar at both 15 and 30dpi.

Table 3.1. ELISA infectivity summary for pepper plants encoding *Pvr1*$^{+}$, *pvr1* and *pvr1*^{2}.

(*pvr1* allele)[a]	TEV-HAT-GFP	TEV-Mex21	TEV-N	TEV-HAT$^{Mex21\text{-}VPg}$-GFP	TEV-HAT$^{N\text{-}VPg}$-GFP
Pvr1^{+}	S	S	S	S	S
pvr1	R	S	R	S	R
pvr1^{2}	R	R	S	R	S

Data are based visual data and on detection of TEV by enzyme-linked immunosorbant assay ELISA using polyclonal antibodies made to TEV. Three to five plants per genotype were screened per virus strain. TEV-HAT-GFP was screened at 15 and 30dpi, TEV-Mex21 and TEV-HAT$^{Mex21\text{-}VPg}$-GFP were sampled at 30dpi, and TEV-N and TEV-HAT$^{N\text{-}VPg}$-GFP were sampled at 15dpi.
[a]**Pepper genotypes**: plants encoding the *pvr1* alleles are represented by the following pepper cultivars: *Pvr1*$^{+}$: 'Habanero' and 'RNaky', *pvr1*: 'CA4' and *pvr1*^{2}: 'Dempsey'.

A.



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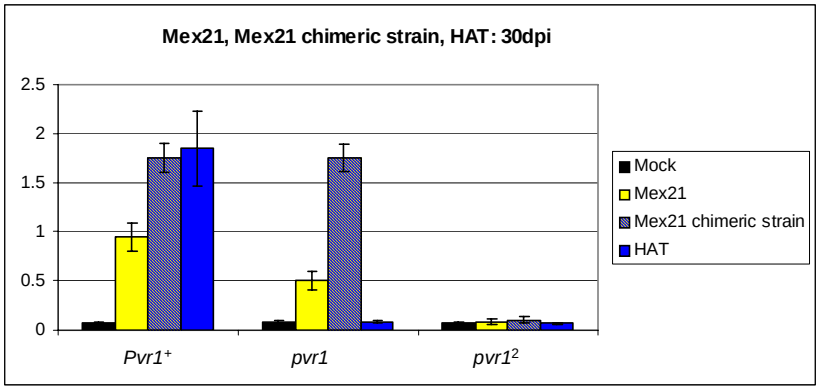


Figure 3.3. TEV infection in *pvr1*, *pvr1*^{2} and *Pvr1*$^+$ leaf tissue determined by enzyme linked immunosorbent assay (ELISA). Values represent average ELISA absorbance values in uninoculated leaf tissue from 4-6 plant samples at 15 or 30 days post inoculation (dpi). Standard error is indicated at the top of each bar. Samples listed as mock are healthy controls and are represented as the first sample bar for each genotype. Pepper cultivars *C. chinense* 'Habanero' (*Pvr1*$^+$/*Pvr1*$^+$), *C. annuum* 'RNaky' (*Pvr1*$^+$/*Pvr1*$^+$), *C. chinense* 'CA4' (*pvr1*/*pvr1*) and *C. annuum* 'Dempsey' (*pvr1*^{2}/*pvr1*^{2}) were tested.

A. TEV-N, TEV-HAT GFP and TEV-HAT$^{N\text{-}VPg}$-GFP ELISA absorbance values, 15dpi. RNaky, CA4 and Dempsey were tested.

B. TEV-Mex21, TEV-HAT GFP and TEV-HAT$^{Mex21\text{-}VPg}$-GFP ELISA absorbance values, 30dpi. Habanero, CA4 and Dempsey were tested.

GFP expression in susceptible genotypes correlates with ELISA data.

Plants infected with GFP labeled viruses, HAT-GFP, HAT$^{Mex21\text{-}VPg}$-GFP and HAT$^{N\text{-}VPg}$-GFP, were monitored by confocal microscopy for virus accumulation at approximately the eight leaf stage (Figure 3.4). Uninoculated leaves from at least three plants per genotype were sampled from each virus inoculation between 7 and 10 dpi; leaves showing mosaic symptoms were preferentially selected when present. GFP fluorescence always correlated with ELISA infectivity data. HAT$^{Mex21\text{-}VPg}$-GFP was detected in uninoculated leaves of *pvr1* plants but was not detected in *pvr1*^{2} plants (Figure 3.4A and data not shown). In contrast, HAT$^{N\text{-}VPg}$-GFP was detected in uninoculated leaves of *pvr1*^{2} plants, but not in uninoculated *pvr1* leaves (Figure 3.4B and data not shown). GFP fluorescence was detected in all *Pvr1*$^+$ leaves examined from each of the three GFP labeled viruses. Consistent levels of fluorescence for each virus strain were observed in *Pvr1*$^+$ leaves. HAT-GFP was never detected in leaves of the resistant genotypes *pvr1* or *pvr1*^{2}. As we observed for *Pvr1*$^+$ leaves, *pvr1* plants infected with HAT$^{Mex21\text{-}VPg}$-GFP showed GFP accumulation throughout the leaf independent of strong visible symptom development, whereas *pvr1*^{2} plants infected with HAT$^{Mex21\text{-}VPg}$-GFP did not accumulate GFP in any of the leaves sampled (Figure 3.4A, and data not shown). In contrast, HAT$^{N\text{-}VPg}$-GFP was detected in *pvr1*^{2} leaves, but not in *pvr1* leaves (Figure 3.4B, and data not shown). All HAT$^{N\text{-}VPg}$ GFP infected *pvr1*^{2} leaves examined showed GFP accumulation; however, unlike all other samples, GFP was not evenly distributed, rather islands of GFP accumulation were observed (data not shown). GFP was observed in all cases in *pvr1*^{2} leaves infected with HAT$^{N\text{-}VPg}$ GFP indicating the virus had accumulated in all leaves sampled. Susceptible host *Pvr1*$^+$ accumulated GFP equally for all three viruses. Based on this assay, GFP being an indicator of viral accumulation, replacement of the HAT VPg with that of N or Mex21 VPg did not appear to have an effect on virus accumulation providing evidence

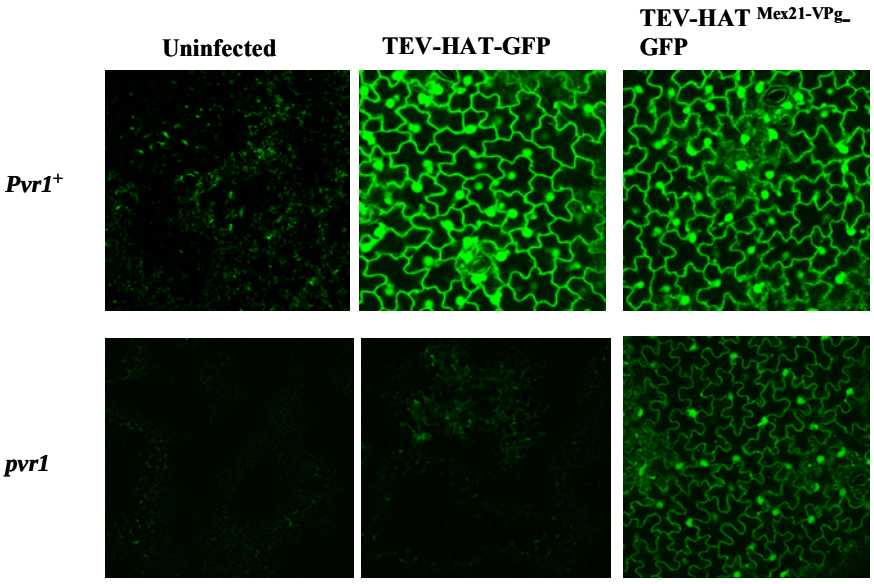
the genomes behaved as expected and produced functional GFP in systemically infected tissue. With the exception of reduced symptom severity for $HAT^{Mex21\text{-}VPg}$-GFP, *Pvr1*$^{+}$ visible symptoms, ELISA absorbance and GFP accumulation appear to indicate that the chimeric virus infectivity was similar to HAT-GFP infectivity when in susceptible genotypes.

Figure 3.4. Infectivity test of chimeric TEV-HAT GFP strains monitored by GFPaccumulation. Confocal microscopy images of GFP accumulation in *pvr1*, *pvr1*^{2} and *Pvr1*$^+$ pepper leaves at 7-10dpi. Uninoculated leaf tissue from at least three inoculated plants per genotype, per virus was examined. Uninfected plants were also included as controls. Images represent the three GFP encoding viruses used in this study: TEV-HAT GFP, TEV-HAT$^{Mex21\text{-}VPg}$ GFP and TEV-HAT$^{N\text{-}VPg}$ GFP. For each virus, the first two images on the left represent leaves from uninoculatedplants; the two on the right represent systemically infected leaf tissue 7-10dpi.

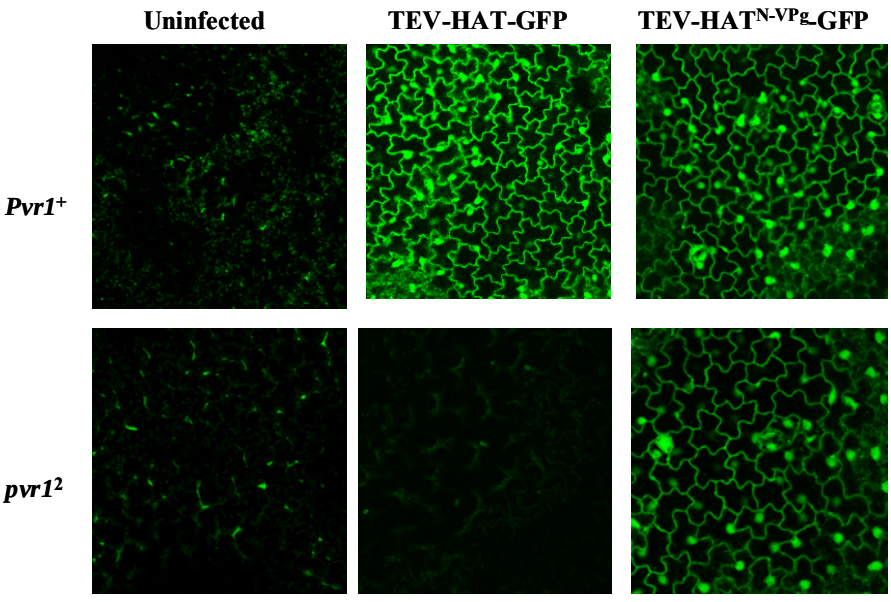
A. TEV-HAT GFP and TEV-HAT$^{Mex21\text{-}VPg}$ GFP fluorescence in *Pvr1*$^+$ and *pvr1*. TEV-HAT-GFP and TEV-HAT$^{Mex21\text{-}VPg}$-GFP: Top and bottom left panels show no GFP accumulation in uninfected leaves of *Pvr1*$^+$ and *pvr1*. Middle and bottom right panels show TEV-HAT GFP fluorescence in systemically infected leaf tissue of TEVHAT GFP infected *Pvr1*$^+$ but no fluorescence in *pvr1* leaves.Top and bottom right panels show TEV-HAT$^{Mex21\text{-}VPg}$ GFP fluorescence in *Pvr1*$^+$and *pvr1*.

B.TEV-HAT GFP and TEV-HAT$^{N\text{-}VPg}$ GFP in *Pvr1*$^+$ and *pvr1*^{2}. TEV-HAT GFP and TEV-HAT$^{N\text{-}VPg}$ GFP: Top left and bottom left panels show no GFP fluorescence in uninfected *Pvr1*$^+$ and *pvr1*^{2} leaves. Middle top and bottom panels show GFP fluorescence in systemically infected leaf tissue of TEV-HAT GFP infected *Pvr1*$^+$ but accumulation in *pvr1*^{2}. Top and bottom panels at right show TEV-HAT$^{N\text{-}VPg}$ GFP accumulation in systemic *Pvr1*$^+$ and *pvr1*^{2} leaf tissue.

A.


Uninfected
TEV-HAT-GFP
TEV-HAT Mex21-VPg-GFP
Pvr1+
pvr1

B.


Uninfected
TEV-HAT-GFP
TEV-HATN-VPg-GFP
Pvr1+
pvr12

N-terminal 60 amino acids of the VPg protein are not required for eIF4E-VPg interaction in Yeast.

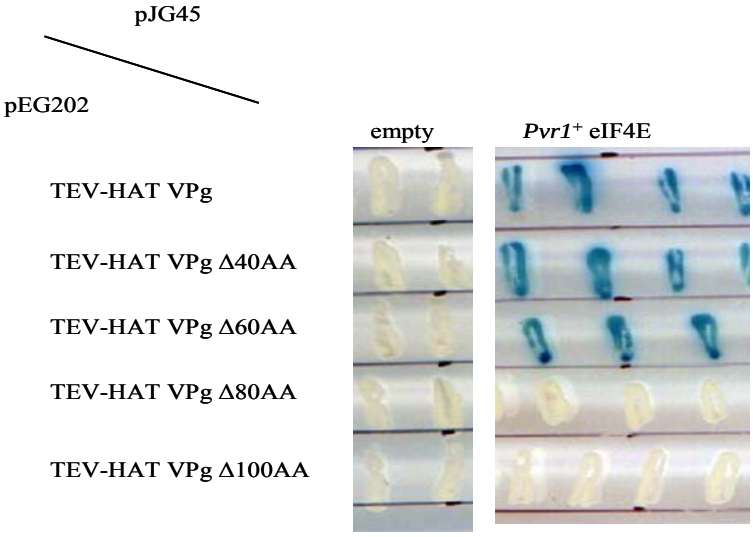
Since the VPg chimera were able to infect *pvr1* and *pvr1*^{2}, an attempt was made to define the region of the VPg responsible for binding to the eIF4E protein encoded by Pvr1$^+$. Yeast two-hybrid was performed with the VPg domain of HAT and susceptible allele Pvr1$^+$. The full-length HAT VPg was tested along with a series of mutants generated by deletion of HAT-VPg which had 40, 60, 80 or 100 amino acids deleted from the N-terminus of the protein: HAT-VPg Δ40AA, HAT-VPg Δ60AA, HAT-VPg Δ80AA, HAT-VPg Δ100AA. VPg interaction with Pvr1$^+$ was detected for the full-length VPg, the HAT-VPg Δ40AA and HAT-VPg Δ60AA amino acid deletion mutants. No interaction was observed between Pvr1$^+$ and the HAT-VPg Δ80AA, HAT-VPg Δ100AA deletion mutants (Figure 3.5). The loss of interaction between VPg and Pvr1$^+$ for HAT-VPg Δ80AA and HAT-VPg Δ100AA indicate that this region of the protein is important for this interaction. Because data indicates that after 80 amino acids were deleted interaction stopped, this could mean that this region of the protein contains the interaction domain, or it could mean that the protein is unstable with such a large deletion and it is therefore no longer capable of interacting in yeast. The VPg sequence alignment shows five out of six amino acid changes, and six out of six amino acid changes occur after amino acid 100 in N and Mex21 VPg, respectively (Figure 3.1). Given that the majority of variation occurs after amino acid 100 and interaction stopped after 80 amino acids were deleted, this region may still be essential for interaction even though the sequence was identical for the three proteins in this region.

Figure 3.5. Interaction between susceptible pepper allele *Pvr1*$^{+}$ and TEV-HAT VPg as shown via yeast two-hybrid β galactosidase assay to determine the region of VPg essential for interaction. VPg used in the study was 1. full length TEV-HAT VPg 2. 120bp deleted 3. 180bp deleted 4. 240bp deleted 5. 300bp deleted. All deletions were made from the 5'end of the VPg coding sequence (5' end of NIa).

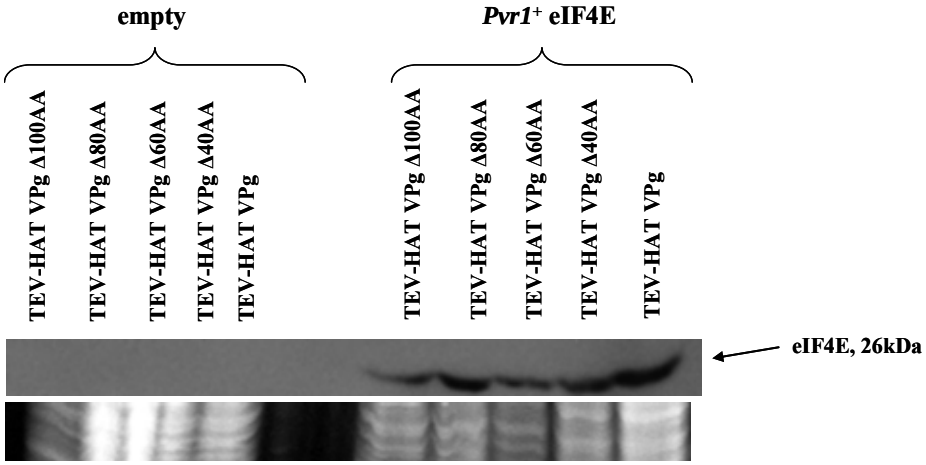
A. Pvr1^{+} interaction with and TEV-HAT VPg. Expression of eIF4E in yeast containing empty vector or eIF4E in pJG45 grown on media lacking leucine and tryptophan

B. Yeast expressing *Pvr1*+ encoded eIF4E protein. Proteins were extracted and run in 15% SDS-polyacrylamide gel and immunoblotted with eIF4E antibody. Immunoblot shows 26kDa eIF4E protein is only present in yeast samples containing Pvr1+ eIF4E in pJG45, not in empty vector. Top image is the immunoblot, lower is SDS PAGE SYPRO Ruby stained gel of total yeast protein.

A.


pJG45
pEG202
empty
Pvr1+ eIF4E
TEV-HAT VPg
TEV-HAT VPg Δ40AA
TEV-HAT VPg Δ60AA
TEV-HAT VPg Δ80AA
TEV-HAT VPg Δ100AA

B.


empty
Pvr1+ eIF4E
TEV-HAT VPg Δ100AA
TEV-HAT VPg Δ80AA
TEV-HAT VPg Δ60AA
TEV-HAT VPg Δ40AA
TEV-HAT VPg
TEV-HAT VPg Δ100AA
TEV-HAT VPg Δ80AA
TEV-HAT VPg Δ60AA
TEV-HAT VPg Δ40AA
TEV-HAT VPg
eIF4E, 26kDa

A three dimensional model provides possible explanation of the phenotype.

Because VPg interaction with eIF4E has been correlated with infectivity, we used protein models to attempt to correlate eIF4E and VPg interaction potential with susceptibility. We focused our effort in generating a 3D model of a fragment of VPg sequence that is expected to interact with eIF4E; this portion of VPg will be referred to as the 'essential region' for this particular interaction. The essential region was based the cap docking region of the mouse eIF4E crystal structure (Protein Data Bank [PDB] code: 1EJ1, monomer A; 190 residues). This region was additionally based on variation observed in the three VPg proteins included in the study (Figure 3.1), as well as the variation in the *pvr1* alleles included in this work. Except for a single variation, all sequence changes in the *pvr1* alleles are predicted to fold inside the binding pocket on the eIF4E models (Figure 3.1). Unlike eIF4E, modeling the active conformation of VPg proteins is a more difficult task, because there is no crystal structure available in the Protein Data bank (PDB) for any potyviral VPg or homologous protein. A recent NMR structure of poliovirus VPg was described (Schein et al. 2006) however, poliovirus VPg is much smaller than TEV VPg, just 22 residues to TEV VPg's 188. As expected given the significant size difference, this structure was not identified as homologous. Furthermore, functional assays, biochemical and biophysical analyses and structure prediction programs all suggest that that potyviral VPg exists in a natively unfolded conformation (Grzela et al. 2008; Rantalainen et al. 2008). Therefore we focused on variation which exist in both eIF4E and VPg. Using the 3D models for the three eIF4E variants and three VPg variants, we used an interactive 3D graphics program to explore possible modes of interaction of the eIF4E and VPg molecules that account for the experimentally observed infectivity (Figure 3.6). Mex21, N and HAT VPgs are highly homologous sequences, without insertions or deletions, in which differences are observed at only nine sequence positions. The

majority of the variations observed among the VPgs are located near the central region of the protein (Figure 3.1B). Furthermore, six of the positions where substitutions occur are concentrated in the region between residues L106 and T118. While the effect of the substitutions at positions 53, 183 and 186 cannot be ruled out, based on the number and on the type of the substitutions (character of the residues), we have made the assumption that the sequence fragment 106-118 is a primary component of the essential region of VPg. Three out of six aa changes in Mex VPg (I107N, P113L and T186M) and one out of five aa changes in N VPg (P113H) can be considered substitutions in which the amino acid properties at the specified site (i.e., charge, hydrophobicity,) are altered substantially. According to secondary structure prediction methods, the VPg fragment 106-118 is likely to be part of a α-helical or partial α-helical conformation (i.e., a broken α-helix). Using the program ECEPPAK (Ripoll 1999), a regular α-helical conformation was built for the VPg fragment, which will be referred to as VPgHX, involving residues I91 to T118.

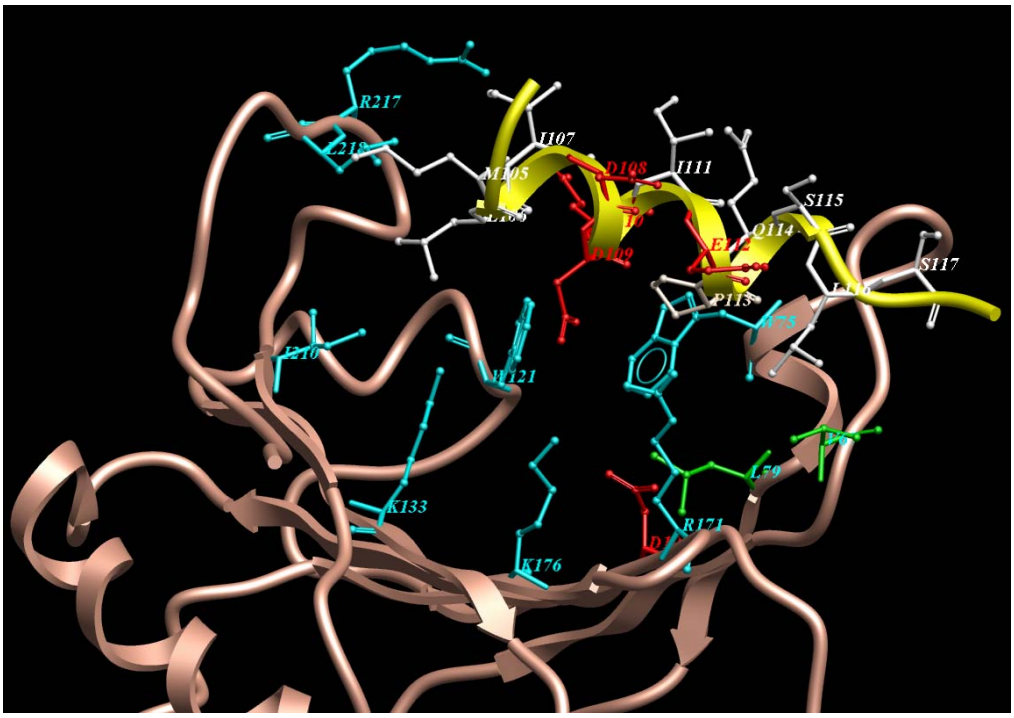
Figure 3.6: Proposed three dimensional eIF4E-VPg interaction models provide a possible explanation for susceptible phenotypes.

A. $Pvr1^{+}$ TEV-HAT VPg proposed interaction model. HAT VPg residue P113 sits in the pocket formed by $Pvr1^{+}$ residue D109 and the conserved $Pvr1^{+}$ residues W75 and W121 interacting favorably with the two aromatic tryptophan residues. HAT VPg residue L116 hydrophobic – hydrophobic interaction with Pvr1+ residue V67 contributes favorably. The net sum of interactions favors Pvr1+ binding and thus susceptibility to HAT.

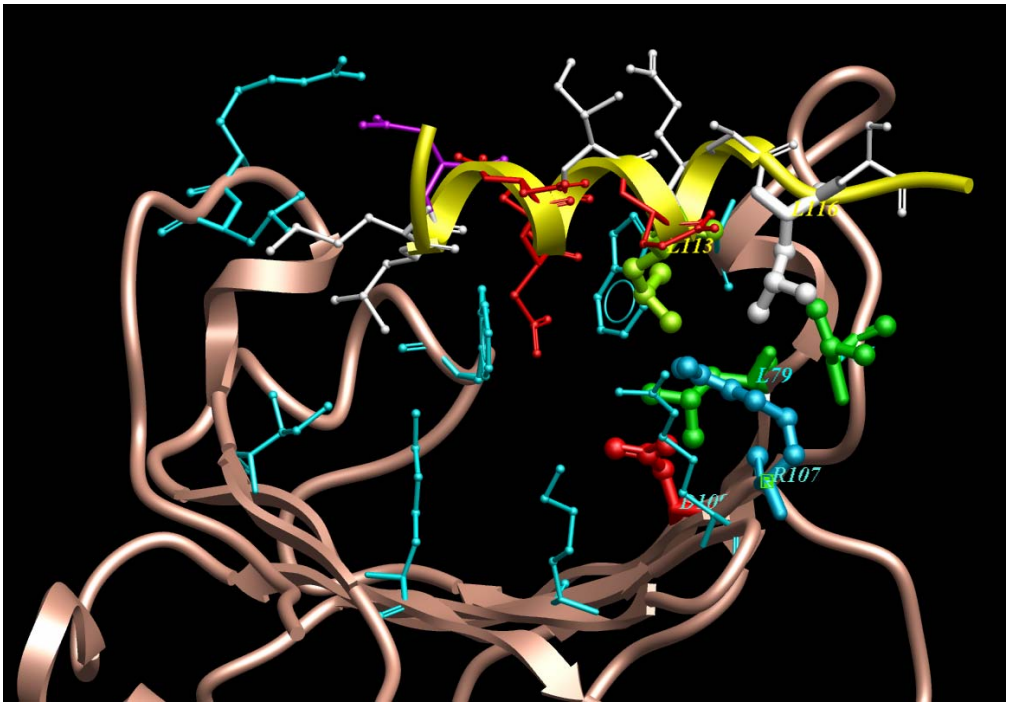
B. pvr1 TEV-Mex21 VPg proposed interaction model. By displacing the pvr1 residue R107 side chain, Mex21 VPg residue L113 is capable of forming a large hydrophobic cluster with pvr1 residues W75, W121, V67, and L79 as well as Mex21 VPg residue L116 that favors the bound state.

C. $pvr1^{2}$ TEV-N VPg proposed interaction model. $pvr1^{2}$ binding pocket near conserved $pvr1^{2}$residues W75 and W121 is polar/charged in character; the walls of this pocket are lined by $pvr1^{2}$ residues N109, E67 and R79. If N VPg residue H113 sits in that pocket, the electrostatic interactions with $pvr1^{2}$ residues E67 and

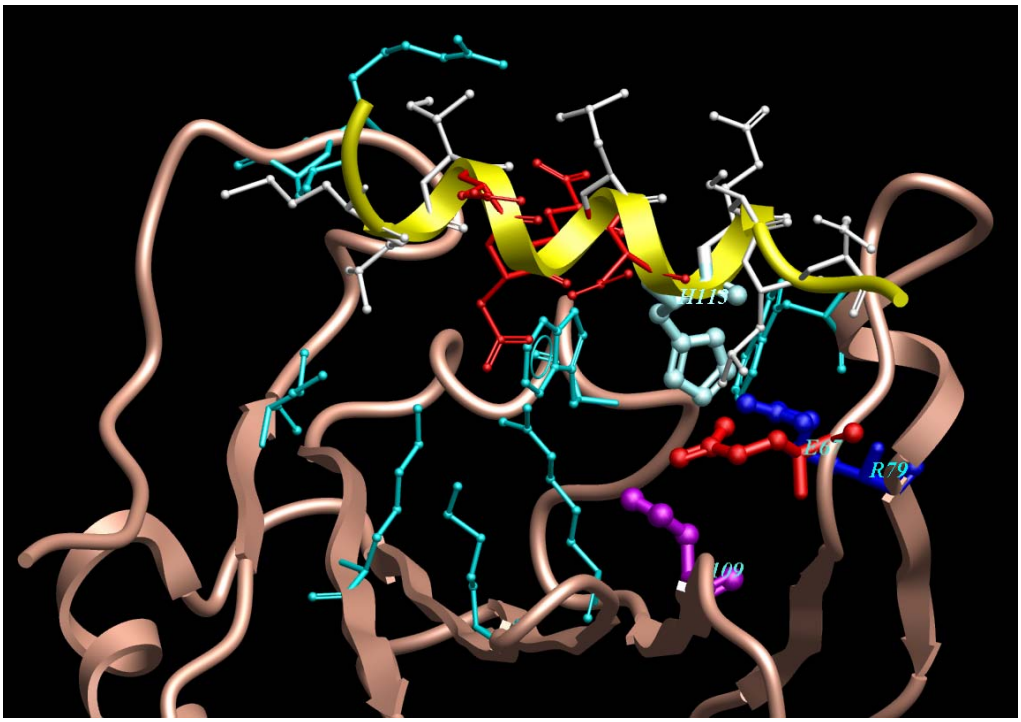
A.


R217
I107
M105
D108
I111
S115
Q114
E112
D109
S117
P113
K73
E110
V121
L79
K133
R171
K176

B.


L116
L113
L79
R107

C.



Among many possible conformations for the complex, a plausible binding model follows which is consistent with our infectivity data. It is possible that VPgHX docks inside the cap-binding cleft of eIF4E with at least two of the three negatively charged residues D108, D109 and E110 in VPg forming salt bridges with K133, K176 and R171 in eIF4E. This electrostatic interaction may provide an anchor point for VPg binding, but additional interacting residues are needed to stabilize the complex between VPg and eIF4E. The N-terminal part of VPgHX (conserved residues M105, L106, and additionally I107 in NVPg and HATVPg) are anchored near I210 and L218 in eIF4E, while the C-terminal portion of VPgHX is directed toward the region of eIF4E where residues W75 and W121 are located. Conserved residue L116 in VPg provides additional favorable interaction when a hydrophobic residue occupies

position 67 in eIF4E. Table 3.2 summarizes the favorable/unfavorable interactions for all eIF4E and VPg combinations.

The susceptible allele, $Pvr1^{+}$ was found to interact favorably with HAT, N and Mex21 VPg proteins (Figure 3.6A and Table 3.2). HAT VPg residue P113 sits in the pocket formed by $Pvr1^{+}$ D109, and the conserved $Pvr1^{+}$ residues W75 and W121 interacting favorably with the two aromatic tryptophan residues. HAT VPg residue L116 hydrophobic – hydrophobic interaction with $Pvr1^{+}$ residue V67 contributes favorably to this interaction (Figure 3.6A). Mex21 VPg also interacts favorably with $Pvr1^{+}$. Mex21 residue L113 sits in the pocket formed by $Pvr1^{+}$ residue D109 and the conserved residues $Pvr1^{+}$ W75 and W121. While the interaction with $Pvr1^{+}$ residue D109 is not quite favorable, Mex21 VPg residue L113 is buried by the two aromatic tryptophans, and additional interactions with $Pvr1^{+}$ residues V67 and L79 and Mex21 VPg residue L116 may lead to a sizable hydrophobic cluster that tends to favor the bound state. N VPg also interacts favorably with $Pvr1^{+}$. N VPg residue H113 interacts favorably in a pocket formed by $Pvr1^{+}$ residue D109 and the conserved $Pvr1^{+}$ residues W75 and W121. In addition, the conserved N VPg residue L116 interacts favorably with $Pvr1^{+}$ residues V67 by hydrophobic – hydrophobic interactions.

Additionally, *pvr1* was predicted to interact favorably with Mex21 VPg providing support for *pvr1* susceptibility to Mex21 (Figure 3.6B and Table 3.2). By displacing the side chain of pvr1 residue R107, Mex21 VPg residue L113 is capable of forming a large hydrophobic cluster with pvr1 residues W75, W121, V67 and L79 and Mex21 VPg residue L116 that favors the bound state. Unlike Mex21, N and HAT VPg are not predicted to favorably interact, corroborating *pvr1* observed resistance to N and HAT. In the case of pvr1 interaction with N VPg, the pocket formed by pvr1 residues D109, W75 and W121 is likely to be occupied by the side chain of pvr1 residue R107 which interacts with pvr1 residue D109. N VPg residue H113 does not

seem to be able to displace pvr1 residue R107 to interact with pvr1 residue D109 and the bound state of the complex is disfavored. Unfavorable interactions provide an explanation for observed *pvr1* resistance to N. As in the case of N VPg, HAT VPg also does not interact favorably with pvr1. HAT VPg residue P113 does not seem to be able to displace pvr1 residue R107 and the bound state of the complex is disfavored.

Models predict that pvr1^2 interacts favorably with N VPg supporting *pvr1*^{2} susceptibility to N (Figure 3.6C and Table 3.2). The pvr1^2 binding pocket near conserved pvr1^2 residues W75 and W121 is much more polar/charged in character; the walls of this pocket are lined by pvr1^2 residues N109, E67 and R79. If N VPg residue H113 sits in that pocket, the electrostatic interactions with pvr1^2 residues E67 and R79 should be quite favorable. In contrast, models indicated an unfavorable interaction between HAT VPg and pvr1^2. HAT VPg residue P113 sitting in the polar/charged pocket of pvr1^2 near pvr1^2 residues W75 and W121 should not have favorable electrostatic interactions with pvr1^2 residues E67 and R79 and will adversely affect the interactions between the last-mentioned two residues. Models also indicate an unfavorable interaction between Mex21 VPg and pvr1^2. As in the case of HAT VPg, the interactions of Mex21 VPg residue L113 with residues in the polar/charged pocket of pvr1^2 should be unfavorable. The presence of Mex21 VPg residue L113 in the pocket will also adversely affect the electrostatic interactions between pvr1^2 residues E67 and R79. These models correlate with previous infectivity data supporting the hypothesis Mex21 and N infect pepper plants encoding *pvr1* and *pvr1*^{2}, respectively.

Table 3.2. Summary of the favorable/unfavorable interactions for all eIF4E and VPg combinations.

VPg \ eIF4E	Pvr1^{+}		pvr1		pvr1^{2}	
	VPg res.	**eIF4E res.**	**VPg res.**	**eIF4E res.**	**VPg res.**	eIF4E res.
N	**I107 L111 D112 H113 S115 N117**	**T51 P66 V67 L79 G107 D109**	**I107 L111 D112 H113 S115 N117**	**A51 T66 V67 L79 R107 D109**	**I107 L111 D112 H113 S115 N117**	T51 P66 **E67 R79** G107 **N109**
HAT	**I107 I111 E112 P113 S115 S117**	**T51 P66 V67 L79 G107 D109**	**I107 I111 E112 P113 S115 S117**	**A51 T66 V67 L79 R107 D109**	**I107 I111 E112 P113 S115 S117**	T51 P66 **E67 R79** G107 **N109**
Mex21	**N107 I111 E112 L113 A115 S117**	T51 P66 V67 L79 G107 D109	**N107 I111 E112 L113 A115 S117**	**A51 T66** V67 L79 **R107** D109	**N107 I111 E112 L113 A115 S117**	T51 P66 **E67 R79** G107 **N109**

Each cell has been divided in two: the left half of the cell lists the variable residues in the VPgHX fragment for the particular VPg variant. The right half of the cell lists the variable residues in the particular eIF4E variant. We have taken the sequences of N VPg and Pvr1^{+} eIF4E as references, and used boldface letters to indicate residues in the particular variant that differ from the reference sequences. Finally, residues that according to our model of the complex provide "key" favorable interactions are highlighted in blue, while those that appear to contribute with interactions that are very unfavorable are colored in red.

Discussion

eIF4E or eIF(iso)4E interaction with VPg has been correlated with virus infectivity in many pathosystems including the *pvr1*-TEV pathosystem (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Kang et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004). The VPg chimera in this study demonstrate that VPg is a virulence determinant in the *pvr1*-VPg pathosystem. Visible symptom development, coat protein and GFP accumulation clearly indicate that N or Mex21 VPg substitutions in the HAT-GFP genome are sufficient to establish infectivity in $pvr1^2$ or *pvr1*, respectively (Figure 3.3, 3.4 and Table 3.1). Based on visible symptoms, it is demonstrated that $HAT^{Mex21\text{-}VPg}$-GFP and Mex21 induced infectivity with similar severity in *pvr1*. $HAT^{N\text{-}VPg}$-GFP induced symptoms which were much less severe than N. While ELISA data showed *pvr1* and $pvr1^2$ plants clearly became infected with the chimeric viruses, ELISA absorbance values could not be comparable to those of the N and Mex21 because the antibody used for analysis binds the TEV coat protein (CP), and variation in the CP of N, Mex21 and HAT is unknown. Therefore, the likelihood the antibody would equally bind the CP of all three strains was not be determined, therefore a comparison of N, Mex21 and HAT absorbance values was not possible. Based on the ELISA absorbance values, we therefore cannot conclude that VPg alone created a strain of equivalent infectivity to that of the VPg donor strain. Additionally, because Mex21-GFP and N-GFP clones were not available we could not determine if there was a change in the level of GFP accumulation in relation to the VPg donor strain. Even though $pvr1^2$ plants infected with $HAT^{N\text{-}VPg}$-GFP showed a reduction in GFP accumulation compared to all other plants, we do not know if this is the nature of N, or a result of compromised infectivity due to the chimeric nature of the clone. On the other hand, HAT-GFP and the chimeras have identical genome identity except for VPg, therefore the absorbance

values and GFP accumulation could be compared for the susceptible genotype *Pvr1*$^{+}$. HAT-GFP absorbance values in *Pvr1*$^{+}$ were very similar to those of HAT$^{Mex21\text{-}VPg}$-GFP and HAT$^{N\text{-}VPg}$-GFP. *Pvr1*$^{+}$ visible symptoms were also similar. Systemic mosaic symptoms were observed for both chimera and HAT-GFP. Mild distortion was also observed for HAT$^{N\text{-}VPg}$-GFP and HAT-GFP; this symptom was not observed for the HAT$^{Mex21\text{-}VPg}$-GFP. This observation could indicate that the replacement of HAT VPg with that of Mex21 VPg reduced HAT-GFP infectivity in *Pvr1*$^{+}$ at visible symptom level. In terms of GFP expression, *Pvr1*$^{+}$ leaves appeared to display equivalent GFP expression upon infection with the chimera as well as HAT-GFP. Even though *Pvr1*$^{+}$ visible symptoms were reduced for HAT$^{Mex21\text{-}VPg}$-GFP compared to HAT-GFP, ELISA absorbance values and levels of GFP expression was similar. Based on these results, it appears the chimera infectivity was similar to HAT-GFP, indicating VPg replacement did not alter infectivity in the susceptible host.

Several studies link nucleotide changes in the central domain of VPg with infectivity (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Leonard et al. 2000; Moury et al. 2004; Rajamaki and Valkonen 1999; Rajamaki and Valkonen 2002). The yeast two-hybrid data presented here provide further support that HAT VPg interacts with susceptible protein Pvr1^{+}, and that the interaction does not require the N-terminal 60 amino acids of VPg. A similar study showed the VPg amino acids 59-93 from *Turnip mosaic virus* were responsible for interaction with *Arabidopsis thaliana* eIF4E isoform eIF(iso)4E in yeast two-hybrid assays (Leonard et al. 2000). Because previous yeast-two hybrid results from our lab indicated pvr1 did not interact with Mex21-VPg (Yeam et al. 2007) and pvr1^{2} did not interact with N-VPg (Jahn lab unpublished results) in spite of susceptibility, site directed mutagenesis of the N and Mex21 VPgs were not completed to determine which VPg amino acid changes were responsible for interaction/susceptibility. Therefore, because we did not test the effect

each amino acid difference in Mex21 and N VPg had on interaction by themselves, we do not know the effect these mutations have on interaction with the pvr1 and pvr1^2, respectively. Future yeast-two hybrid experiments using the susceptible allele Pvr1$^+$ and HAT-VPg with site specific deletions at the specific locations where the amino acid differences present in Mex21 and N VPgs occur to determine which amino acid(s) are responsible for eliminating interaction with Pvr1$^+$ may answer these questions. Additionally, because interaction stopped prior to the region where this variation occurred, it could not be determined if the region containing the specific amino acid differences was essential for interaction. Further yeast-two hybrid experiments could also include the use of VPg proteins with deletions at both termini of the protein to determine if the interaction would still occur with a fragment of the VPg protein consisting of the region containing the majority of sequence variation such as amino acids 60-117. Another study would include full length VPg proteins which differed only in the specific amino acid changes present in the three proteins.

In order to better understand the effect the specific amino acid differences present in VPg and eIF4E, we also looked at the potential interaction of VPg central domains of Mex21, N and HAT with the eIF4E encoding alleles *Pvr1*$^+$, *pvr1*, and *pvr1*^{2} to correlate favorable protein-protein interactions with infectivity. The *Pvr1*$^+$, *pvr1* and *pvr1*^{2} eIF4E variants are highly homologous sequences; their alignments show no insertions/deletions, and the differences are localized in six positions in the sequence (Figure 3.1). Previously we reported that sequence variation in the eIF4E proteins encoded by the *pvr1* and *pvr1*^{2} alleles was concentrated in the mRNA binding pocket structure (Kang et al. 2005; Yeam et al. 2007). To better understand the ability of eIF4E and VPg to interact, predicted three dimensional models of eIF4E and the VPg from each of the three viruses were constructed. Based on the structural models, a favorable protein-protein interaction was predicted for those VPg – eIF4E

combinations that resulted in a systemic infection of the plant. pvr1 was predicted to interact favorably with Mex21 VPg, supporting *pvr1* susceptibility to Mex21 and $HAT^{Mex21\text{-}VPg}$-GFP (Figure 3.6). Additionally, VPg models from both N and HAT, which could not infect *pvr1* plants, were predicted to interact unfavorably with pvr1 (Table 3.2). $pvr1^2$, which was susceptible to N and $HAT^{N\text{-}VPg}$-GFP, was also predicted to favorably interact with N VPg, and did not interact favorably with the VPg from Mex21 and HAT (Figure 3.6 and Table 3.2). Model data shows favorable interactions for fully susceptible $Pvr1^+$ plants with all three VPg proteins (Figure 3.6 and Table 3.2). Since the models predicted the interactions of a few key amino acids interacted, site directed mutagenesis could be used to test if they are indeed essential for infectivity. Our results are supported by a study which used VPg chimera to demonstrate six codon changes in the central and C-terminus of PVY VPg, and five codon changes in the same region of PVA VPg, allowed infectivity in pepper plants encoding *pvr1* alleles, $pvr1^1$ and $pvr1^2$, respectively (Moury et al. 2004).

In the current study, we focused on two alleles of the *pvr1* locus, *pvr1* and $pvr1^2$. Using differential infectivity conferred by *pvr1* and $pvr1^2$, we were able to determine that TEV VPg was the virulence determinant using TEV VPg chimeras. This study also supports prior evidence that the central domain is important for strain specific infectivity. Identifying variation in the VPg domain of resistance breaking strains like Mex21 and N can provide the reason for susceptibility. Because susceptibility is linked to VPg interaction with eIF4E or eIF(iso)4E, identifying the specific amino acids responsible for interaction with VPg it may be possible to engineer new eIF4E alleles that are unable to interact with VPg and subsequently confer resistance to one or more viruses. Previously, we showed that overexpression of the *pvr1* resistance allele in tomato conferred potyvirus resistance (Kang et al. 2007). If a new strain of TEV is identified which can overcome all *pvr1* resistance

alleles, it would be possible to mutate a *pvr1* allele to eliminate interaction with the VPg of the resistance breaking strain and thereby confer resistance. The synthesis of new eIF4E or eIF(iso)4E transgenic resistance alleles could potentially be developed to confer resistance to any resistance breaking strain. This could be a powerful tool for engineering new sources of resistance to viruses which encode a VPg.

Material and methods

Plants, viruses and populations for genetic studies

C. annuum 'NuMex RNaky' (RNaky) was obtained from Asgrow Seed Co. (San Juan Bautista, CA). *C. annuum* 'Dempsey' was provided by C. M. Deom, University of Georgia, Athens, GA, USA. *C. chinense* PI 159234 (CA4) was obtained from the USDA Southern Regional Plant Introduction Station (Griffen, GA). *C. chinense* 'Habanero' was obtained from Tomato Growers Supply Co. (Fort Myers, FL).

Plant virus strains used in this study included TEV-HAT, TEV-N and TEV-Mex21. TEV-HAT and TEV-N were obtained from T. Pirone, University of Kentucky, Lexington, KY. TEV-Mex21was obtained from J. F. Murphy, Auburn University, Auburn, AL. Each virus was maintained by mechanical passage in *Nicotiana tabacum* 'Kentucky 14'. TEV-HAT GFP was obtained from J. Carrington (Oregon State University, Corvallis, OR, USA.

VPg cloning and sequence alignment

Total RNA was extracted from flash frozen 'Kentucky 14' non-inoculated leaf tissue infected with the respective virus using a Qiagen RNeasy Plant Mini kit (Qiagen, Valencia, CA), following the manufacturer's instructions. cDNA was synthesized as described previously (Kang et al. 2005) using primers TEV-Mex21 VPg reverse primer 5'-CCCTTCAAACATCAACTCCTC-3' for TEV-Mex21 RNA and TEV-N VPg reverse primer 5'-CCCTTCAAACGTCAACTCCTC-3' for TEV-N RNA. The

VPg coding region was cloned using a pGEM-T Easy kit (Promega). Clones including TEV-Mex21 VPg and TEV-N VPg as well as TEV-HAT GFP and both viral chimeras TEV-HAT$^{N\text{-}VPg}$ and TEV-HAT$^{Mex21\text{-}VPg}$ GFP were aligned using Seqman software (DNASTAR Inc., Madison, WI, USA) as described previously (Kang et al. 2005). Primers used for chimera sequencing are listed in Supplemental Table 3.1.

Megaprimer to replace VPg using mega primers

The chimera were synthesized using the megaprimer method and transcribed *in vitro* as described (Kang et al. 2005; Perez et al. 2006; Schaad, Lellis, and Carrington 1997). In the initial reactions, a 564bp VPg DNA fragment was amplified. TEV-Mex21 forward primer 5'-CAAGGGAAGAAGAATCAGAAG-3' and reverse primer 5'-CCCTTCAAACATCAACTCCTC-3' were used to amplify TEV-Mex21 VPg. TEV-N VPg was amplified using TEV-N forward primer 5'-CAGGGGAAGAAGAATCAGAAG-3' and reverse primer 5'-CCCTTCAAACGTCAACTCCTC-3'. For the second step of mega-primer amplification, the manufacturer's protocol was followed for use of the Quikchange XL kit (Stratagene, La Jolla, CA) with the modification of using 100 ng VPg megaprimers, 10 ng of pTEV-HAT GFP plasmid, 2.5 U *Pfu* DNA polymerase, 50 µM dNTPs as described previously (Perez et al. 2006). Following this PCR reaction, a digestion with 10 U *Dpn*I was performed at 37 ^{o}C for 1 hr and transformed into *E.coli*. Clones were verified for VPg replacement by PCR, restriction enzyme digestion, and DNA sequencing analysis.

In vitro transcription and biolistic inoculation

TEV-HAT GFP, TEV-HAT$^{N\text{-}VPg}$ and TEV-HAT$^{Mex21\text{-}VPg}$ RNA transcripts were prepared as described previously (Kang et al. 2005; Schaad, Lellis, and Carrington 1997), and used to inoculate 'Kentucky 14'plants at the 4-6 leaf stage using a microprojectile bombardment gun (Biorad, Hercules, CA, USA). The infected tobacco

plants were subsequently used as sources of inoculum throughout the study. For each virus, 15-20 plants were inoculated. Leaf tissue from symptomatic plants at 7-10 dpi served as inoculum for additional 'Kentucky 14' plants at the 4-6 leaf stage. These plants were used as inoculum for pepper plants.

Virus inoculation and resistance test

Pepper seeds were allowed to germinate at 30 C in petri dishes containing water-saturated sterilized germination paper. Germinated seed were sown in plastic trays containing the soilless potting medium Cornell Mix. Plants were inoculated at approximately the six leaf stage. Virus inoculum was rub inoculated onto at least two of the youngest leaves big enough to inoculate, approximately $3cm^2$ in size, after the leaves were dusted with Carborundum. Inoculum was prepared by grinding systemically infected 'Kentucky 14' tissue in 50 mM potassium phosphate buffer, pH 7.5 (approximately 1 g tissue : 20 mL buffer). Mortars and pestles were chilled at -20 C prior to use and then maintained on ice throughout the inoculation process. Genotypes pvr1, pvr12 and Pvr1+ were always included. For the TEV-Mex21, TEV-HAT-GFP and TEV-HAT$^{Mex21\text{-}VPg}$ GFP test *Pvr1*$^{+}$ genotype *C. chinense* Habanero was included so it could be compared to *C. chinense* CA4. For the TEV-HAT GFP, TEV-N, TEV-HAT$^{N\text{-}VPg}$ GFP test and TEV-HAT$^{Mex21\text{-}VPg}$ test Pvr1+ genotype *C. annuum* RNaky was included so it could be compared to *C. annuum* Dempsey. Mock-inoculated controls were always included. Plants were arranged so each tray contained a single genotype infected with a single virus; trays were arranged so plants did not touch each other to avoid cross-contamination. Plants were monitored routinely for the appearance of symptoms. Leaf tissue was tested for the presence of virus using antigen plate-coating indirect ELISA, as described previously (Kang et al. 2005). TEV polyclonal anti-viral immunoglobulins were obtained from Agdia, Inc. (Elkhart, IN, USA). At least three uninoculated leaves were sampled and combined per plant at 15

and30 dpi. Tissue was sampled in identically sized leaf punches per plant. ELISA absorbance values were determined at 405 nm. Samples were considered positive if their absorbance value was above the mean of the mock inoculated controls plus three standard deviations. Samples are divided in Figure 3.3 based on sampling date and ELISA experiment. All samples included in each graph were inoculated and loaded into ELISA plates on the same day. Multiple 96well plates were included for each ELISA date. Mock inoculated controls were sampled for ELISA for genotype for each sampling date.

GFP imaging

GFP fluorescence resulting from virus infection was monitored using a Leica TCS SP2 scanning confocal microscope (Leica, Wetzlar, Germany). At 7-10dpi, 4-5 uninoculated leaves were sampled from inoculated plants for viewing. At least 3 plants per genotype were sampled per virus and monitored for GFP accumulation. Leaves showing signs of infection were preferentially selected in all cases when available.

Yeast two-hybrid analysis

Yeast two-hybrid analysis using yeast strain EGY48 and vectors pEG202, pJG4-5 and pSH18-34 was carried out as described (Kang et al. 2005; Yeam et al. 2007). Yeast and vectors were provided by G. Martin (Boyce Thompson Institute, Ithaca, NY). Full length and deleted TEV-HAT VPg was fused into pEG202 and prey plasmid, pJG4-5, expressed full length *Capsicum* eIF4E Pvr1^{+}. Yeast western blot analysis with anti-eIF4E (New England Biolabs) assured equivalent expression for all constructs. eIF4E antibody was produced as previously described (Kang et al. 2005).

Modeling of the VPg and eIF4E proteins

PSIPRED and SAM-T02 were used to develop secondary structure predictions of VPg, and the program ECEPPAK was used to build the VPgHX fragment (Ripoll 1999). The program MODELLER (Sali 1995; Sali and Blundell 1993; Sanchez and

Sali 2000) was used to generate the 3D models for the sequences of the *pvr1* alleles. Final adjustment of the alignment between the Capsicum-eIF4E and the yeast sequence was performed manually with the help of graphic tools included in the commercial programs ICM (MOLSOFT) and DS-Modeling (Accelrys). The pairwise alignments of the sequences of Capsicum variants and the template 1AP8 were generated by using the program BLAST (Altschul 1997).

Table 3.3. Primer sequences used in this study.

Primer ID	bp covered	F	R
1 TEV HAT	1-600	ggcaagagacgcaaagtttc	ttgcaaatgtcttccactgc
2 TEV HAT	601-1200	gtgcaattgttcgcaagtgt	tgcctcaaaactaggccact
3 TEV HAT	1201-1800	agggtgagtcgggagaaagt	gccagtacgtgtgtgaatgg
4 TEV HAT	1801-2400	tcggatctaaagcacccaac	cctttgcatcctcttccttg
5 TEV HAT	2401-3000	atgcgcaggtaattttggac	ttgcggagaatttttccaac
6 TEV HAT	3001-3600	cctgaagggaaccatctcaa	ttgtcccactcgctctcttt
7 TEV HAT	3601-4200	ggggcacttcatggagttta	atcctcaaagttgggaagca
8 TEV HAT	4201-4800	gagcggtgcagtacaacaaa	agcttcagtggcaaccattt
9 TEV HAT	4801-5400	tgctggaaaatgccacatta	atgccagcatcctctgctat
10 TEV HAT	5401-6000	agggcaatatgaggttgcag	tcaataggtgcgtttgtgga
11 TEV HAT	6001-6600	cgagcaccatttgtcatttg	ggaaatggtgggaaatcctt
12 TEV HAT	6601-7200	tcagcatcgaatttcaccaa	aggctcttcaggtttgctca
13 TEV HAT	7201-7800	gaaagcagctatgggagcac	cacgcaaactttaccagcaa
14 TEV HAT	7801-8400	ggtggacaacacactcatgg	tgtccctggtggtacagtca
15 TEV HAT	8401-9000	tcagcacggaacaaactctg	gcctgctcagcgactttatc
16 TEV HAT	9001-9494	acgctatgcgttcgacttct	gcatgttacggttcacatcg

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CHAPTER 4

PEPPER RECESSIVE RESISTANCE GENE *PVR1*^{2} CONFERS DOMINANT NEGATIVE RESISTANCE TO *POTATO VIRUS Y* IN POTATO[a,b]

Abstract

Potato infecting viruses such as potyvirus *Potato virus Y* (PVY) continue to limit production despite breeding efforts to confer resistance. Transgenic approaches to confer virus resistance in potato are particularly significant because new varieties gain popularity infrequently and variety mainstays are often susceptible to PVY. In this study, we transformed widely cultivated and PVY susceptible variety Russet Burbank with one of four alleles of the naturally occurring pepper potyvirus resistance gene *pvr1*, which encode variants of eukaryotic translation initiation factor eIF4E. Naturally occurring mutations in eIF4E provide virus resistance in many systems. Enzyme-linked immunosorbant assay (ELISA) data indicate Russet Burbank lines overexpressing the *pvr1*^{2} or the *pvr1*^{1} allele from pepper were resistant to at least one of three PVY strains tested: PVY$^{N\text{-}Wi}$, PVY^{O}, and PVY^{NTN}. The majority of *pvr1*^{2} lines were resistant to all strains of PVY and plants grown from tubers of the inoculated resistant lines were virus free. *pvr1*^{1} plants grown from tubers of inoculated plants were virus free in nearly all cases. This technology could be extremely valuable for the industry given potato is tuber propagated and controlling PVY costs seed producers and growers a great deal of income each year.

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[b]Byoung-Cheorl Kang and Molly Jahn conceived of the transgenic plants included in this study. Kari Perez completed all the experiments except for the two potato transformations experiments which were completed by Joyce Van Eck and Sandra Austin-Phillips. Kari Perez wrote this document with assistance from Inhwa Yeam, Stewart Gray and Molly M. Jahn.

Introduction

As the world's fourth most important crop, potato (*Solanum tuberosum*) has a tremendous impact on the global food market. In fact, the Food and Agriculture Organization (FAO) of the United Nations named 2008 'The Year of the Potato', stating the hardy crop could provide much needed food security worldwide (FAO 2008). Small scale or subsistence farmers, representing 500 million in China, 500 million in India and 400 million in Africa (Toenniessen, O'Toole, and DeVries 2003), could benefit from increased potato productivity for many reasons. Potato is traded locally so prices are determined by the local market rather than the global market, like grain crops, whose prices have recently soared due to increased demand for cereals for biofuel production and animal feed (FAO 2008). Additionally, potato is highly nutritious containing potassium, phosphorus, folate, magnesium, Vitamins C, B1, B3 and B6 as well as antioxidants and fiber, and has the highest protein to calorie ratio of any other tuber (FAO 2008; Mih and Atiri 2001).

While potato can be produced in less than ideal environmental growing conditions, there are many viruses which have a tremendous impact on production (CIP 2007). Among them is *Potato virus Y* (PVY), an economically devastating virus found in all potato production areas which ravages potato yields worldwide (Crosslin et al. 2006; Ellis, Stace-Smith, and de Villiers 1997; Khurana 2004; Thottappilly 1992). Isolates are differentiated as PVY^{O}, the common strain, or as isolates of PVY^{N}, the necrotic strain, which causes necrosis in tobacco and sometimes in potato (Beczner et al. 1984; de Bokx and Huttinga 1981). The four PVY strains were used in this study: PVY^{N}, PVY^{O}, PVY^{NTN} and $PVY^{N\text{-}Wi}$. PVY^{NTN} was first identified in Hungary for its ability to cause tuber necrotic ringspot disease (Beczner et al. 1984). PVY^{NTN} is now commonly found worldwide (Blanco-Urgoiti et al. 1998). $PVY^{N\text{-}Wi}$ was first identified in Poland (Chrzanowska 1991) and has also been identified in potato

growing regions throughout the world (Blanco-Urgoiti et al. 1998; Chikh Ali, Maoka, and Natsuaki 2007; Crosslin et al. 2006; Kerlan et al. 1999). Molecular analysis of isolates PVY^{NTN} and $PVY^{N\text{-}Wi}$ revealed they were actually recombinant isolates of PVY^{O} and PVY^{N} (Glais, Tribodet, and Kerlan 2002). Mechanical transmission, as well as non-persistent transmission by aphids, particularly the green peach aphid, *Myzus* (Nectarosiphon) *persicae* (Sulzer), can quickly spread the virus through a field (Radcliffe and Ragsdale 2002). Oils as well as insecticides are used to control aphid populations (Martín-López 2006) and both can have a detrimental impact on the environment (Kleter 2007).

The virus can also be transmitted to the tuber, making virus free seed tubers essential since potatoes are tuber propagated. Unfortunately, widely cultivated potato varieties such as Russet Burbank, which represented over 23 per cent of all potato acreage grown in the US in 2008, more than any other potato cultivar, are susceptible to PVY (Minnesota Certified Seed Potato Growers Association 2008). In the U.S., the incidence of PVY has been a major problem for potato seed certification programs which tolerate at most very low levels of virus incidence. Potato tuber seed is first multiplied first by Foundation Seed producers, then by Certified Seed producers who either save seed for recertification or sell to growers who produce for consumption. Seed growers are allowed a limited amount of virus in their fields which are monitored by state run seed certification programs such as the New York Seed Improvement Project (NYSIP) or the Idaho Crop Improvement Association. In New York, all varieties produced for potato seed need to be approved by NYSIP; currently they do not allow PVY asymptomatic cultivars in the program (Westra personal communication, 2009). The amount of virus mosaic permitted in the field increases the further the seed moves away from Foundation Seed. If mosaic symptoms are too high in the field, the seed will downgraded and likely sold for a lower price. The price

is dependent on fluctuations in the market price for tubers; in some years the table stock price will be high enough that certified seed growers will sell their harvest rather than putting it into storage (Westra personal communication, 2009). The strictest guidelines exist for Foundation Seed which is produced from tissue culture plantlets. These plants are subsequently grown in the field for multiplication. In New York, there is a 0.5% tolerance policy for visible mosaic symptoms in Foundation Seed fields (Westra personal communication, 2009). After Foundation Seed multiplication, seed is multiplied in certified fields which have a higher level of mosaic allowed in fields. There is a 5% tolerance policy in NY State for certified seed (Westra personal communication, 2009). In Idaho, which has had a tuber seed testing program since 1913 (Potato Country November, 2005), the Idaho Crop Improvement Association allows 0.2 to 2.0% virus mosaic symptoms during a field inspection (Nolte et al. 2004). Most states have a 5% tolerance policy for foliar mosaic symptoms including Maine, Michigan and Wisconsin, while Colorado allows 10% and Nebraska 8% (Oregon Seed Certification Service 2009). These symptoms could be due to PVY, PVX or PVA or other viruses which induces foliar mosaic symptoms. Usually the mosaic symptoms are clearly distinguishable by eye, but if there is uncertainty they will be tested for virus infection in the laboratory by an independent source (Westra personal communication, 2009). Last year in New York, all questionable plants tested to confirm virus infection were infected with PVY; no other viruses were identified (Westra personal communication, 2009). If the amount of virus in the field is still too high, the seed can only be sold table stock. Some years the seed price has been close to the table stock, other years there is a significant difference in price. In these years growers can stand to lose a significant profit due to virus infection. Additionally, if plants are infected with virus the tubers will harder to sell (Westra personal communication, 2009).

In the Northwest, the investment to control viruses is about 100 million dollars annually, with at least a quarter of that due to PVY (Brown 2009, personal communication). In the developing world, certified seed tubers produced in the home country are often unavailable or do not meet the requirements of the growers due to difficult growing conditions (Vreugdenhil 2007). Seed tubers are often exported from the U.S., Europe and Canada around the world to meet these needs (Vreugdenhil 2007). PVY susceptible Russet Burbank and Shepody are among top exported varieties from the U.S., making virus free seed essential for the global market (Vreugdenhil 2007). In addition to Russet Burbank, Shepody and Russet Norkotah are also PVY susceptible (Minnesota Certified Seed Potato Growers Association 2008; Potato Country November, 2005). In 2008, out of over 300 certified varieties produced in the U.S., Russet Burbank was the number one followed second by Russet Norkotah which made up 9.8%; Shepody ranked seventh and made up 3.2% (Minnesota Certified Seed Potato Growers Association 2008). These two cultivars are particularly problematic because they exhibit latent PVY infection so growers do not know the plants are infected until it is too late (Hane and Hamm 1999). Even though they do not show strong foliar symptoms, the virus has been shown to reduce yield nearly 80% for Shepody and 65% for Russet Norkotah (Hane and Hamm 1999). Unfortunately, latent PVY syndrome is not limited to Shepody and Russet Norkotah. Many new varieties also do not show strong symptoms (Brown 2009, personal communication). These cultivars serve as a source of infection for an entire field and seriously impact yield.

Natural sources of resistance to PVY include the single dominant genes *Ry* and *Ny*, which confer complete resistance or hypersensitivity, respectively (Solomon-Blackburn and Barker 2001a). The Ny_{tbr} gene, identified from *S. tuberosum*, confers hypersensitivity only to the PVY^{O} strains not the PVY^{N} strains (Solomon-Blackburn and Bradshaw 2007; Solomon-Blackburn and Barker 2001a). *Ry* resistance genes have

all been identified from wild species (Solomon-Blackburn and Barker 2001a). The *Ry*$_{sto}$ gene, isolated from *S. soloniferum*, has been introgressed into *S. tuberosum* (Ross 1958). To date, cultivars encoding this gene are resistant to all strains of PVY (Baldauf, Gray, and Perry 2006; Solomon-Blackburn and Barker 2001a). Even though *Ry*$_{sto}$ resistance is complete, clones such as Eva are not widely cultivated due to variety preferences in the industry (Brown 2009, personal communication; Minnesota Certified Seed Potato Growers Association 2008). In fact, over the past 100 years, few new potato cultivars have had success in the marketplace, even though over 250 cultivars have been developed (Douches 1996). Potato breeding is not a closely targeted affair; sexual progeny are so variable that it is difficult to achieve an array of traits that make a potato a success (Brown 2009, personal communication). This is because many desirable characteristics must be combined in each cultivar: high yield, high horticultural quality and resistance to a range of pests and pathogens are all required (Solomon-Blackburn 2001b). Transgenic approaches can solve this problem because genes of interest can be integrated into any cultivar of interest without affecting quality. Transgenic strategies have been employed to control potato viruses since the first transformation of potato. The PVY coat protein was the first transgene used to transform potato (Newell et al. 1991). Other potato transgenes have included the *Potato leafroll virus* movement protein, an antibody that binds the PVY NIa protein, the PVY P1 and coat protein, *cryIIIA* (which produces toxin against pests such as the Colorado potato beetle) and a pathogen-inducible glucosyltransferase (TOGT) to confer PVY resistance (Gargouri-Bouzid et al. 2006; Kaniewski et al. 1990; Lawson et al. 1990; Mäki-Valkama et al. 2001; Matros and Mock 2004; McDonald 1997; Newell et al. 1991; Perlak et al. 1993; Tacke, Salamini, and Rohde 1996; Visser et al. 1991). Additionally, the BASF corporation recently released another transgenic potato called Amflora after waiting nearly 12 years long for the European Food Safety

Authority (EFSA) to approve it (Burger and Steitz 2009). The potato is designed for industrial purposes rather than human consumption as the tuber starch has been altered to produce 100% amylopectin, and does not produce amylose which drastically alters the starch as well makes the tuber inedible (Burger and Steitz 2009; Visser et al. 1991). Until the approval of the amyflora potato, only *Bt* maize was grown in Europe by seven of the 27 countries in the EU: Portugal, Spain, Germany, Czech Republic, Slovakia and Romania (James 2008).

The Monsanto Corporation commercialized Russet Burbank lines encoding *Bt* gene and PVY resistance as NewLeaf Y as well as NewLeaf Plus which also encoded *Bt* as well as PLRV resistance in Canada and the United States in 1998 and 1999 (Kaniewski 2004). The transgenic lines required fewer insecticide sprays, produced disease free tubers and was preferred by consumers for their superior quality and competitive pricing (Kaniewski 2004). Even with positive consumer feedback, McDonald's, whose french fries are made from Russet Burbank potatoes, would not purchase the potatoes prompting the downfall of the transgenic potato market and Monsanto dropped the product in 2001 (Kaniewski 2004). To date, all transgenic approaches to control PVY in potato have encoded pathogen derived sources of resistance (PDR). It is possible that using a plant derived gene rather than a pathogen derived gene could improve acceptance of transgenic crops. This hypothesis is supported by a 2002 survey from Lusk et al. which found 59.6% of consumers surveyed would consume plant genes as transgenes, while just 14.3% would consume viral genes as transgenes (Lusk 2002).

In this study, we have used a pepper gene as a transgene to confer PVY resistance. Pepper plants encode recessive resistance alleles at the *pvr1* (*potyvirus resistance gene 1*) locus conferring resistance to several potyviruses (Kang et al. 2005). *pvr1* encodes eukaryotic translation initiation factor eIF4E, which is part of translation

initiation complex, eIF4F, made up of eIF4A, eIF4E and eIF4G (Browning 2004; Gingras, Raught, and Sonenberg 1999). This complex is responsible for translation initiation which includes mRNA 5' cap recognition, mRNA unwinding and recruitment to the 40S ribosomal subunit (Browning 2004; Browning 1996; Gingras, Raught, and Sonenberg 1999; Kawaguchi and Bailey-Serres 2002; Nicaise et al. 2007). eIF4E has been correlated with resistance in several pathosystems In susceptible plants, eIF4E has been shown to interact with potyviral protein VPg, which is linked to the 5'end of the potyviral genome (Bruun-Rasmussen et al. 2007; Kang et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Yeam et al. 2007). In fact, VPg has been identified as the virulence determinant in a number of diverse hosts (Borgstrom and Johansen 2001; Bruun-Rasmussen et al. 2007; Murphy et al. 1991; Murphy et al. 1996; Rajamaki and Valkonen 1999; Rajamaki and Valkonen 2002; Revers et al. 1999). We have shown previously that just one amino acid substitution mutation in the *pvr1* resistance allele is sufficient to eliminate the interaction with tobacco etch virus VPg and confer resistance (Yeam et al. 2007).

Recessive resistance alleles at the *pvr1* locus have been employed in pepper breeding programs for over 50 years (Greenleaf 1956). Currently the *pvr1*^{2} (also cited as pvr22) allele is more widely used than the *pvr1* or the *pvr1*^{1} alleles in pepper breeding programs, making it the most important allele to the industry (Czaplewski 2009, personal communication). The resistance conferred by the *pvr1*^{2} allele has proven adequate in most areas. If it is overcome, the level of infection has not been overwhelming or recurring in the same area in subsequent years (Czaplewski 2009, personal communication). Fifty years later, *pvr1*^{2} has not been replaced by other genes to any extent (Czaplewski 2009, personal communication). Because *pvr1*^{2} mediated resistance has proven durable in pepper, we transformed Russet Burbank with the full length open reading frame (ORF) of the *pvr1*^{2} allele. We also transformed the same

variety with the ORF of the other *pvr1* alleles, *pvr1*, *pvr1*^{1} and *Pvr1*$^+$. Previous pepper PVY infectivity studies have shown peppers encoding the *pvr1*^{2} confer the broadest spectrum of PVY resistance (Kyle and Palloix 1997). PVY isolates which infect *Capsicum* have been named for their ability to overcome recessive resistance alleles *pvr1*, *pvr1*^{1} and *pvr1*^{2} (Kyle and Palloix 1997), the last two alleles are also cited in the literature as *pvr2*^{1} and *pvr2*^{2} (Moury et al. 2004; Ruffel et al. 2002), but have been determined to be alleles of the *pvr1* locus (Kang et al. 2005). A virus survey conducted in pepper fields found isolates PVY-0 and PVY-0,1 most frequently (Palloix 1994). Isolate PVY-0 is resisted by all three alleles, *pvr1*, *pvr1*^{1} and *pvr1*^{2}, while PVY-0,1 and PVY-0,1,2 can infect *pvr1*^{1} plants, and PVY-0,1,2 can infect *pvr1*^{2} (Kyle and Palloix 1997). Because PVY-0, PVY-0,1 and PVY-0,1,2 have not been sequenced, and were not included in this study, we could not correlate previous pepper *pvr1* infectivity data with *pvr1* infectivity data generated in this study for the four potato infecting strains we tested for pepper infectivity, PVY^N, PVY^O, PVY^{NTN} and PVY$^{N\text{-}Wi}$.

Previous studies show that transgenic overexpression of a modified version of eIF4E can confer resistance to potyviruses. A study from Schaad et al. (1997) produced transgenic Arabidopsis overexpressing naturally occurring eIF4E mutants which confer resistance in other plant systems. When these plants were inoculated with *Tobacco etch virus* (TEV) virus, they accumulated the virus at levels much lower than wild type (Schaad, Lellis, and Carrington 1997). In our lab, we have shown that *S. lycopersicum* 'MicroTom' plants overexpressing the *pvr1* gene were resistant to several strains of TEV as well as *Pepper Mottle Virus* (Kang et al. 2007). This study reports several Russet Burbank transgenic lines overexpressing the pepper *pvr1*^{2} resistance gene were resistant to all strains of PVY tested.

Results

Pepper cultivars homozygous for the *pvr1*^{2} or *pvr1*^{1} alleles are resistant to PVY.

Pepper cultivars homozygous for one of the four *pvr1* alleles, *Pvr1*$^+$, *pvr1*, *pvr1*^{1} and *pvr1*^{2}, were tested for their susceptibility to isolates of four different strains of PVY (PVY$^{N\text{-}Wi}$, PVY^O, PVY^{NTN} or PVY^N). At least one cultivar per *pvr1* allele was inoculated with each PVY strain and infectivity was assessed by visible symptom development and ELISA. Symptoms were never observed in plants encoding the *pvr1*^{2} and *pvr1*^{1} inoculated by any of the four virus isolates; leaves looked identical to virus free controls (Figure 4.1A). Plants homozygous for *pvr1* occasionally developed very mild mosaic symptoms, but most plants remained symptomless when infected with any of the isolates. Mosaic symptoms were observed on plants encoding the susceptible allele *Pvr1*$^+$ by 20 days post infection (dpi) with PVY^O, PVY^{NTN} and PVY^N (Figure 4.1A). In contrast, *Pvr1*$^+$ plants infected with PVY$^{N\text{-}Wi}$ displayed severe yellow mosaic symptoms, necrotic lesions and leaf deformation. (Figure 4.1A). PVY^O and PVY^{NTN} were tested in the fall, PVY^N in the winter and PVY$^{N\text{-}Wi}$ in the spring. Since the inoculations took place at different times of year, variation in day length and temperature could have accounted for symptom severity for the PVY$^{N\text{-}Wi}$ screen.

Young leaves that developed after the plants were inoculated with PVY were sampled 39-45 dpi and tested by ELISA using an antibody that detects all PVY strains. Virus antigen was not detected in leaves from any of the inoculated *pvr1*^{2} or *pvr1*^{1} plants. PVY antigen was detected in both *Pvr1*$^+$ and *pvr1* plants inoculated with each of the four PVY isolates (Table 4.1).

Table 4.1. PVY susceptibility in pepper plants differing at the *pvr1* locus.

Cultivar[a]	*pvr1* allele	(PVY^{O})[b]	PVY^{O} symptoms[c]	(PVY^{NTN})[b]	PVY^{NTN} symptoms[c]	($PVY^{N\text{-}Wi}$)[b]	$PVY^{N\text{-}Wi}$ symptoms[c]	(PVY^{N})[b]	PVY^{N} symptoms[c]
RNaky	*Pvr1*$^{+}$	11/17	M	28/28	M	10/10	SM, NL, LD	5/5	M
HAB	*Pvr1*$^{+}$	7/8	M	NS	M	6/6	SM	NS	M
5502	*pvr1*	7/8	MM	7/8	MM	5/5	MM	5/5	MM
CA4	*pvr1*	5/5	MM	9/15	MM	5/5	MM	NS	NS
YY	*pvr1*[1]	0/6	none	0/13	none	0/5	none	0/4	none
DP	*pvr1*[2]	0/8	none	0/6	none	0/6	none	0/4	none

[a]Cultivars included in the study are NuMex RNaky, Habanero (Hab), 5502, CA4, Yolo Y (YY) and Dempsey (DP).

[b]Data are number of plants which tested positive for PVY by double-antibody sandwich enzyme-linked immunosorbant assay. PVY screens include: $PVY^{OME\text{-}11}$ which was sampled at 42days post inoculation (dpi), PVY^{NTN} sampled at 41dpi, $PVY^{N\text{-}Wi}$ sampled at 39dpi and PVY^{N} sampled at 45dpi. NS were not included in the screen. 2 uninoculated plants were sampled and screened per each cultivar included in each inoculation except for the PVY^{N} screen where one uninoculated plant per cultivar was sampled. All uninoculated plants had abs values at or near zero (data not shown).

[c]Visible symptoms were monitored until sampling date: None - no visible symptoms ever developed, MM mild mosaic, M - yellow mosaic, SM - severe yellow mosaic, NL - necrotic lesions, LD - leaf deformation.

Potato plants expressing the pepper *pvr1*^{2} or *pvr1*^{1} allele are resistant to PVY.

Given that pepper plants encoding *pvr1*^{1} and *pvr1*^{2} had broad spectrum resistance to PVY, and that overexpression of the *pvr1* pepper allele in transgenic tomato conferred resistance to other potyviruses (Kang et al. 2007), we hypothesized that expression of *pvr1*^{1} or *pvr1*^{2} in transgenic potato may confer broad spectrum PVY resistance. To test this hypothesis, the PVY susceptible *Solanum tuberosum* cultivar Russet Burbank was transformed with each of three *pvr1* alleles: *pvr1*^{1}, *pvr1*^{2}, and *Pvr1*$^+$.

An initial transformation experiment generated five Russet Burbank lines transformed with *Pvr1*$^+$, five lines transformed with *pvr1*^{1} and three lines transformed with *pvr1*^{2}. T_0 transformants were multiplied by cuttings and at least two plants derived from each line were inoculated with each strain of virus, PVY^O, PVY$^{N\text{-}Wi}$, or PVY^{NTN} (Table 4.2). PVY^N was not included because it was found infrequently in potato viral surveys. Infectivity was monitored by the appearance of visible symptoms and ELISA. Plants derived from each of the three *pvr1*^{2} lines did not develop visible symptoms when inoculated with any of the three strains and virus antigen was not detected in leaves sampled 21 to 22 dpi (Table 4.2). A second experiment on additional cuttings from two of these three lines also indicated that all the plants were resistant to PVY infection when tested at 44 days post infection. Infectivity results of *pvr1*^{1} lines were more complicated. All *pvr1*^{1} plants derived from lines 5, 21 and 25 were resistant to PVY^O and PVY$^{N\text{-}Wi}$ in two separate experiments; however some of the plants from these lines were susceptible to PVY^{NTN}. Plants derived from line 23 were susceptible to all three PVY isolates, but the results were not consistent between experiments. Plants derived from line 27 were susceptible to PVY$^{N\text{-}Wi}$ and PVY^{NTN}, but appeared resistant to PVY^O, although only a small number of plants were tested (Table 4.2). Plants derived from each of the four *Pvr1*$^+$ lines and the non-transgenic

Russet Burbank plants that were inoculated with each of the three PVY strains displayed yellow mosaic and leaf deformation symptoms. Virus antigen was detected by ELISA in all symptomatic plants, but not in the few asymptomatic plants which were considered escapes, not resistant plants.

To determine if the resistance to virus infection identified in the foliage also was functional in the tubers, tubers were harvested from a subset of plants used in the first inoculation experiment. Following an appropriate dormancy period, the tubers were sprouted and planted in the greenhouse, and the foliage was subsequently tested by ELISA (Table 4.2). All tubers from *pvr1*^{2} plants were virus free, whereas at least one tuber from each of the infected non-transgenic control plants tested positive for virus. Tubers harvested from *pvr1*^{1} plants which tested positive for infection in the infectivity test were always positive, but tubers produced from one plant that had tested negative by ELISA, a plant from line 21 infected with PVY^{NTN}, produced infected daughter plants.

To confirm resistance data from the previous transformation, a second transformation experiment using a different Russet Burbank clone produced more *pvr1*^{2} and *Pvr1*$^+$ lines, as well as lines transformed with another resistance allele *pvr1* and the *GUS* reporter gene. Since all of the lines transformed with *pvr1*^{1} were susceptible to at least one PVY strain in the previous experiment, this allele was not included in the second experiment. Plants from the second transformation were propagated by cuttings from T_0 tissue culture plants for PVY infectivity tests. Twenty-four *pvr1*^{2}, five *Pvr1*$^+$, five *pvr1,* and four *GUS* transgenic lines as well as non-transgenic controls were tested for resistance to three PVY strains, PVY^{N-Wi}, PVY^O and PVY^{NTN}. An additional eight *pvr1*^{2} and seven *GUS* lines were tested for resistance to at least one of the three strains. Plants inoculated with PVY^{N-Wi} and PVY^O were tested in the same experiment, whereas time and space constraints forced plants

inoculated with PVY^{NTN} to be tested in a separate experiment. A minimum of two plants were inoculated for each transgenic line, but typically five plants per line were inoculated with each virus strain. Infectivity was determined by visible symptom development and ELISA.

All resistant *pvr1*^{2} lines failed to show any visible symptoms of infection (Figure 4.1B). Leaf symptoms in the non-transgenic, *GUS*, *pvr1*, *Pvr1*$^+$ and *pvr1*^{2} plants which were determined to be infected by ELISA were clearly visible 20-30dpi (Figure 4.1B). Symptom type and severity were similar in plants inoculated with $PVY^{N\text{-}Wi}$ and PVY^{O}. Symptoms included mosaic and leaf deformation, while PVY^{NTN} induced only minor mosaic symptoms. Plants were infected under the same conditions; however, the PVY^{NTN} evaluation was completed several months prior to the PVY^{O} and $PVY^{N\text{-}Wi}$ screens. The variation in visible symptoms severity could possibly be attributed to variation in day length, temperature variations, etc.

Figure 4.1. Potato Virus Y (PVY) symptoms. Evaluation of PVY resistance in pepper and transgenic and non-transgenic potato plants. Transgenic plants include plants expressing the *GUS* reporter gene as well as recessive resistance genes $pvr1^2$, *pvr1* and $Pvr1^+$ from *Capsicum*.

A. PVY symptoms in pepper leaves. Pepper plants encoding $Pvr1^+$ or $pvr1^2$ alleles inoculated with PVY^{NTN} or $PVY^{N\text{-}Wi}$. $Pvr1^+$ inoculated with PVY^{NTN} shows yellow mosaic symptoms at 52dpi. $Pvr1^+$ inoculated with $PVY^{N\text{-}Wi}$ shows leaf deformation, severe yellow mosaic and necrotic lesions while $pvr1^2$ shows no symptoms. $PVY^{N\text{-}Wi}$ images were taken at 56dpi.

B. PVY symptoms in potato leaves. T_0 transgenic potato progenies from individual T_0 plants previously assessed for the presence their respective transgenes were inoculated with PVY^{O}, $PVY^{N\text{-}Wi}$. Images of all inoculations were taken at approximately 63dpi. Leaves of non-transgenic samples (RBIDA) show leaf deformation and yellow mosaic, which encode the $pvr1^2$ transgene (ED3 lines) do not show symptoms.

A.

Pvr1^{+} (RNaky) PVY^{NTN}

Pvr1^{+}(RNaky) uninoculated

Pvr1^{+} (RNaky) PVY $^{N-Wi}$

Pvr1^{+} (RNaky) PVY $^{N-Wi}$

pvr1^{2}/*pvr1*^{2} (Dempsey) PVY $^{N-Wi}$

B.

RBIDA PVY^{N-Wi} vs. ED3 25 PVY^{N-Wi}

GUS 66 PVY^{O} vs. ED3 21 PVY^{O}

Table 4.2. *pvr1*[1], *pvr1*[2] and *Pvr1*[+] transgenic potato PVY infectivity summary, preliminary transformation.

Genotype	Line number	(PVY^{O})[a]: 1st screen	(PVY^{O})[a]: 2nd screen	Positive tubers[b]	($PVY^{N\text{-}Wi}$)[a]: 1st screen	($PVY^{N\text{-}Wi}$)[a]: 2nd screen	Positive tubers[b]	(PVY^{NTN})[a]: 1st screen	(PVY^{NTN})[a]: 2nd screen	Positive tubers [b]
pvr1[1]	5	0/5	0/6	0/14	0/5	0/5	0/1	1/6	2/6	4/7
pvr1[1]	21	0/3	0/10	0/7	0/4	0/12	0/10	0/5	1/12	2/5
pvr1[1]	23	2/5	0/10	0/8	3/7	0/11	5/11	0/7	1/11	0/5
pvr1[1]	25	0/3	0/10	0/8	0/4	0/10	NS	0/5	3/10	0/5
pvr1[1]	27	0/3	NS	0/2	2/3	NS	0/1	1/4	NS	0/3
pvr1[2]	8	0/3	NS	NS	0/6	NS	NS	0/4	NS	0/2
pvr1[2]	57	0/3	0/4	0/7	0/5	0/4	NS	0/4	0/4	0/4
pvr1[2]	59	0/3	0/12	0/4	0/4	0/12	0/8	0/4	0/12	0/4
Pvr1[+]	63	3/3	3/3	NS	3/4	1/2	NS	2/4	4/4	NS
Pvr1[+]	65	2/2	NS	NS	2/2	NS	NS	2/2	NS	NS
Pvr1[+]	67	3/3	NS	NS	4/4	NS	NS	4/5	NS	NS
Pvr1[+]	79	3/3	NS	NS	4/4	NS	NS	4/4	NS	NS
Pvr1[+]	81	3/3	NS	NS	3/3	NS	NS	2/5	NS	NS
Non-transgenic	NTRB	9/9	7/7	7/7	10/10	8/12	NS	8/12	9/9	2/4

[a]Data are number of plants which tested positive for PVY by double-antibody sandwich enzyme-linked immunosorbant assay. PVY screens include: PVY^{O} 1st and second screens were sampled at 22dpi and 44dpi, respectively; $PVY^{N\text{-}Wi}$ screens were sampled at 42 and 41dpi; PVY^{NTN} screens were sampled at 21 and 45dpi, respectively. NS were not included in the screen. At least 2 uninoculated plants were sampled and screened per genotype for each inoculation except for *Pvr1*[+] which only 1 plant was sampled (data not shown). All uninoculated plants had abs values at or near zero.

[b]Total number of tubers tested represents tubers sprouted from individual plants included in the infectivity test. One to three sprouted tubers were tested by ELISA per inoculated plant harvested from the first virus screen.

Table 4.3. *pvr1*^{2}, *pvr1*, *Pvr1*$^+$ and *GUS* transgenic potato PVY infectivity, second transformation.

Genotype	Line number	(PVY^O)^{a,b}	(PVY$^{N\text{-}Wi}$)^{a,c}	(PVY^{NTN})^{a,d}
non-transgenic	Mock RBIDA-1	0/2	0/3	0/3
GUS	Mock *GUS*	0/2	0/4	0/12
pvr1^2	Mock ED3	0/2	0/23	0/23
pvr1	Mock CA4	0/5	0/2	0/2
Pvr1$^+$	Mock RNOIY	0/5	0/2	0/2
pvr1^2	ED3-11	0/5	1/5	NS
pvr1^2	ED3-15	**0/5**	**0/5**	**0/4**
pvr1^2	ED3-21	**0/5**	**0/4**	**0/4**
pvr1^2	ED3-24	**0/5**	**0/5**	**0/4**
pvr1^2	ED3-25	**0/5**	**0/5**	**0/4**
pvr1^2	ED3-29	NS	NS	1/2
pvr1^2	ED3-37	0/4	4/5	NS
pvr1^2	ED3-40	**0/5**	**0/5**	**0/4**
pvr1^2	ED3-44	**0/5**	**0/5**	**0/4**
pvr1^2	ED3-49	**0/5**	**0/6**	**0/4**
pvr1^2	ED3-52	**0/5**	**0/5**	**0/2**
pvr1^2	ED3-53	0/5	2/5	0/5
pvr1^2	ED3-56	2/2	0/2	1/2
pvr1^2	ED3-60	0/4	1/5	0/2
pvr1^2	ED3-61	3/3	1/5	NS
pvr1^2	ED3-62	**0/5**	**0/4**	**0/2**
pvr1^2	ED3-64	**0/5**	**0/5**	**0/2**
pvr1^2	ED3-66	0/5	2/4	NS
pvr1^2	ED3-68	NS	NS	2/4
pvr1^2	ED3-70	**0/2**	**0/2**	NS
pvr1^2	ED3-75	**0/5**	**0/3**	**0/3**
pvr1^2	ED3-76	NS	NS	1/4
pvr1^2	ED3-77	**0/5**	**0/5**	**0/4**
pvr1^2	ED3-82	**0/5**	**0/5**	**0/2**
pvr1^2	ED3-85	4/4	3/3	3/3
pvr1^2	ED3-86	**0/5**	**0/3**	**0/3**
pvr1^2	ED3-87	NS	NS	2/2
pvr1^2	ED3-95	4/4	3/3	3/3
pvr1^2	ED3-97	4/4	3/3	3/3
pvr1^2	ED3-98	0/5	3/3	3/3
pvr1^2	ED3-110	4/4	3/3	3/3
pvr1^2	ED3-111	0/5	0/3	1/3
pvr1^2	ED3-112	**0/3**	**0/3**	**0/2**
pvr1	CA4-39	3/3	3/3	2/2
pvr1	CA4-49	5/5	3/3	2/2
pvr1	CA4-68	5/5	4/4	3/3
pvr1	CA4-111	5/5	3/3	3/3
pvr1	CA4-242	5/5	3/3	3/3
Pvr1$^+$	RNOIY104	5/5	3/3	3/3
Pvr1$^+$	RNOIY180	5/5	3/3	3/3
Pvr1$^+$	RNOIY186	5/5	4/4	3/3
Pvr1$^+$	RNOIY191	5/5	3/3	3/3
Pvr1$^+$	RNOIY203	4/4	3/3	3/3

Table 4.3 Continued

GUS	GUS7	NS	NS	4/4
GUS	GUS32	4/4	5/5	3/3
GUS	GUS37	5/5	4/4	3/3
GUS	GUS40	NS	NS	4/4
GUS	GUS46	3/3	4/4	3/3
GUS	GUS53	5/5	5/5	NS
GUS	GUS64	NS	NS	3/3
GUS	GUS66	4/4	4/4	4/4
GUS	GUS68	NS	NS	3/3
GUS	GUS76	NS	NS	4/4
GUS	GUS82	NS	NS	2/2
Non-transgenic	RBIDA-1	21/21	13/13	9/9
Non-transgenic	RBIDA-2	6/6	6/6	6/6

[a]Data are number of plants which tested positive for PVY by double-antibody sandwich enzyme-linked immunosorbant assay. *pvr1*[2,], *GUS* and non-transgenics (RBIDA-1) were sampled at 61dpi PVY^{O} 61dpi, 51dpi $PVY^{N\text{-}Wi}$, and 31dpi PVY^{NTN}. *Pvr1*[+], *pvr1* and non-transgenics (RBIDA-2) were sampled at 41dpi PVY^{O}, 42dpi $PVY^{N\text{-}Wi}$ and 46dpi PVY^{NTN}. All infected *pvr1*[2] lines as well as *Pvr1*[+], *pvr1*, *GUS* and non-transgenic plants displayed mild mosaic symptoms for PVY^{NTN} and yellow mosaic and leaf deformation symptoms for PVY^{O} and $PVY^{N\text{-}Wi}$. Samples listed as mock were uninoculated controls. NS were not included in the screen. Two uninoculated non-transgenic plants were also sampled as controls for the *pvr1*, *Pvr1*[+] screen for each of the three viruses; all had abs values at or near zero (data not shown).

[b,c]PVY^{O} and $PVY^{N\text{-}Wi}$ symptoms: Symptom type and severity were similar in plants inoculated with $PVY^{N\text{-}Wi}$ and PVY^{O}. Symptoms included mosaic and leaf deformation (Figure 4.1B). Non-trangenic plants, *GUS*, *pvr1* and *pvr1*[2] transgenic lines which tested positive by ELISA for PVY infection displayed symptoms which were similar in type and severity.

[d]PVY^{NTN} symptoms: PVY^{NTN} induced only minor mosaic symptoms. As PVY^{O} and $PVY^{N\text{-}Wi}$, PVY^{NTN} induced similar symptoms in all positive samples. Plants were infected under the same conditions; however, the PVY^{NTN} evaluation was completed several months prior to the PVY^{O} and $PVY^{N\text{-}Wi}$ screens. The variation in visible symptoms severity could possibly be attributed to variation in day length, temperature variations, et

Table 4.4. *pvr1*^{2}, *Pvr1*$^+$ and non-transgenic tuber PVY infectivity test, second transformation.

Genotype	Line	PVY^{Oa}		PVY$^{N\text{-}Wi\ a}$		PVY$^{NTN\ a}$	
		Plants Infected	Tubers Infected	Plants Infected	Tubers Infected	Plants Infected	Tubers Infected
Non-transgenic	Mock RBIDA	0/2	0/2	0/2	0/2	0/2	0/2
Pvr1$^+$	Mock RNOIY	0/5	0/2	0/2	0/2	0/2	0/2
pvr1^2	Mock ED3	0/13	0/2	0/2	0/2	0/2	0/2
pvr1^2	ED3-24	0/5	0/15	0/3	0/7	0/3	0/10
pvr1^2	ED3-25	0/5	0/12	0/3	0/6	0/3	0/8
pvr1^2	ED3-40	0/5	0/12	0/3	0/9	0/3	0/10
pvr1^2	ED3-44	0/5	0/11	0/3	0/9	0/3	0/10
pvr1^2	ED3-52	0/5	0/10	0/3	0/7	0/3	0/8
pvr1^2	ED3-85	4/4	NS	3/3	NS	3/3	NS
pvr1^2	ED3-86	0/5	0/14	0/3	0/6	0/3	0/9
pvr1^2	ED3-95	4/4	NS	3/3	NS	3/3	NS
pvr1^2	ED3-97	4/4	NS	3/3	NS	3/3	NS
pvr1^2	ED3-98	4/5	NS	3/3	NS	3/3	NS
pvr1^2	ED3-110	4/4	NS	3/3	NS	3/3	NS
pvr1^2	ED3-111	0/5	0/2	0/3	0/2	1/3	0/5
pvr1^2	ED3-112	0/3	0/4	0/3	0/3	0/2	0/4
Pvr1$^+$	RNOIY104	5/5	NS	3/3	NS	3/3	9/9
Pvr1$^+$	RNOIY186	5/5	NS	4/4	8/8	3/3	NS
Pvr1$^+$	RNOIY191	5/5	4/4	3/3	NS	3/3	NS
Non-transgenic	RBIDA	6/6	4/4	6/6	10/10	6/6	10/10

[a]Data is number of plants which tested positive for PVY by ELISA. *Pvr1*+, *pvr1*^{2} and non-transgenics (RBIDA) were sampled at 41dpi for PVY^O, 42dpi for PVY$^{N\text{-}Wi}$ and 46dpi for PVY^{NTN}. Samples listed as mock were uninoculated controls. Infectivity data for *pvr1*^{2} lines 24, 25, 40, 44 and 52 represents the second screen for these lines; previous data is listed in Table 4.3. All other screen data is repeated from Table 4.3.

[b]For the PVY^{NTN} and PVY$^{N\text{-}Wi}$ tests, all tubers were sprouted from three plants of transgenic lines 24, 25, 40, 44, 52, and 86. For the same six lines, tubers were sprouted from all plants included in the PVY^O test, between four to five plants. At least two tubers per plant were sprouted per plant. A limited number of tubers were harvested for *pvr1*^{2} lines 111 and 112; tubers from one plant were harvested from the PVY$^{N\text{-}Wi}$ test for both 111 and 112, tubers from three 112 plants and one 111 plant were sprouted from the PVY^O screen, tubers from three 111 plants and two 112 plants were harvested from the PVY^{NTN} screen. All plants sprouted from *pvr1*^{2} tubers were virus free. Tubers were harvested from three *Pvr1*$^+$ plants from line 191 from the PVY^O screen, three plants of *Pvr1*$^+$ line 186 from the PVY$^{N\text{-}Wi}$ test, and from three plants of *Pvr1*$^+$ line 104 for the PVY^{NTN} test. Tubers from two non-transgenic plants were harvested from the PVY^O screen and five non-transgenic plants from the PVY^{NTN} and PVY$^{N\text{-}Wi}$ screens. All *Pvr1*$^+$ and non-transgenic tubers sprouted PVY infected plants

Upon completion of the infectivity tests, plants were sprouted from tubers of inoculated plants to test for PVY by ELISA (Table 4.4). For the PVY^{NTN} and PVY^{N-Wi} tests, tubers were sprouted from three plants of *pvr1*^{2} transgenic lines 24, 25, 40, 44, 52 and 86 included in the each of the two infectivity tests. For the PVY^{O} test, tubers from the same six lines were spouted from all plants included in the test, between four to five plants. For all three infectivity tests, at least two tubers were sprouted and tested by ELISA per plant. A limited number of tubers were also sprouted from *pvr1*^{2} resistant line 112, as well as susceptible *pvr1*^{2} line 111; because tubers from these lines sprouted up to a week later than other lines making sufficient tissue unavailable at sampling time. Tubers from one 111 plant and one 112 plant were harvested from the PVY^{N-Wi} test, tubers from three 112 plants and one 111 plant were sprouted from the PVY^{O} screen and tubers from three 111 plants and two 112 plants were harvested from the PVY^{NTN} screen. During the infectivity test, one 111 plant became infected with PVY^{NTN}, plant 111-1. Two tubers were produced by plant 111-1 and both were sprouted; they both tested negative for virus accumulation. In fact, all plants sprouted from *pvr1*^{2} tubers were virus free; ELISA absorbance values were similar to plants sprouted from uninfected control tubers. In contrast, tubers harvested from three *Pvr1*$^+$ lines 104, 180 and 191, as well as several non-transgenic plants all tested positive for PVY. Tubers were harvested from three *Pvr1*$^+$ plants from line 191 from the PVY^{O} test, three plants of *Pvr1*$^+$ line 186 from the PVY^{N-Wi} test, and from three plants of *Pvr1*$^+$ line 104 for the PVY^{NTN} test. Tubers from two non-transgenic plants were harvested from the PVY^{O} test and five non-transgenic plants from the PVY^{NTN} and PVY^{N-Wi} tests.

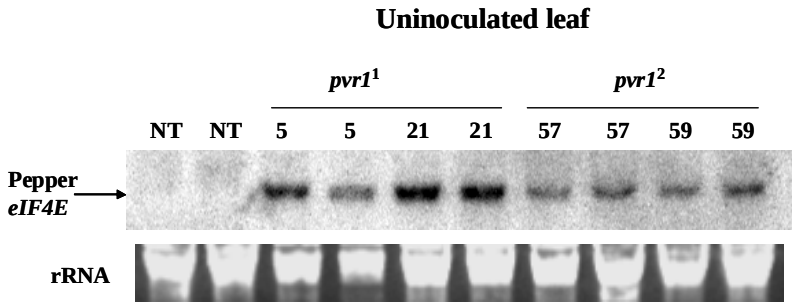
While the majority of transgenic material included in this study appeared identical to non-transgenic plants, all three *pvr1*^{2} transgenic lines from the first transformation, as well as seven *pvr1*^{2} lines from the second transformation grew

abnormally compared to non-transgenic plants. These plants were stunted and produced cupped leaves. Interestingly, all three abnormal *pvr1*^{2} lines from the first transformation were resistant to all strains of the virus, while the seven abnormal *pvr1*^{2} lines from the second transformation became infected with PVY: lines 29, 85, 56, 60, 61, 76 and 85. Two *GUS* and one *pvr1* line from the second transformation also exhibited this phenotype. The remaining transgenic material did not show this phenotype; they appeared identical to non-transgenic plants.

Resistant *pvr1*^{2} transgenic potato lines show high level of Capsicum *eIF4E*.

Northern analysis to determine RNA expression of the transgene was conducted on transgenic and non-transgenic plants from both transformation experiments. Varying levels of *pvr1*^{2} and *pvr1*^{1} transgene mRNA were detected in virus infected and uninoculated transformed plants (Figure 4.2A, lanes 3-10 and Figure 4.2B, lanes 3-8), but endogenous eIF4E mRNA was not detected in virus infected or uninoculated non-transgenic plants (Figure 4.2A, lanes 1-2 and Figure 4.2B, lanes 1-2). Two *pvr1*^{1} lines, 5 and 21, show *eIF4E* mRNA levels that are consistently higher in three separate Northern blots than the non-transgenics plants where expression was barely discernible from empty wells on the blot. *pvr1*^{2} lines 57 and 59 were confirmed in four separate Northern blots; each blot included multiple sample replicates of each line and each time the same results were obtained. All lines included in the study were included in one blot and the same result was obtained for all transgenic lines. Additionally, transgene mRNA levels were independent of inoculation. Non-transgenic, *pvr1*^{1} and *pvr1*^{2} inoculated leaves were sampled three days after inoculation with PVY^O, PVY$^{N\text{-}Wi}$ and PVY^{NTN} (Figure 4.2B). Inoculation appeared to have no effect on the level of *pvr1* mRNA in both transgenic and non-transgenic plants when compared to uninoculated mRNA expression (Figure 4.2A and 2B).

A.



B.

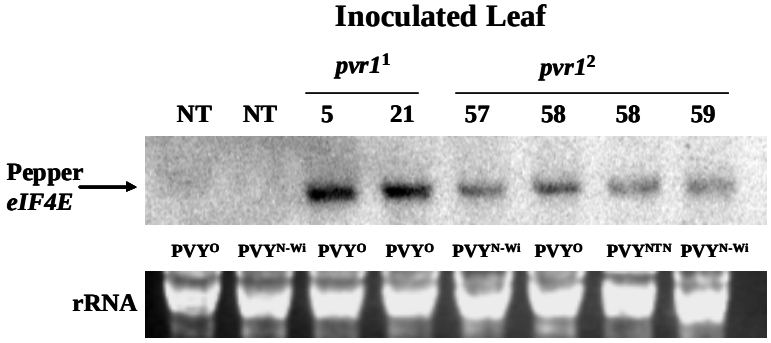


Figure 4.2. *eIF4E* RNA expression in transgenic plants, preliminary transformation. Total RNA was isolated to test for *eIF4E* accumulation using radiolabeled pepper *eIF4E* cDNA as a probe. RNA was extracted from both inoculated and uninoculated T_0 *pvr1*[1]and *pvr1*[2] transgenics, as well as non-transgenic plants.

A.Uninoculated leaf tissue samples. NT (non-transgenic), *pvr1*[1]: 5 and 21, *pvr1*[2]: 57 and 59.

B. 3dpi inoculated leaf tissue samples. NT (non-transgenic potato) - PVY^{O} and $PVY^{N\text{-}Wi}$, *pvr1*[1]: 5 - PVY^{O}, 21 - PVY^{OZ}**,** *pvr1*[2]: 57 - $PVY^{N\text{-}Wi}$, 58 - PVY^{O} and PVY^{NTN}, 59 - $PVY^{N\text{-}Wi}$.

pvr1^2 transgenic lines produced from the second transformation experiment were also analyzed for *pvr1*^{2} mRNA expression by Northern blot (Figure 4.3). Fifteen resistant and nine susceptible *pvr1*^{2} lines were included; RNA samples from each line were included except for resistant line 86 and susceptible lines 85, 95, 97, 98, 110 and 111. Of the 15 transgenic samples, *pvr1*^{2} expression was not detected in one sample, line 61, which appeared identical to the non-transgenic and *GUS* samples also included in the blot (Figure 4.3). Unlike all other samples included in the study, line 61 leaf tissue was of poor quality at sampling time which could have been the cause for this result. Other than this sample, Northern analysis shows pepper *pvr1*^{2} mRNA expression was high in all *pvr1*^{2} transgenic samples. eIF4E mRNA was not detected in the non-transgenic and *GUS* transgenic samples which could have been due to the sequence dissimilarity to potato eIF4E mRNA, or the low level of expression of the endogenous gene compared to transgenic expression.

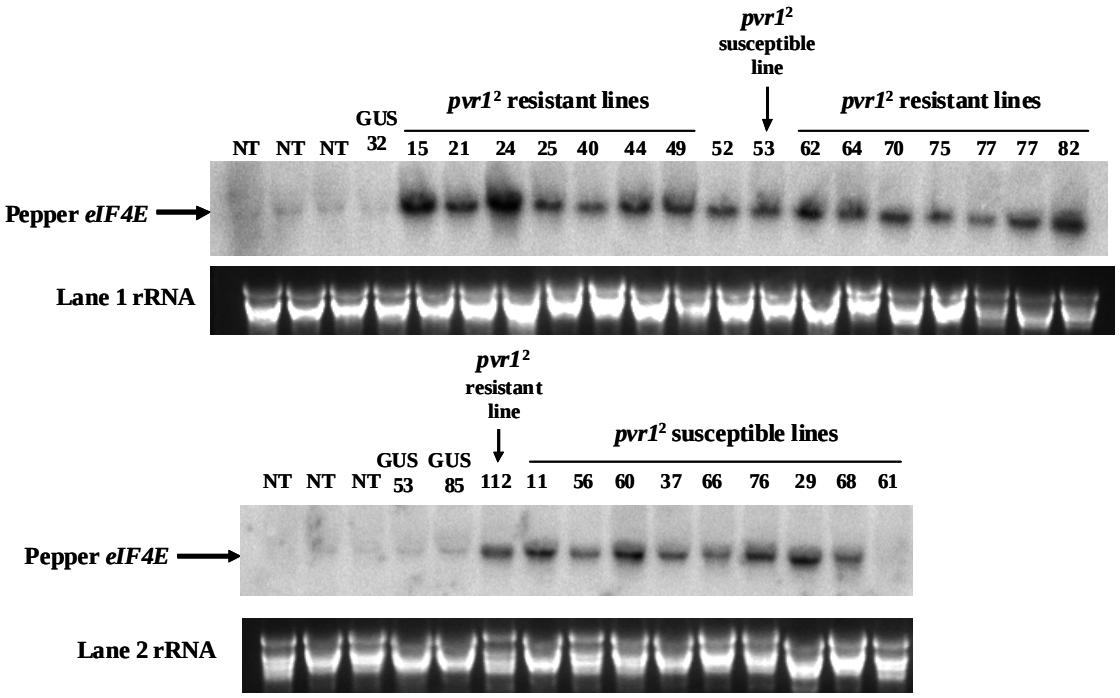


Figure 4.3. ***eIF4E*** **RNA blot using *pvr1*^{2}-pepper *eIF4E* probe, second transformation**. eIF4E blot shows elevated levels of pepper *eIF4E* RNA in transgenic *pvr1*^{2} lines, not in non-transgenic samples or in *GUS* transgenic lines. Total RNA was isolated to test for *eIF4E* accumulation using radiolabeled pepper cDNA as a probe. All lane one *pvr1*^{2} samples were isolated from plants of lines resistant to all strains of PVY screened. Lane two *pvr1*^{2} samples were isolated from plants which had at least one plant from their lines become infected with at least one PVY strain, except for line *pvr1*^{2}112 which was resistant to all three strains. With the exception of the sample 15 in lane two, susceptible and resistant lines show nearly equivalent *eIF4E* RNA expression. Lane 1 samples: NT (RBIDA), NT, (RBIDA), NT, (RBIDA), GUS32, *pvr1*^{2} lines: 15, 21, 24, 25, 40, 44, 49, 52, 53, 62, 64, 70, 75, 77, 77 and 82. Lane 2 samples: NT (RBIDA), NT (RBIDA), NT (RBIDA), GUS53, GUS85, *pvr1*^{2} lines: 112, 11, 56, 60, 37, 66, 76, 29, 68 and 61

To better understand the composition of the *eIF4E* mRNA pool, RT-PCR using *eIF4E* primers which amplify both pepper and potato *eIF4E* followed by sequence analysis were completed on three *pvr1*^{2} resistant transgenic lines, as well as two non-transgenic plants and one *GUS* transgenic line (Figure 4.4). A total of 31 clones from three independent resistant lines were sequenced, 10 each from *pvr1*^{2} lines 15 and 25, and 11 from *pvr1*^{2} line 21. Additionally, eight clones were sequenced from a *GUS* plant, and 21 clones from two non-transgenic plants. All 31 clones sequenced from the *pvr1*^{2} lines were identical to pepper *pvr1*^{2} coding sequence. The sequences of clones from the non-transgenic and *GUS* plants all represented one of three *eIF4E* potato alleles which are aligned in Figure 4.4 with pepper *pvr1*^{2} eIF4E. From this analysis, one potato *eIF4E* allele appeared to be predominant, however, due to small sample size conclusions could not be drawn on relative abundance of the three alleles. Potato *eIF4E* alleles are 89% homologous at the DNA level to pepper *eIF4E* (Figure 4.4).

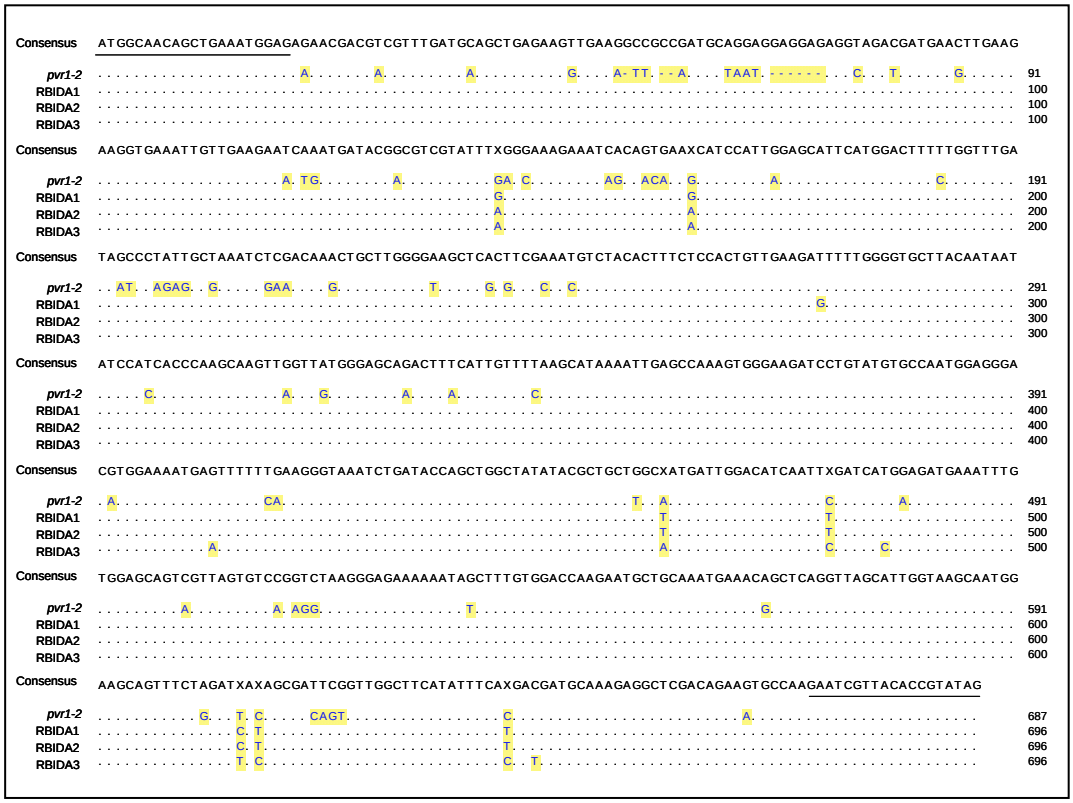


Figure 4.4. Sequence alignment of *eIF4E* cDNA cloning from transgenic and non-transgenic potatoes. RNA was isolated for *eIF4E* cDNA synthesis from *pvr1*^{2} resistant lines 15, 21 and 25 as well as from two non-transgenic plants and one *GUS* transgenic line; all transgenics were developed in the second transformation. *eIF4E* cloned from non-transgenics and the *GUS* line indicates there are three potato *eIF4E* alleles present while only transgenic pepper *pvr1*^{2} was isolated from resistant transgenic lines. Dots represent consensus sequence, dashes in the *pvr1*^{2} sequence represent nucleotides present only in the RBIDA potato sequence missing sequence in the alignment with the RBIDA alleles. Forward and reverse primer sequences are underlined in the consensus sequence at the used for cDNA synthesis and cloning are underlined in the consensus sequence.

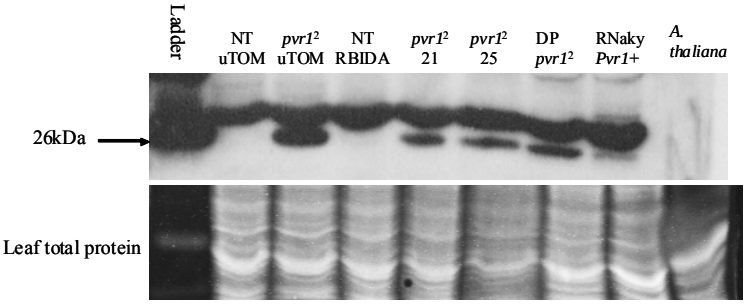
Immunoblot analysis using pepper eIF4E antibodies bound pepper eIF4E protein in transgenic *pvr1*^{2} potato plants and non-transgenic pepper plants, but not in non-transgenic potato plants (Figure 4.5A). Our transgenic samples clearly show a 26kDa band, the expected size of pepper eIF4E, present for *pvr1*^{2} transgenic resistant lines 21 and 25 (Figure 4.5A). This result was replicated in six blots; all six contained line 25, and three of the six also contained line 21 (data not shown). The 26kDa pepper eIF4E band was also observed for *pvr1*^{2} transgenic tomato (MicroTom) as well as non-transgenic pepper lines RNaky (*Pvr1*$^+$) and Dempsey (*pvr1*^{2}). This protein is clearly not present in non-transgenic tomato (MicroTom) or non-transgenic potato (RBIDA). The *pvr1*^{2} transgenic potato plants that were sampled for these experiments were not the same plants that were inoculated for the PVY infectivity test. Another immunoblot was completed to determine the level of *pvr1*^{2} transgenic protein in the plants which became infected with PVY. Figure 4.5B shows that transgenic eIF4E protein was not detected in susceptible *pvr1*^{2} plants, but was detected in resistant *pvr1*^{2} plants. This immunoblot included leaf tissue harvested from susceptible plants from lines 37, 61 and 66. While eIF4E transgenic protein appeared to be either inconsistent or absent in susceptible lines, eIF4E in resistant lines such as 21 and 25 was consistent as evidenced by replicated immunoblot analysis and plant resistance. Because samples included in the Western blot in 5B had been in a -80°C freezer for fourteen months, we included control samples to test for eIF4E degradation during storage. Two pepper samples and one transgenic tomato sample were included as controls: one sample had been in the same freezer for six months, and the other sample had been in the freezer for one day (Figure 4.5B). eIF4E was no longer detected in the pepper sample which had been in storage for six months, but was detected in freshly harvested tissue. A protein of slightly lower molecular weight than eIF4E was detected in the frozen pepper sample, however, in addition to the size difference, this protein could not have

been pepper eIF4E given it was also detected in non-transgenic potato sample. Unlike the pepper sample, storage did not seem to have an effect on the detection of eIF4E protein in transgenic potato and tomato plants. Transgenic protein was still detected in the *pvr1*^{2} tomato sample and in resistant *pvr1*^{2} transgenic potato samples; however, the protein was not detected in susceptible potato plants. We frequently found the level of eIF4E protein to be lower in pepper samples compared to the transgenic samples (Figure 4.5). Storage could have also degraded eIF4E protein in the transgenic samples, but this degradation was clearly apparent in the pepper sample due to the comparatively smaller amount of the eIF4E protein present.

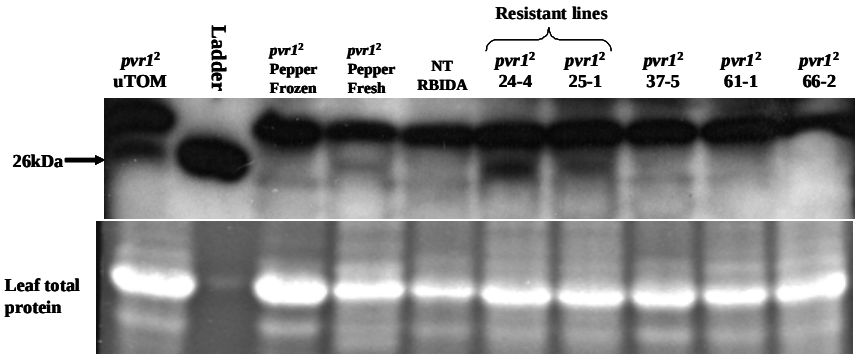
Figure 4.5. eIF4E immunoblot shows PVY resistant *pvr1*^{2} transgenic potato plants produce pvr1^2 protein.
A. 26kDa pepper eIF4E protein is present only in pepper and transgenic potato and tomato samples. Total protein was isolated from equal amounts of leaf tissue from non-transgenic and transgenic plants. Non-transgenic plants included: non-transgenic potato (NT Russet Burbank cv. RBIDA), tomato variety MicroTom (uTOM), pepper lines RNaky (*Pvr1*$^+$) and Dempsey (*pvr1*^{2}/*pvr1*^{2}), and *Arabidopsis thaliana*. Transgenic samples included: PVY resistant transgenic potato lines from the second transformation 21 and 25, as well as *pvr1*^{2} transgenic tomato (uTOM). Top image is the immunoblot using pepper eIF4E antibody, lower is SDS-PAGE SYPRO Ruby stained gel of leaf total protein. Lanes in both immunoblot and gel from left: 1 – ladder, 2 – non-transgenic (NT) tomato (uTOM) 3 – *pvr1*^{2} transgenic tomato (uTOM), 4 – non-transgenic (NT) potato (RBIDA) 5 – *pvr1*^{2} transgenic potato line 21, 6 – *pvr1*^{2} transgenic potato line 25, 7 – non-transgenic pepper Dempsey (*pvr1*^{2}/*pvr1*^{2}) 8 – non-transgenic pepper RNaky (*Pvr1*$^+$) 9 – non-transgenic *Arabidopsis thaliana*.

B. 26kDa pepper eIF4E protein is present only in pepper, transgenic tomato and resistant transgenic potato samples. Total protein was isolated from *pvr1*^{2} transgenic potato and non-transgenic (NT RBIDA) potato leaves inoculated with PVY$^{N\text{-}Wi}$ or PVY^{OZ} at 70 dpi. All *pvr1*^{2} potato samples included in this blot were developed during the second potato transformation experiment. Leaf tissue samples harvested from non-transgenic potato and *pvr1*^{2} transgenic lines were kept in -80°C storage for 14 months before this Western blot was completed. Pepper and *pvr1*^{2} transgenic tomato samples were kept in the -80°C freezer for six months prior to the completion of this Western blot. The pepper frozen sample shows degradation of the eIF4E protein has occurred after six months in the freezer compared to the fresh pepper sample. There is no evidence of degradation in transgenic tomato, which was harvested at the same time, or resistant transgenic potato samples, which were harvested eight months prior to the pepper and transgenic tomato (uTOM) samples. Top image is the immunoblot, lower is SDS-PAGE SYPRO Ruby stained gel of leaf total protein. Lanes in both immunoblot and gel from left: 1 – *pvr1*^{2} transgenic tomato (uTOM) after four months in the -80°C freezer, 2 – ladder, 3 – non-transgenic *pvr1*^{2} pepper Dempsey after four months in -80°C freezer, 4 – non-transgenic Russet Burbank inoculated with PVY$^{N\text{-}Wi}$, 5 - non-transgenic pepper Dempsey (*pvr1*^{2}) after one day in -80°C freezer, 6 – resistant *pvr1*^{2} transgenic plant 24-4 inoculated with PVY$^{N\text{-}Wi}$, 7 – resistant *pvr1*^{2} transgenic plant 25-1 inoculated with PVY$^{N\text{-}Wi}$, 8 – susceptible *pvr1*^{2} transgenic plant 37-5 inoculated with PVY$^{N\text{-}Wi}$, 9 – susceptible *pvr1*^{2} transgenic plant 61-1 inoculated with PVY^{OZ}, 10 – susceptible *pvr1*^{2} transgenic plant 66-2 inoculated with PVY$^{N\text{-}Wi}$.

A.


Ladder
NT uTOM
pvr1² uTOM
NT RBIDA
pvr1² 21
pvr1² 25
DP pvr1²
RNaky Pvr1+
A. thaliana
26kDa
Leaf total protein

B.


Resistant lines
pvr1² uTOM
Ladder
pvr1² Pepper Frozen
pvr1² Pepper Fresh
NT RBIDA
pvr1² 24-4
pvr1² 25-1
pvr1² 37-5
pvr1² 61-1
pvr1² 66-2
26kDa
Leaf total protein

Discussion

Overexpression of pepper-*pvr1*^{2} in potato conferred resistance to multiple PVY strains.

This study transformed the popular potato cultivar Russet Burbank with a pepper resistance gene *pvr1*^{2}, an eIF4E homolog, to confer PVY resistance. Overexpression of *pvr1*^{2} in potato conferred the same resistance spectra observed in *pvr1*^{2} pepper plants. Replicated experiments show half of all *pvr1*^{2} transgenic potato lines produced were extremely resistant to all three of the PVY strains screened: PVY$^{N\text{-}Wi}$, PVY^{O}, and PVY^{NTN}. Except for line 112, which based on our limited sample size, produced tubers which sprouted slower than non-transgenic plants, these lines were just as vigorous as the non-transgenic plants and did not display defects in vegetative or tuber growth and development. All resistant transgenic lines from the second transformation did not display the aberrant phenotype observed in several lines included in this study. We believe somaclonal variation, which is frequently reported in transgenic experiments, was the cause of this phenotypic dissimilarity (Davidson 2002; Heeres et al. 2002; Meiyalaghan et al. 2006; Phillips 1994).

The infectivity screen was stringent enough that not a single escape was allowed; all *pvr1*, *Pvr1*$^+$, *GUS* and non-transgenic plants became infected. Among 24 *pvr1*^{2} lines tested for all three strains, 15 displayed resistance against all PVY strains, while at least one plant became infected from each of the remaining nine lines. Additionally, at least one plant became infected from an additional eight *pvr1*^{2} lines which were only screened for one or two strains. Only five lines encoding the *pvr1*^{1} gene were tested for this study and the results were inconsistent. Screening more *pvr1*^{1} lines may be necessary to identify a line that can produce a consistent phenotype. Only with sufficient lines would we be able to conclude this gene functions against the virus. This is supported by the result from the *pvr1*^{2} infectivity test.

Virus free tubers are crucial for the control of viruses such as PVY since tubers are planted by potato farmers for propagation. It was therefore essential tubers produced during the inoculation tests were sprouted and tested for virus infection to determine the ability for the transgene to control virus accumulation in all parts of the plant not only the leaves. The results show that all the *pvr1*^{2} tubers we tested from the infectivity test produced virus free plants. All tubers sprouted and tested from non-transgenic and *Pvr1*$^+$ transgenic plants included in the infectivity test sprouted PVY infected plants, demonstrating the high stringency of the infectivity assay. An additional field experiment is planned to determine if the transgene is able to control the virus in the presence of PVY inoculum and the aphid vector as it did in our greenhouse inoculation studies. If resistance is maintained under these conditions, the *pvr1*^{2} gene could potentially be used to transform any potato cultivar, particularly the asymptomatic cultivars, such as Shepody and Russet Norkotah, whose latent PVY symptoms have cost the potato industry millions of dollars (Brown 2009, personal communication; Hane and Hamm 1999). Furthermore, it is possible that transgenic expression of the *pvr1*^{2} gene in potato provides resistance against other viruses, since *pvr1*^{2} confers resistance not only to PVY, but also to *Tobacco etch virus* and *Pepper mottle virus* in pepper (Kang et al. 2005; Yeam et al. 2007). *pvr1*^{2} transgenic lines should be tested for resistance to other important potato viruses such as *Potato virus A* (PVA) and *Potato virus X* (PVX) which also adversely impact the industry with crop losses as well as costs due to clean seed production. Like PVY, PVA has a VPg which has been identified as the virulence determinant in *Nicandra physaloides* (Rajamaki and Valkonen 1999). PVX does not have a VPg, but it does have a 5' M7Gppp cap (Sonenberg 1978). *Cucumber mosaic virus*, which also has a cap, has been shown to be effectively controlled by eIF4E knock-out mutations in *Arabidopsis thaliana*

(Yoshii et al. 2004). These studies suggest PVX and PVA could also be controlled using the *pvr1*^{2} transgene.

Because PVY strains which currently infect potato were our focus, we did not correlate PVY infectivity to previous data in pepper PVY infectivity data which includes PVY-0 and PVY-1, and PVY-1,2 because sequence data are not available for these isolates (Kyle and Palloix 1997). It remains unknown if PVY isolate PVY-1,2, which was previously reported to infect *pvr1*^{2} (Kyle and Palloix 1997), is a problem for the potato industry. From our work, it is clear that the *pvr1*^{2} gene, both in pepper and in transgenic potato, is highly effective against the PVY strains currently infecting potato. Moury et al. (2004) identified the PVY VPg domain essential for interaction with *pvr1*^{2}. If a strain of the virus was capable of overcoming *pvr1*^{2} mediated resistance, the VPg of that strain, as well as the VPg of the three strains included in this study, should be cloned and sequenced. Studies have shown that VPg - eIF4E interaction correlates with infectivity (Bruun-Rasmussen et al. 2007; Kang et al. 2005; Kuhne et al. 2003; Leonard et al. 2000; Moury et al. 2004; Yeam et al. 2007), therefore, engineering eIF4E transgenes which eliminate this interaction could provide effective means of virus control.

Transgene expression analysis

We believe the resistance observed in transgenic *pvr1*^{2} potatoes is conferred by a dominant negative mechanism where transgenic eIF4E overwhelms the expression of the endogenous potato eIF4E. Northern analysis indicated that *pvr1*^{2} eIF4E mRNA levels were high in all but one of the *pvr1*^{2} samples; even samples from susceptible lines showed high levels of eIF4E transgenic RNA (Figure 4.3). However, samples included in all Northern blots were not included in the inoculation tests, even those samples of inoculated leaves were not the same plants which were tested by ELISA. Therefore, our Northern data indicates only that it was possible for each transgenic

line to express high levels of transgenic eIF4E RNA and do not represent the level of transgenic RNA in infected transgenic plants. Northern blot analysis using tissue from plants which became infected during the infectivity test could indicate eIF4E RNA levels were lowered or negligible compared to resistant plants. In addition to Northern blot analysis, the only *eIF4E* cDNA cloned and sequenced from three resistant *pvr1*^{2} transgenic potato lines was pepper *eIF4E*; no potato *eIF4E* was sequenced during cloning. On the other hand, three potato *eIF4E* alleles were sequenced from non-transgenic potato and *GUS* transgenic lines. Potato allele sequences are aligned with the *pvr1*^{2} sequence in Figure 4.4. None of these three potato alleles were identified in the transgenic *pvr1*^{2} samples. This result clearly indicates the predominant form of *eIF4E* present in transgenic plants is that of pepper *eIF4E*. In addition to RNA analysis, all immunoblot experiments showed resistant *pvr1*^{2} lines contained pepper eIF4E protein (Figure 4.5). eIF4E protein was only identified in resistant plants and was not identified in susceptible plants, providing proof that the presence of the eIF4E protein correlates with resistance (Figure 4.5B).

pvr1^2 line variation

In previous transgenic potato projects encoding genes for pathogen resistance, very few transgenic lines of the total developed were resistant to the targeted pathogen (Solomon-Blackburn 2001b), and considerable variations in plant phenotype have been reported (Heeres et al. 2002). An example of this is clearly illustrated by Monsanto's transgenic potato lines expressing the PVY and *Potato virus X* coat proteins to try to achieve resistance to both viruses (Lawson et al. 1990). Out of the sixteen lines produced, just one line showed complete resistance to both viruses (Kaniewski et al. 1990; Lawson et al. 1990). Transgenic studies have shown transgene expression can be unstable and vary by organ and tissue stage (Tennant et al. 2001), particularly when the gene is overexpressed with the 35S promoter which we used in

this study (Davidson 2002; Pauk et al. 1955). Additionally, studies have shown that transgene expression can increase with plant development. For this study, plants were inoculated at the earliest possible stage of development for the infectivity tests. A study in transgenic rapeseed, also using the 35S promoter, showed that the transgene was much more active in older tissue rather than in meristematic buds (Pauk et al. 1955). This was further supported by another study in papaya, also using the 35S promoter, which found older plants were resistant to certain virus isolates which were able to infect younger plants of the same genotype (Tennant et al. 2001). We did not inoculate older plants, which could have shown a reduction in susceptibility. We also only sampled very young leaves for RNA and protein expression analysis so gene expression in older leaves is unknown. An additional Western blot of the same susceptible *pvr1*^{2} plants from the infectivity test using older leaf tissue rather than young tissue as we used for analysis in Figure 4.5B, may have shown the presence of transgenic protein.

Transgene multicopy number has also been associated with an overall reduction in phenotype activity (Flavell 1994; Hobbs, Warkentin, and DeLong 1993). Our results show that the level of transgenic protein in some lines is inconsistent, and it is possible that this observation could be associated with increased copy number. Copy number was not determined for any transgenic plants used in this study. Southern blot analysis may provide a possible explanation for observed susceptibility. In addition to copy number, studies have shown that flanking host DNA can adversely affect introduced sequences. Transgene modification or association of the DNA with chromatin proteins prior to integration have also been cited as possible explanations (Peach and Velten 1991). Methylation is another frequent side effect of transformation which can sometimes cause undesired variations in plant phenotype (Phillips 1994). Studies in transgenic petunia have shown have shown that 35S promoter methylation,

which can occur at anytime during development and can effect isolated parts of the plant, caused unstable transgene expression (Linn et al. 1990). Researchers found methylation can occur on single branches and not others, and can occur early or late in development (Meyer et al. 1992). Methylation of the 35S promoter could provide a possible explanation for the phenotype variation observed in this study. For the infectivity screens, at least three leaves at the plant apex were inoculated per plant. If the gene was not stably expressed in each of these leaves, perhaps due to methylation, the virus could have infected that leaf and spread to any new leaves which were not expressing the gene. Another possible reason for differences in phenotype could be due to post-translational modification. This has been the subject of review in recombinant protein expression studies and includes modifications due to glycosylation, degradation or folding and assembly (Ann R. Kusnadi 1997). Because we have not looked at the transgenic protein present in all the susceptible transgenic lines, we do not know if the level of protein will be lower as we showed in Figure 4.5B, or if the susceptibility was due to some type of post-translation modification. Therefore we do not know if the protein has been modified in some way which would allow eIF4E to interact with VPg. As we have shown in the past, slight modifications in eIF4E can determine infectivity (Kang et al. 2005; Yeam et al. 2007).

Concluding remarks

pvr1^2 mediated PVY resistance would benefit the potato industry in several ways. Most importantly, our results show that over half of the transgenic lines were resistant to all three of the predominant PVY strains identified in cultivated potato fields. In an industry dominated by very few varieties which are often susceptible to PVY, transgenic resistance would allow growers to produce their favorite varieties which maintain the plant and tuber phenotype they expect. Transgenic expression did not appear to have an adverse effect on Russet Burbank plant growth and development.

Future field studies of the material will determine if what we observed in the greenhouse is consistent with what we see in the field. Additionally, PVY resistance may decrease the amount of insecticides growers spray which are used as a means to control aphid populations in potato cultivation (Guenthner et al. 1999; Radcliffe and Ragsdale 2002). While insecticides are not particularly effective against the control of virus spread by aphids, particularly for non-persistently transmitted viruses such as PVY (Perring, Gruenhagen, and Farrar 1999; Radcliffe and Ragsdale 2002), certain applications such as synthetic pyrethroids have been shown to increase growth and yield of plants infected with potyviruses (Thomas M. Perring and Farrar 1993) and have been shown to reduce PVY transmission (Gibson 1982). These transgenics would diminish the need for such products which have been shown to have a damaging impact on human health, as well as on the environment (Pimentel 1992). Studies have found that there is a decrease in the impact of transgenic plants on the environment versus classically bred crops due to an overall decrease in the pesticide usage (Kleter 2007).

Alleles of the *pvr1* gene have successfully conferred resistance in both tomato and potato (Kang et al. 2007). Using this dominant negative strategy, any plant gene could potentially be transferred across sexual hybridization boundaries to disrupt the activity of a wild type protein. In our system, we have shown this strategy even works with a recessive gene. This strategy provides a great resource since certain pathogens are more destructive in some plant species compared to others. For example, overexpression of an eIF4E mutant in the stone fruits could provide much needed control the industry has been searching for against *Plum pox virus*. Furthermore, an endogenous eIF4E allele from any cultivar currently ravaged by potyviruses could be mutated and transgenically expressed to confer resistance. This strategy could not only

be an effective means of virus control, but consumer surveys suggest these products would enjoy the greatest amount of consumer acceptance (Lusk 2002).

Materials and Methods

Plant materials and viral cultures

Pepper lines which differ at the *pvr1* locus were used in infectivity studies as well expression analysis. *C. annuum* $Pvr1^{+}$ line NuMex RNaky (RNaky), $pvr1^{1}/pvr1^{1}$ line Yolo Y, $pvr1^{2}/pvr1^{2}$ line Dempsey and *pvr1*/*pvr1* breeding line 5502 were obtained from Asgrow Seed Co. (San Juan Bautista, CA) except 'Dempsey' which was provided by M. Deom, University of Georgia, Athens, GA, USA.

Solanum tuberosum cultivar Russet Burbank was used for all *pvr1* transformations, infectivity studies and transgenic expression analysis. Two different Russet Burbank clones were transformed, RB and RBIDA. *In vitro*-plantlets used for the first transformation, the RB transformation, were obtained from Sandra Austin-Phillips at the University of Wisconsin-Madison Biotechnology Center, where the transformation was completed. The second transformation used Russet Burbank clone RBIDA which was received from Deb Baer at the North Dakota State Seed Department, Fargo, ND. The RBIDA transformation was carried out by the Joyce Van Eck lab at the Boyce Thompson Institute for Plant Research, Ithaca, NY.

PVY strains used in this study were previously identified in a survey of US cultivated potato: $PVY^{O\text{-}ME11}$, an isolate of the ordinary strain PVY^{O}, as well recombinant strains $PVY^{N\text{-}Wi}$ and PVY^{NTN} which are recombinants of PVY^{O} and necrotic strain PVY^{N} (Blanco-Urgoiti et al. 1998; Glais, Tribodet, and Kerlan 2002). Viruses used for infectivity tests include PVY strains: PVY^{N} (252), $PVY^{Wi\text{-}N}$ (209), PVY^{O} isolates PVY^{OZ} and $PVY^{O\text{-}ME11}$ isolates, and PVY^{NTN} (312) were obtained from Stewart Gray (Cornell University, Ithaca, NY, USA). All PVY strains were maintained on *Nicotiana tabacum* cv. 'NN' and were inoculated onto potato at 15-20

days post inoculation (dpi). The same PVY^{N-Wi} and PVY^{NTN} isolates were used to infect pepper and potato, however, a different PVY^{O} isolate, PVY^{O-ME11} was used in the pepper inoculations because PVY^{OZ} inconsistently infected susceptible control RNaky. Out of five PVY^{O} isolates, PVY^{O-ME11} was the only isolate identified which consistently infected RNaky. In addition to PVY^{OZ} and PVY^{O-ME11}, PVY^{O-MT7}, $PVY^{O-NY557}$, PVY^{O-MN20} were also tested.

Plant Material and Transformation

The first transformation consisted of a 2-day co-cultivation with the *Agrobacterium tumefaciens* (LBA4404) followed by selection on 25 mg/l kanamycin using the regeneration protocol previously described (Cearley 1997). This transformation produced a total of five $pvr1^{1}$ events, three $pvr1^{2}$ events and five $Pvr1^{+}$ events. All events were screened for resistance to at least one strain of PVY. eIF4E cloning was completed as described previously (Kang et al. 2005). Constructs were constructed using vector pBI121 and full length eIF4E as previously described (Kang et al. 2007).

For the second transformation experiment, approximately 100 stem internode segments of 0.5 - 1 cm in length were excised from 6-week-old *in vitro*-grown plants, and incubated in 50 ml of *Agrobacterium tumefaciens* strain LBA4404 containing either pG-CHa/s or p35S-CHa/s for 10 min. They were blotted on sterile filter paper and transferred to a callus induction medium (CIM) which contained Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) (Caisson Laboratories, Sugar City, ID), 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.4 mg/l thiamine, 0.25 mg/l folic acid, 0.05 mg/l D-biotin, 100 mg/l myo-inositol, 30 g/l sucrose (grade II; PhytoTechnology Laboratories, Shawnee Mission, KS), 1 mg/l benzyladenine (BA), 2 mg/l naphthaleneacetic acid (NAA) (added after autoclaving), and 6 g/l Agar/Agar (SIGMA, St. Louis, MO). The pH of the medium was adjusted to 5.6 before the

addition of the Agar/Agar. One hundred explants were cultured per 100 x 20 mm Petri plate. All cultures were maintained at 24 $\pm$ 1C under a photoperiod of 16h (light)/8 h (dark) at 74 μE $m^{-2}s^{-1}$.

After 48 hours, the internode segments were transferred to selective plant regeneration medium containing MS salts, 1 mg/l thiamine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 100 mg/l myoinositol, 30 g/l sucrose, 0.1 mg/l indole-3-acetic acid (IAA) (added after autoclaving), 3.4 mg/l zeatin riboside (added after autoclaving), 500 mg/l carbenicillin (Phytotechnology Laboratories) (added after autoclaving), 75 mg/l kanamycin monosulfate (added after autoclaving), and 8 g/l Agar/Agar. The pH of the medium was adjusted to 5.9 before the addition of the Agar/Agar. Twenty-five internode segments were cultured per 100 x 20 mm Petri plate and the plates were sealed with 0.5 inch Micropore Tape (3M HealthCare, St. Paul, MN). Explants were transferred weekly for 1 month to fresh selective plant regeneration medium, then every 10 - 14 days after the 1-month period. All cultures were maintained at 24 $\pm$ 1C under a photoperiod of 16 h (light)/8 h (dark) at 74 μE $m^{-2}s^{-1}$.

When regenerants were approximately 0.5 - 1 cm in length, they were excised and transferred to selective rooting medium which contained MS salts, 0.4 mg/l thiamine, 0.1 mg/l myoinositol, 500 mg/l carbenicillin (added after autoclaving) and 75 mg/l kanamycin (added after autoclaving). Five regenerants were cultured per GA7 Magenta box (Phytotechnology Laboratories). For extended maintenance of the transgenic lines, the shoot tip from each plant was transferred to rooting medium without kanamycin and carbenicillin in test tubes.

Four *GUS*, five *Pvr1*$^{+}$ events, five *pvr1* events, and 24 *pvr1*^{2} lines were screened for resistance to three strains of the virus. An additional seven *GUS* and eight *pvr1*^{2} lines were screened for resistance to one or more stains of the virus.

Virus inoculation and resistance test

Pepper seed was germinated on water saturated sterilized germination paper at 30°C until seed sprouted. Germinated seed were sown in plastic trays containing Cornell Mix potting medium. Potato cuttings were made as described previously (Chapman et al., 1961) dipped in Hormex Rooting Powder No.1 and potted in plastic trays filled with 1:5 mix of fine grade vermiculite to Cornell potting medium. Plants were allowed to root on a mist bench for 7-10 days at 21ºC before they were put in the greenhouse and allowed to acclimate for approximately 7 days. Once visible new growth appeared cuttings were transplanted into approximately 15cm pots so they could produce tubers which we could later harvest and screen for PVY infection. Plants were maintained in a temperature-controlled Guterman greenhouse, Cornell University, Ithaca NY, USA. Ambient air temperatures in the greenhouse were maintained at 23ºC day/20ºC night. Watering procedures were carried out routinely by greenhouse personnel.

Potato and pepper plants were inoculated with three PVY strains: PVY^{O}, $PVY^{N\text{-}Wi}$ and PVY^{NTN}. Pepper plants and the preliminary transformation were also inoculated with PVY^{N}. All potato plants were inoculated with the PVY^{OZ} isolate of PVY^{O}, while pepper plants were inoculated with $PVY^{O\text{-}ME11}$ because PVY^{OZ} was not consistently infectious in pepper. Plants were arranged in rows by genotype. Separate benches divided the different strains in the same greenhouse, including the uninoculated plants which were always included and kept on a separate bench in the same greenhouse. Uninoculated plants were sampled along with the virus infected plants to test for presence of PVY to ensure starting material was virus free. Inoculum was prepared by grinding systemically infected tobacco variety 'NN' tissue in 50 mM potassium phosphate buffer, pH 7.5 (approximately 1 g tissue: 20 mL buffer). Mortars and pestles were chilled at -20 C prior to use and then maintained on ice throughout

the inoculation process. Pepper plants were inoculated at approximately the six leaf stage. Virus inoculum was rub inoculated onto at least two of the youngest leaves big enough to inoculate, approximately $3cm^2$ in size, after the leaves were dusted with Carborundum. Once the new cuttings had developed at least 6 new leaves at least $3cm^2$ in size, at least three of them were inoculated. These leaves developed approximately three to four weeks after the cuttings had been transplanted to the greenhouse. For both pepper and potato, inoculum was allowed to dry and was reapplied once to the same leaves approximately one hour after the first inoculation. Plants were monitored routinely for the appearance of symptoms.

For ELISA screening, 0.3-0.4g of fresh pepper or RBIDA potato leaf tissue was squeezed in 2mL General Extract Buffer (Agdia). Pepper was sampled at 39-42dpi and potato plants was sampled between 40-61dpi. Only uninoculated leaves were sample for ELISA; inoculated leaves showed inconsistent infection in the initial screening process at 7 and 14dpi (data not shown), therefore, only non-inoculated leaves were sampled. Before harvesting leaves for ELISA, the entire plant was scanned for PVY symptoms (yellow mosaic and leaf deformation). Leaves which displayed PVY symptoms were always sampled when identified. For all RBIDA transgenic screens, at least 5 leaves were included per plant. These leaves were weighed and broken up until their combined weight was 0.3-0.4g. Agdia PVY Pathoscreen Kits (PSA 200001) were used for all screens according to Agdia protocol (both coat and conjugate antibody were included). Sampled leaf tissue was pressed through a motorized leaf squeezer along with 2mL General Extract Buffer and sap was collected (Agdia Buffer GEB).

All RB transformants were screened differently than RBIDA transformants. For the first ELISA screen of RB transformations, two leaf discs were sampled per plant, each approximately 1cm in diameter. This tissue was ground and liquid nitrogen

and weighed approximately 200mg per sample. ELISA was also carried out using the aforementioned Agdia PVY Pathoscreen kit (PSA 200001). PVY^{N} was sampled at 22dpi in the first screen and was not included in the second screen. PVY^{O} was sampled at 22dpi in the first screen, and 44dpi for the second screen. PVY^{NTN} was screened at 21dpi and 45dpi, for the first and second screens, and $PVY^{N\text{-}Wi}$ was sampled at 43dpi and 41dpi for the first and second screens. The second RB ELISA screens was carried out identical to the RBIDA protocol 41-45dpi.

For all ELISA screens, average absorbance values at wavelength 405nm of the uninoculated plants were at or near zero, samples were therefore considered positive if the absorbance values reached 0.09 or above.

Tubers were put into cold storage for approximately four months, left at room temperature for approximately 2 weeks and subsequently treated with Rindite in the case of the RB plants or carbon disulfide in the case of the RBIDA plants to induce sprouting. Tubers were then green sprouted. For the first *pvr1*[1] screen, tubers were planted in the greenhouse and leaves were sampled once the plants produced large enough leaves for sampling approximately one month after transplant. For all the RBIDA lines in this study, tubers were also allowed to green sprout. As soon as tubers produced approximately 0.3g of leaves (approximately 3 mature leaves), the tissue was harvested for ELISA.

Immunoblot analysis

Two leaf discs each approximately 1cm in diameter were flash frozen and ground in liquid nitrogen. The powder was suspended in 100ul GTEN extraction buffer: 10% glycerol, 25mM Tris (pH 7.5), 1mM EDTA, 150mM NaCl, 1% protease inhibitor (Sigma Aldrich) and 10mM DTT, and 200uL SDS-PAGE loading buffer: 12.5% 1M Tris-Cl (pH 6.8), 10% glycerol, 1% SDS, 2.5ml 2-Mercaptoethanol and 0.125% bromophenol blue . Samples were boiled 10 minutes and immediately spun at

12,0000g for 10min. 20uL was loaded onto a 12% SDS-PAGE Tris-HCL gel (BioRad 161-1102). Proteins were transferred to PVDF membrane and blocked overnight in 5% non-fat milk resuspended in 1XPBS at 4°C. Anti-eIF4E (Spring Valley Laboratories, Inc.) produced as previously described (Kang et al. 2005), was added for one hour at room temperature. Membrane was washed 2x5min in 1XPBS, 2x5min in 1XPBS 5% Tween 20 and 2x5min 1XPBS. The secondary antibody, anti-rabbit (GE Healthcare), was added to 5% non-fat milk resuspended in 1XPBS. Proteins were detected using ECL Plus Western Blotting Detection kit (GE Healthcare) according to manufacture's instructions on Kodak BioMax XAR film. The blot in Figure 4.5A is representative of a result which was obtained six times, the blot in Figure 4.5B was not repeated.

DNA extraction

Potato genomic DNA was extracted using standard CTAB protocol as described previously (Doyle 1987). All transgenics were confirmed by PCR using *eIF4E* specific primers in all cases except *GUS* which were confirmed via PCR using *nptII* primers. 35SF 5'-GCTCCTACAAATGCCATCATTGCG-3' and eIF4E SacR 5'-GAGCTCCTATACGGTGTAACGATT-3' primers were used for eIF4E transgenic lines. nptII F 5'-GGTGGAGAGGCTATTCGGC-3' and nptII R 5'-CGGGAGCGGCGATACCGTAAAGC-3' primers confirmed GUS transgenic lines.

RNA extraction, RNA blotting and cDNA synthesis

Total RNA was extracted from two flash frozen leaf discs approximately 1cm in diameter using RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. 14uL of RNA was loaded per sample and run according to RNeasy recommendations. RNA was transferred to Hybond-XL membrane according to manufacture's Northern blotting recommendations by neutral transfer with 10 x SSC. Transgenic *pvr1*^{2} genomic DNA served as template for *eIF4E* PCR using eIF4E

primers: eIF4E-F 5'-CCCGGGATGCAACAGCTGAAATGGA-3' and eIF4E-R 5'-GAGCTCCTATACGGTGTAACGATTC-3' were purified using Qiaquik gel purification kit (Qiagen). eIF4E primers were designed using pepper eIF4E cDNA sequence. 50ng of purified *eIF4E* PCR product was labeled with ^{32}P dCTP using Amersham Ready-To-Go DNA Labelling Beads (-dCTP) and purified through MicroSpin^{TM} G-25 columns according to manufacturer's instructions. Membranes were hybridized in Ambion ULTRAhyb hybridization buffer according to manufactuer's instructions and placed on a phosphoimager and visualized 3-5 days depending on signal strength.

First strand cDNA was synthesized as described previously (Kang et al. 2005) using the same eIF4E-R primer listed above. *eIF4E* PCR products using the same primers listed above eIF4E-F and eIF4E-R, were purified using Qiaquik gel purification kit (Qiagen) and cloned into pTOPO TA Cloning System (Invitrogen). *eIF4E* was cloned from 3 *pvr1*^{2} resistant transgenic lines, 2 non-transgenic RBIDA plants and 1 GUS transgenic line. 32 *pvr1*^{2} clones were sequenced from the three *pvr1*^{2} lines and 29 clones were sequenced from the 2 RBIDA plants and the *GUS* plant. Sequences were aligned using Seqman software (DNASTAR Inc.) as described previously (Kang et al. 2005).

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CHAPTER 5

CONCLUSIONS

This thesis presents a series of studies that shed light on the determinants of plant-virus interactions. Virus stain or isolate variation can be due to reassortment, recombination or mutation and can occur in any region of the viral genome. My data suggest that at least in the interactions under investigation in this work, VPg variation is the key to understanding why certain viral stains are able to infect a previously resistant host. Our previous data (Kang et al. 2005; Yeam et al. 2007)indicated that interaction of VPg with eIF4E correlated with susceptibility in the *pvr1*-TEV pathosystem. The work included in these three chapters and Appendix One describes the evolution of our understanding of the *pvr1*-potyvirus pathosystem. In Chapter 2, I described the technique employed to identify VPg as the virulence determinant in the *pvr1*-TEV pathosystem identified in Chapter 3. The identification of the VPg as the specific domain required for susceptibility supports this interaction data and shows us that VPg determines infectivity. Since we identified VPg as the virulence determinant and we previously showed this interaction correlated with infectivity, we developed transgenic plants which aimed to knock out this interaction to confer resistance described in Chapter 4. We have shown that susceptible potato plants encoding the pepper *pvr1*^{2} resistance allele were resistant to all strains of PVY tested. Because the pepper eIF4E transgene was overexpressed using a constitutive promoter, we hypothesized that endogenous potato eIF4E was overwhelmed by pepper-encoded eIF4E in the cell. Future protein interaction studies to determine if the pvr1^2 and pvr1^1 resistance alleles interacted with PVY VPg should be completed. Based on infectivity data, we also hypothesized that PVY VPg would not interact with pvr1^2 or pvr1^1, but would interact with pvr1 and Pvr1$^+$. Because many mutated forms of eIF4E have been

identified, there is a huge potential for the application of this technique to confer resistance to a wide array of viruses. Additionally, this technique could potentially be used to combat resistance-breaking strains by using site directed mutatagenesis to specifically mutate eIF4E so as to inhibit VPg interaction and thus potentially confer a more stable form of resistance even in a highly divergent species.

Many factors in the translation initiation complex could be essential to initiate viral translation and/or replication in addition to eIF4E. Previously we have shown that the pepper pvr1 resistance alleles, pvr1, pvr1^1 and pvr1^2, can all still interact with arabidopsis eIF4G in yeast, even though they may have reduced cap binding ability (Kang et al. 2007; Yeam et al. 2007). In addition to eIF4E interaction, eIF4G interacts with initiation factors eIF4A and eIF3 as well as the poly(A)-binding protein (PABP) (Browning 1996; Korneeva et al. 2000; Le et al. 1997). Because eIF4E is the only factor overexpressed in the transgenic plants we developed, there is a limited amount of all the other initiation factors, such as eIF4G, in the cell. Previous eIF4E-eIF4G interaction data in yeast suggests that *pvr1^2*-encoded eIF4E can still interact with endogenous potato eIF4G, which would presumably maintain interaction with the other components of the complex, eIF4A, eIF3 and the PABP. Our eIF4E cloning results showed the amount of transgenic pepper eIF4E is much more abundant than endogenous potato eIF4E in transgenic plants. Therefore, we hypothesize other initiation factors would be bound to pepper eIF4E and would not be available to interact with endogenous potato eIF4E. This resistance mechanism could potentially work for any one of the factors in the translation initiation complex. Other factors could potentially be mutated and overexpressed to block interaction and potentially confer resistance. Two candidates are eIF4G (or isoform eIF(iso)4G) and the PABP which have been the focus of several studies. In addition to eIF4E based resistance, eIF4G and eIF(iso)4G mutants in *Arabidopsis thaliana* and rice have been found to

confer resistance to both *Cucumber mosaic virus* and *Rice yellow mottle virus*, respectively (Albar 2006; Yoshii et al. 2004). In rice, transformation with the eIF(iso)4G susceptible allele resulted in susceptibility in a previously resistant host (Albar 2006). eIF4G has been linked to potyviral translation via direct interaction with the potyviral internal ribosome entry site (IRES) which is responsible for cap-independent translation (Gallie and Browning 2001; Ray et al. 2006). Overexpression of a mutated eIF4G which could not interact with the IRES element could potentially confer resistance by interfering with translation. In a human virus, a rotavirus, it has been shown that a rotaviral RNA-binding protein binds eIF4G and subsequently takes the place of PABP on eIF4G (Piron 1998). A mutated form of eIF4G which would inhibit interaction with the RNA-binding protein could inhibit viral accumulation. In addition to eIF4G, overexpression of a mutated PABP could potentially confer resistance. PABP was found to interact with VPg *in planta* (Leonard et al. 2004), demonstrating that other protein-protein interactions may be essential in addition to eIF4E interaction with VPg. Additionally, the PABP from cucumber was shown to interact with the RNA-dependent RNA polymerase (RdRp) of *Zucchini yellow mosaic virus* in vitro, providing evidence for PABP involvement in viral replication (Wang, Ullah, and Grumet 2000). In poliovirus infection, human PABP interaction with both the viral poly(A)-tail and ribonucleoprotein complexes at the ends of the viral genome have been shown to be essential for viral replication (Herold and Andino 2001). Clearly eIF4E is just one of many proteins involved in viral translation/replication, demonstrating the potential for many different factors to be overexpressed to confer resistance.

Transgene overexpression strategies, as we used in Chapter 4, are frequently employed to confer resistance using constitutive promoters such as the 35S promoter. By overexpressing the mutated pepper eIF4E in potato, we were able to disrupt the

activity of wild type potato eIF4E in the virus infection cycle. This strategy is often referred to as a 'dominant negative' transgenic where a mutated gene is overexpressed to disrupt the activity of a wild type proteins (Herskowitz 1987). Both pathogen and plant genes have been overexpressed to confer resistance. Several studies have overexpressed mutated forms of viral genes to interfere with wild type viral gene function. Overexpression of a *Tomato yellow leaf curl virus* (TYLCV) replicase mutant conferred resistance by inhibiting transcription of the TYLCV C1 domain of the virus (Lucioli et al. 2003). Another study overexpressed a dysfunctional form of the *Tobacco mosaic virus* (TMV) movement protein which interfered with TMV movement and conferred resistance (Lapidot 1993; Malyshenko et al. 1993). In *Nicotiana benthamiana,* overexpression of a 29 amino acid peptide of unknown origin was found to interfere with the nucleocapsid protein of several tospoviruses (Rudolph, Schreier, and Uhrig 2003).

In addition to viral genes, plant genes have been constitutively expressed to confer resistance or tolerance as we showed in chapter 4. Many of these studies focused on genes which trigger a defense response (Cao, Li, and Dong 1998; Chen and Chen 2002; Deslandes et al. 2002; Friedrich et al. 2001; He et al. 2001; Li, Brader, and Palva 2004; Malnoy 2007; Marchive et al. 2007; Oldroyd and Staskawicz 1998; Park et al. 2001; Seo et al. 2006; Tang et al. 1999). In these studies, the transgene frequently turns on the salicylic acid or jasmonic acid defense pathways to stimulate the production of plant defense compounds such as salicylic acid, ethylene, and hydrogen peroxide. For example, a full length *Arabidopsis thaliana* resistance gene was overexpressed in a susceptible arabidopsis plant to confer resistance to a bacterial pathogen (Deslandes et al. 2002). Just as in our study, the gene was a recessive resistance gene which, when overexpressed, behaved as a dominant gene in transgenic plants. The resistance conferred in this case was salicylic acid dependent suggesting

similar signaling pathways to those controlled by resistance genes in a gene for gene interaction. Tolerance to *Rice tungro bacilliform virus* (RTBV) was conferred by overexpressing two plant transcription factors important for viral promoter activity (Dai et al. 2008). Researchers hypothesized that the overexpression of wild type transcription factors RF2a and RF2b, which are important for RTBV promoter activity, conferred tolerance to the virus because the factors, which were previously monopolized by the virus, became available for transcription of defense related genes (Dai et al. 2008). Additional research has focused on plant genes which inactivate a stage of the pathogen life cycle, rather than triggering a defense pathway. Overexpression of a ribosome inactivator gene from the cell walls of polkweed in both *N. benthamiana* and potato conferred some resistance to PVX and PVY (Lodge, Kaniewski, and Tumer 1993). Researchers hypothesized the gene functioned by inhibiting viral translation (Lodge, Kaniewski, and Tumer 1993). This paragraph needs something to tie it back to your work

Of all the studies identified in this literature search which encoded plant transgenes, our study was the only plant transgenic strategy which conferred complete resistance without triggering a defense pathway. Because consumer acceptance of plant transgenes is higher than acceptance of pathogen transgenes (Lusk 2002), transgenic strategies using plant transgenes should be the direction of transgenic development. Since three genera of plant viruses require eIF4E or eIF(iso)4E for the virus infection cycle (Robaglia and Caranta 2006), and plants from five plant families confer virus resistance by naturally occurring eIF4E or eIF(iso)4E mutants, transgenic strategies using these genes could have a broad impact to confer complete viral resistance worldwide (Kang et al. 2005; Kanyuka et al. 2005; Nicaise et al. 2003; Nieto et al. 2006; Ruffel et al. 2005; Ruffel et al. 2002; Stein et al. 2005).

Genetic engineering is obviously a very important tool to confer pathogen resistance in many plant systems. The technique has been extremely important given sexual incompatibility prevents plant breeders from introgressing genes from distantly related species. In addition to genetically modified organisms (GMOs), advancements in non-transgenic breeding methodologies such as marker assisted selection including precision breeding have greatly reduced the number of generations required to develop new varieties. Genes of interest can be selected early to make sure important traits such as disease resistance are maintained in the breeding population. Since resistant plants can be selected early, or in some cases even before they are planted based on DNA marker analysis of the seeds, these strategies can dramatically increase desirable gene frequencies in each field generation and can reduce the number of plants that have to grown. Precision breeding uses molecular markers to identify and track genetic regions responsible for favorable genetic traits to expedite breeding improved varieties (Stuber, Polacco, and Senior 1999). This was the strategy employed to create flood tolerant rice which allows rice to survive flooding preventing crop loss in adverse conditions (Xu et al. 2006). Like molecular markers, genetic engineering represents a tool available to plant breeders to improve crops. Crops are frequently genetically modified through chemically induced mutations. Approximately 2250 new plant varieties have been developed through mutation breeding methods, most commonly by radiation (Ahloowalia, Maluszynski, and Nichterlein 2004). Of the 2250, approximately 1575 were released directly, while the remaining varieties were included in breeding programs (Ahloowalia, Maluszynski, and Nichterlein 2004).

Public perception of agricultural biotechnology ranges from a belief that it can save agriculture or it could destroy the planet. Ironically, transgenics are currently being employed to detoxify pollution in the air, soil and water through a technique known as phytoremediation which utilizes transgenes to aide in environmental clean-

up (Macek, Macková, and Kás 2000; Macek et al. 2008; Meagher 2000). In addition, there are several advancements that genetic engineering has made to the to the biofuels industry, such as increasing energy yield by improving biomass cell wall deconstruction and decreasing toxins required for pretreatment (Chen and Dixon 2007; Himmel et al. 2007; Koslowsky 2008; Sticklen 2006). However, even with all the advances researchers have made in using transgenics to help the environment, transgenics do pose a real environmental risk as they must be contained and tested to prevent transgene flow and ensure safety. Transgenic contamination in native maize landraces in Oaxaca, Mexico has been reported (Quist and Chapela 2001). However, the validity of these results have been seriously questioned (Christou 2002), and the results could not be repeated in subsequent studies (Ortiz-Garcia et al. 2005). Nevertheless, the initial results provoked a government moratorium on GM planting which could be the reason the results were not repeatable in subsequent studies conducted by other laboratories (Marris 2005; Ortiz-Garcia et al. 2005). Even though this result was severely questioned, it sparked a backlash around the world because Mexico is the maize center of origin and contamination of the native varieties with transgenes could contaminate unique native species and detrimentally affect the earth's ecosystem.

Another important example of transgene contamination occurred with the release of StarLink corn by the Aventis Corporation. Starlink encodes the Cry9C protein making the plants toxic to the European corn borer as well as other insect pests (Lin 2003). StarLink was initially released only for use in animal feed and industrial uses due to concerns about possible allergenicity of the Cry9C protein (Lin 2003). This limited release was a grave mistake given the strict tolerance policy for unapproved transgenics in food products in the U.S., Japan and South Korea (Lin 2003) . Ultimately, Starlink cost the industry a great deal of money due to transgene

contamination in the human food supply. The contamination in corn shipments was determined to be between 5-10% during the 2000 – 2001 marketing year (Lin 2003), and the transgene was identified in several processed corn food products between 2000 and 2001 (Schmitz 2005). These products were subsequently taken off the market (Schmitz 2005). The appearance of StarLink in food products caused Japan to stop all corn imports from the U.S. (Schmitz 2005). The USDA was forced to start a program whereby growers were paid an additional 25 cents per bushel to ensure they would only feed the corn to their animals (Lin 2003). On top of crop losses, multi-million dollar settlements were awarded to consumers who claimed to have suffered allergic reactions after consuming processed corn products produced with StarLink corn, even though no physical evidence of an allergic reaction was ever demonstrated (Schmitz 2005). Other multimillion dollar lawsuits were awarded to growers who did not grow StarLink, but claimed they had lost income due to crop losses and property contamination (Schmitz 2005). Overall, StarLink cost growers, Aventis, the U.S. government and food processors such as Kraft Foods a great deal of money. It is unknown if the contamination occurred in the field or during storage and/or processing. (Lin 2003). It was unrealistic to believe the crop could have been separated from corn intended for human consumption given the same storage and processing facilities were used for the two crops (Lin 2003). Additionally, extremely sensitive testing procedures such as PCR were used for transgene detection (Lin 2003). Therefore, even if DNA samples were pooled together to expedite screening, just one transgenic seed in a pool could make the entire the pool positive. The StarLink disaster proved it is nearly impossible for grain crops to be kept separate in the grain marketing system due to additional costs associated with separation (Lin 2003).

While issues of gene flow are of potential importance, the risk transgenes have on human health need to be realistically questioned. To date, GMO crops have not had

an adverse effect on health, undoubtedly due to thorough safety testing requirements (Ronald 2008). On the other hand, conventionally bred crops, which are not subject to the same rigorous safety testing, have caused health problems. There are many naturally occurring toxins, or natural toxicants, which can be present in food. Transgenics suffer poor public opinion because their introduction was not accompanied by a consumer education campaign; instead there was a strong anti-GMO campaign started by environmental watch organizations such as Greenpeace. Education campaigns focused on communicating the safety of transgenic plants would undoubtedly show consumers that many fears surrounding these products are simply unfounded and the benefits of these crops including reduced pesticide usage and increased yield justify their continued regulated production. Genetic engineering represents a valuable tool for agriculture to produce enough food for an ever-growing world population. This work demonstrates plant transgene overexpression strategies can provide broad spectrum virus resistance in a divergent species, even against viruses which are responsible for significant crop losses. Future field trial experiments including *pvr1*^{2} transgenic potato lines developed in this study will hopefully demonstrate the PVY resistance is in fact durable in the field. Given PVY continues to cause crop losses in potato worldwide, this technology could reduce losses and provide growers with a means to grow their favorite varieties without worrying about PVY. There seems to be no limit to the usage of strategies such as this one to control potyvirus infection worldwide.

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APPENDIX 1

ABSPII: AGRICULTURAL BIOTECHNOLOGY SUPPORT PROJECT II, APPLICATION OF BIOTECHNOLOGY TO THE TOMATO VIRUS CRISIS IN WEST AFRICA

Establishment of regional capacity for screening virus resistant tomato germplasm in West Africa.

'Tomato leaf curl disease' refers to symptoms caused by *Begomoviruses* both in West Africa and elsewhere in the world. In West Africa, there are three tomato-infecting *Begomoviruses* that have been associated with the disease: *Tomato leaf curl Mali virus* (TLCMV), *Tomato yellow leaf curl Mali virus* (TYLCMV) and *Pepper yellow vein Mali virus* (PYVMV) (Zhou et al. 2008). These are each distinct virus species, but often occur in mixed infections (Gilbertson 2006, personal communication). Using PCR primers, ABSPII collaborators at the Gilbertson lab at UC Davis have determined all three viruses are present in the region. TLCMV was identified most often, but PYVMV was also common, and was the most common in Ghana (Gilbertson 2006, personal communication). TYLCMV was less common, but was frequently identified in Burkina Faso (Gilbertson 2006, personal communication). All three viruses are important, depending on location.

TLCMV, TYLCMV and PYVMV are distinct viral species from those previously identified in other parts of the world and not strains of *Tomato yellow leaf curl virus* (TYLCV). TYLCMV is a recombinant between TYLCV and other unknown *Begomoviruses* making a unique strain (Zhou et al. 2008). Therefore, tomato cultivars encoding some resistance to tomato leaf curl disease must be screened in West Africa to specifically identify sources of resistance.

The objective of this project was to establish capacity for *Begomovirus* resistant tomato germplasm screening in West Africa to identify sources of resistance which would stand up against West African viruses. Three years of trials were conducted. For the first year, over 40 tomato varieties obtained from seed companies and public institutions were collected with several sources of TYLCV resistance sources represented: TY172 based resistance (which includes at least 3 genes from *L. peruvianum*), TY-1 (one major gene with two modifier genes from *L. chilense*) and TY-2 (source which includes 2 epistatic genes from *L. hirsutum*). Seminis, Harris Moran, Enza Zaden, DeRuiter Seeds, Takii, Nunhems and Hazera, as well as the Volcani Institute, all donated seed for the project. Tomato lines were trialed by National Agricultural Research and Extension Institute (NARES) partners in Benin, Burkina Faso, Togo, Ghana, Mali, Niger and Senegal. In addition to seed collection, we worked very closely with our Mali based AVRDC collaborators to plan the NARES trials by providing plot designs as well as infectivity scoring materials.

A wide array of TYLCV resistance sources were represented in the first year's trial designed to select only those resistance sources which held up well against West African strains of the virus. Trials were conducted for three consecutive years. The first year testing was comprised of a preliminary trial which was not replicated. In the second year, another non-replicated preliminary trial was conducted including new lines, and an advanced trial of the 10 best lines from the previous season was conducted using a triple replicate randomized complete block design. The third year, an advanced trial with the best material from the previous year's preliminary trial was conducted. In addition, a multi-location trial was conducted with the four to six of the best varieties from the previous year's advanced trial which included three replicates per location in two ecologically distinct locations per country. A total of 115 varieties were trialed at least once, but some were only trialed in Mali. Approximately 70

varieties were trialed in replicated multi-location trials. In the first two years, the trials were conducted in Benin, Burkina Faso, Ghana, Mali, Niger, Senegal and Togo. In the third year, Burkina and Senegal didn't participate because of funding restrictions.

The West African Seed Alliance is trying to develop a small-scale seed distribution industry in West Africa to make begomovirus resistant tomato seed directly available to farmers. They are currently working with three companies that donated seed to the trials, De Ruiter, Enza Zaden and Seminis.

Production of transgenic virus resistant tomatoes

The goal of this breeding program was to create a virus resistant tomato which would be resistant to both *Begomovirues* and *Potyviruses*. *Potyvirus Pepper veinal mottle virus* has been shown to infect tomato in West Africa (Konate 1999). Previously, a virus survey of all pepper producing regions throughout Senegal, which includes seven locations throughout the country, showed that all pepper plants encoding the pepper *Potyvirus* resistance allele *pvr1* were resistant to *Pepper veinal mottle virus* (PVMV) (Mbaye 1999). Recently we showed that MicroTom plants overexpressing the *pvr1* gene were resistant to several strains of *Tobacco etch virus* (TEV) as well as *Pepper Mottle Virus* (Figure 1) (Kang et al. 2007). A population was therefore developed by crossing transgenic *S. lycopersicum* MicroTom containing the *pvr1* transgene with a Seminis TYLCV resistant hybrid, GemPride.

GemPride was selected for the breeding program because it was previously found to hold up well in West Africa against *Begomovirus* infection when it was trialed in Mali (Thera 2002). Additionally, it was included in the three years of trials in project 1 and was included in the set of four varieties that every participating country trialed during the multi-location trials in the third year of project 1. NARES partners conducting the trials found it was notably tomato leaf curl disease resistant and a good producer. In addition to TYLCV resistance, GemPride is also resistant to verticillium

wilt race 1 and fusarium wilt races 1 and 2 (Scott 2005). GemPride is an F1 hybrid with a large determinant plant which produces medium square fruit with extended set for processing or fresh market (Scott 2005).

MicroTom encodes the DWARF (d) gene making it a brassinosteroid mutant as well as a recessive miniature gene (Scott 2005, personal communication). The *pvr1* transgene, due to constitutive expression driven by the 35S promoter, behaves as a dominant gene. Transgene segregation ratios therefore followed the single dominant gene pattern of inheritance. This was illustrated by TEV infectivity data which confirmed F1 progeny (*pvr1* MicroTom by TEV susceptible GemPride) were resistant to TEV (Figure 2). In order to eliminate all dwarf traits and bring the F1 back to the GemPride phenotype, three backcross generations followed by three generations of selfing were completed. Backcross breeding was carried out as described previously (Briggs and Allard 1953). The breeding strategy is outlined below and diagramed in Figure 3.

Backcross Breeding Plan:

F1 and BC1F1: F1 seed was obtained from the initial cross: *pvr1* transgenic MicroTom (male) by GemPride (female). GemPride was the female parent for the entirety of this breeding program. Forty F1 plants confirmed by PCR for the *pvr1* transgene, which segregated in a 1:1 ratio, were planted along with 70 plants of the recurrent parent GemPride in Cornell greenhouses (Figure 4). Pollen from the 10 F1 plants most horticulturally similar to GemPride were selected and backcrossed to GemPride. At least 4500 seed were collected from this generation.

BC2F1: Approximately 4000 seed were planted in Ithaca greenhouses and confirmed transgenic by kanamycin resistance (Figure 4) (Weide, Koornneef, and Zabel 1989). Again, given that the *pvr1* transgene behaves as a dominant gene,

segregation would be expected to be approximately 1:1. Of the 4000 seedlings grown to approximately the six leaf stage in the greenhouse, 2296 plants were confirmed transgenic by kanamycin resistance and planted in Ithaca fields, summer, 2006. Seventy-two GemPride plants were planted alongside the BC1F1 plants so selections could be made more accurately. Three plant breeders separately evaluated the field to make selections which were based on plant likeness to the recurrent parent. Twenty-nine confirmed transgenic BC1F1 plants were selected based on superior horticultural characteristics such as fruit shape and size, number of fruit per plant and plant habit (Figure 5). These 29 plants were backcrossed to the recurrent parent; and a minimum of two fruit were produced per BC1F1 selection. Twenty-nine BC2F1 lines were obtained.

BC3F1: Twelve BC2F1 plants were planted for each of the 29 lines (348 plants) in Cornell greenhouses. Two confirmed transgenic plants from each of the 29 BC2F1 lines were transplanted to large pots to allow plants to fully mature so they could be evaluated based on the same horticultural characteristics: fruit shape and size, number of fruit per plant and plant habit before backcrossing to maintain only the most superior lines for the next generation. The lines were grown alongside the recurrent parent GemPride and some were found to be horticulturally superior to GemPride based on the aforementioned criteria. The best two plants from the 29 BC2F1 lines were selected to backcross to produce BC3F1 seed.

Brix BC3F1 and fruit images: Total soluble solids (brix) was determined on BC3F1 fruit using a hand-held refractometer as described previously (Tanksley and Hewitt 1988). Soluble solids was determined for four fruit from each plant and averaged. The four selected fruit were representative in size, shape, quality and color of all the fruit produced by the plant. Brix was also determined for GemPride and

tomatoes purchased from the local Ithaca grocery store for comparison. Fruit with the highest brix are shown in Figure 6, and listed in Table 1.

BC3F2: Twelve BC3F1 lines which had the highest brix were selected out of the original 29 lines to produce the BC3F2 generation. Statistically, one in 16 plants should be homozygous for the *pvr1* transgene (Hanson 1959). A minimum of 17 confirmed transgenic plants were therefore planted and manually self pollinated to insure adequate seed set. Three separate plant breeders phenotyped all the lines after the plants had set fruit. If a plant continued to maintain any of the dwarf MicroTom characteristics, the line was dropped. Two lines were dropped for this reason.

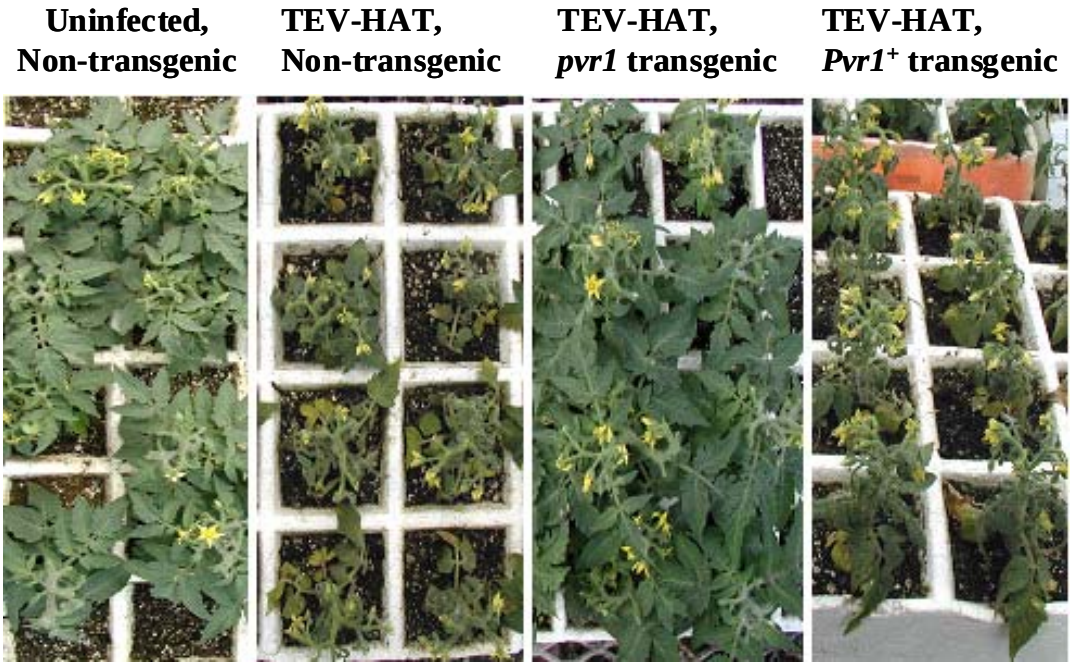
BC3F3: 50 BC3F3 seed were planted from each line and sprayed at the four to six leaf stage with kanamycin. Any BC3F3 line which showed absolutely no bleaching was confirmed homozygous for the transgene. Homozygous lines are listed in Table 2. This BC3F2 seed was sent to Bob Gilbertson to test for resistance to TYLCV (Table 3).

Final BC3F3 selections: Final selections of this breeding program are shown in Figure 6. Selections were based on homozygosity for the *pvr1* transgene, TYLCV resistance, brix and the aforementioned horticultural traits: fruit shape and size, number of fruit per plant and plant habit.

Conclusions and recommendations for future research

Mali has recently lifted the ban on transgenic cultivation (Shotkoski 2009, personal communication), and Burkina Faso is currently growing transgenic cotton. Pending approval from our West African collaborators, selected material will be sent to Mali for further evaluation and selection. Three backcross generations have created tomato lines which are nearly 95% back to the GemPride genotype. We originally thought several generations of intercrossing may be necessary to adapt the variety for

the region, but given the recurrent parent GemPride consistently performed well in all the countries which trialed the variety, these selected lines should be perform well in the region. This breeding material represents a great resource to combat multiple virus infections in West Africa and steps will hopefully be taken to make the material part of the West African tomato germplasm.



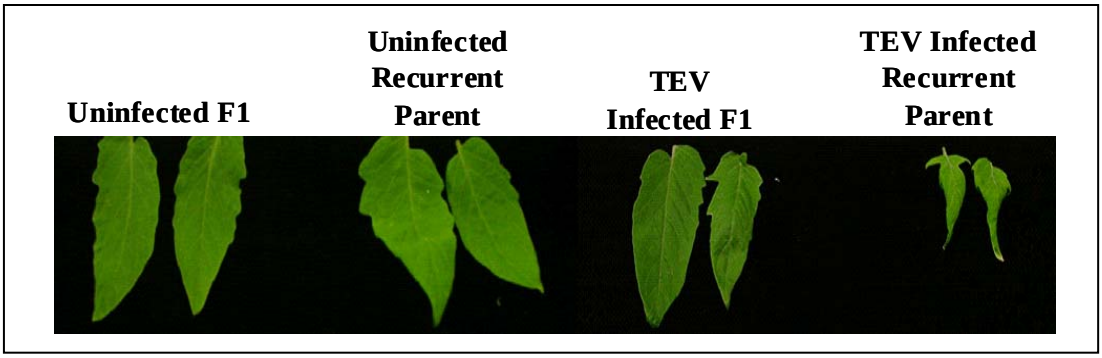
Appendix Figure 1. *pvr1* transgenic MicroTom is resistant to TEV-HAT. Our previous work confirmed the *pvr1* transgene conferred TEV resistance in susceptible MicroTom.(Kang et al. 2007).

Appendix Figure 2. F1 (*pvr1* transgenic MicroTom x GemPride) is extremely resistant to *Tobacco etch virus* isolate HAT (TEV-HAT).

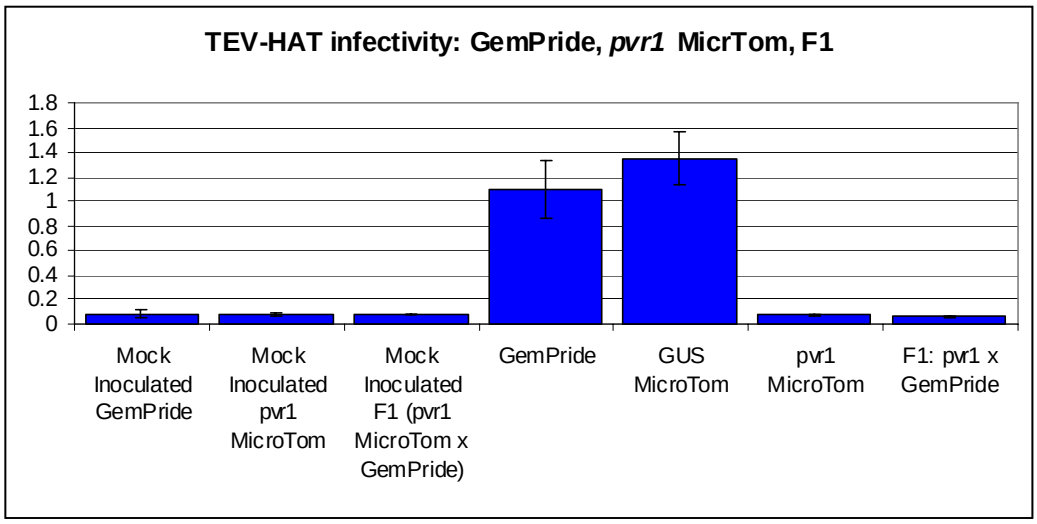
A. TEV-HAT induced symptoms: Recurrent parent GemPride displays symptoms characteristic of TEV infection such as leaf curling and leaf dwarfing approximately 15 days post inoculation. Below are uninocualted leaves from inoculated plants approximately 20dpi.

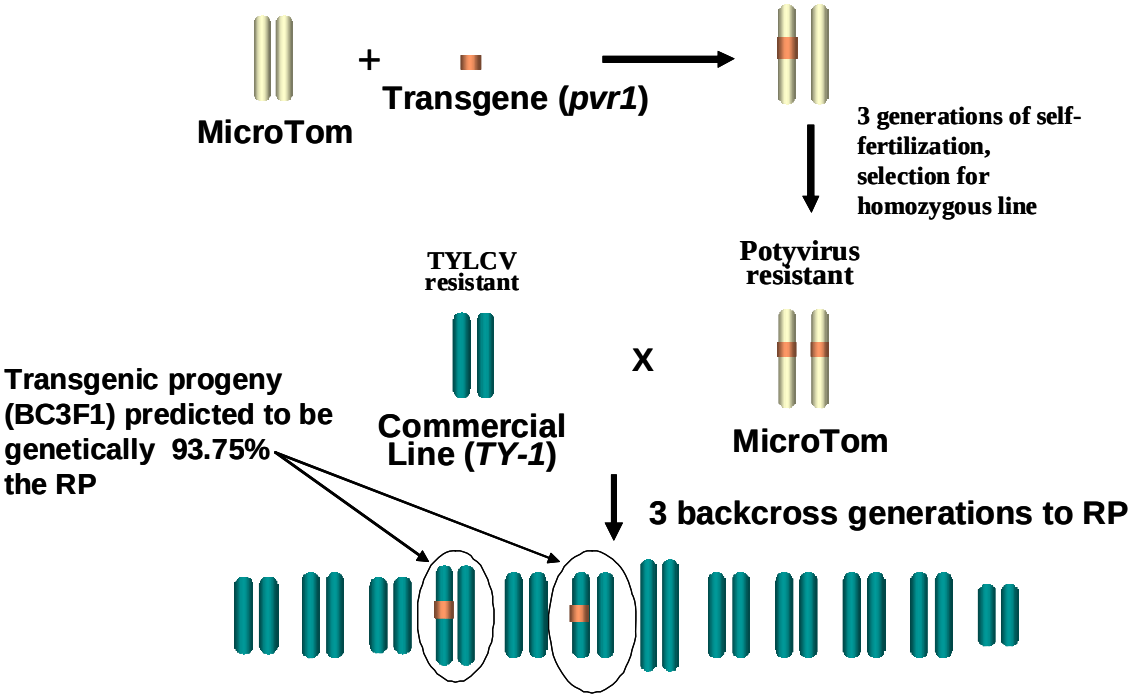
B. TEV-HAT ELISA results: Enzyme linked immunosorbant assay (ELISA) average absorbance values represent uninoculated leaf tissue sampled from 3-12 plants per genotype at 32 days post inoculation. ELISA absorbance values in F1 tomato progeny indicate plants are extremely resistant to TEV-HAT while GemPride is fully susceptible, along with transgenic MicroTom encoding the *GUS* reporter gene. Plants from five F1 lines were included and averaged. All samples had absorbance values similar to mock inoculated plants. Error bars represent standard error.

A.


Uninfected F1
Uninfected Recurrent Parent
TEV Infected F1
TEV Infected Recurrent Parent

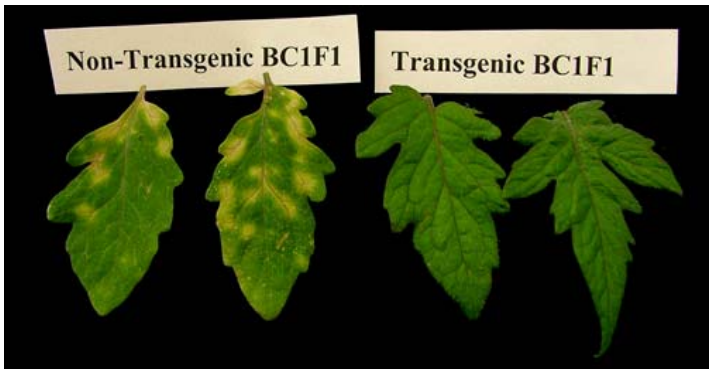
B.


TEV-HAT infectivity: GemPride, pvr1 MicrTom, F1
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mock Inoculated GemPride
Mock Inoculated pvr1 MicroTom
Mock Inoculated F1 (pvr1 MicroTom x GemPride)
GemPride
GUS MicroTom
pvr1 MicroTom
F1: pvr1 x GemPride

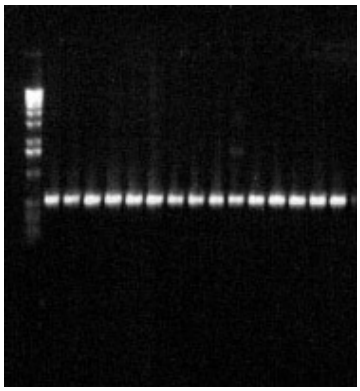


Appendix Figure 3. Transgenic Breeding Plan. A three generation backcross breeding program is diagramed below; GemPride is recurrent parent. This was followed by 3 generations of selfing to identify *pvr1* homozygotes.

Kanamycin bleaches non-transgenics



PCR confirms plants are transgenic



Appendix Figure 4. Transgene confirmation methods. Tomato progeny were selected using kanamycin antibiotic sprays. Plants were sprayed for three consecutive days with 300mg/L kanamycin solution to test for presence of the *nptII* gene, the selectable marker of the transformation vector which confers kanamycin resistance. Foliar bleach spots appeared on non-transgenic approximately three days after the last spray. For the F1 selection, unbleached plants were additionally genotyped for presence of the *pvr1* transgene by PCR using *pvr1* specific primers.

Appendix Figure 5. BC1F1 field season: Ithaca, NY. 2296 BC1F1 plants along with 72 GemPride plants in Ithaca fields, summer 2006. Selections were made based on plant habit, fruit shape, fruit size and the number of fruit per plant.

Appendix Table 1. BC3F1 fruit with highest total soluble solids (TSS) or brix.

Tomato line/Variety	Brix	se
Wegman's beefstake tomatoes	**4.13**	0.05
Wegman's vine ripened tomatoes	**4.10**	0.07
GemPride	**4.86**	0.13
7-1-3	**4.98**	0.04
18-1-1	**5.02**	0.02
21-1-1	**5.02**	0.02
1-2-10	**5.04**	0.02
29-2-1	**5.08**	0.03
11-2-1	**5.10**	0.10
9-2-2	**5.40**	0.04
8-1-2	**5.70**	0.18
17-1-2	**6.24**	0.15
Grape tomatoes	**7.50**	0.47

Two selections were made from each of the 29 lines and planted in Cornell greenhouses. Brix was determined for at least one of the two selections for each of the 29 lines. All selected lines had higher brix than the recurrent parent. Commercially available tomatoes were included for comparison. Brix values represent an average refractometer reading from four fruit per plant.

Appendix Table 2. Homozygous BC3F3 according to brix in descending order.

BC3F3 homozygous lines:
17-1-2-13
17-1-2-14
17-1-2-2
8-1-2-16
8-1-2-4
8-1-2-7
8-1-2-7
9-2-2-10
9-2-2-4
9-2-2-5
11-2-1-10
11-2-1-12
29-2-1-5
29-2-1-9
1-2-10-10
1-2-10-2
18-1-1-11
18-1-1-2
21-1-1-17
21-1-1-7
7-1-3-3
7-1-3-6

Appendix Table 3. BC3F3 TYLCV resistance screening of selected homozygous lines.

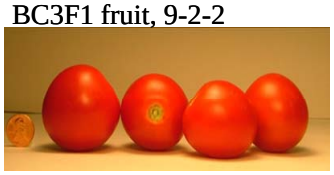
Resistant lines: A total of 6 lines were resistant to TYLCV. No symptoms in any of the inoculated plants
17-1-2-2, 9-2-2-5, 9-2-2-4, 9-2-2-10, 11-2-1-12, and 7-1-3-6
Moderately resistant: Six lines showed moderate resistant; plants showed a combination of no symptoms or very mild to mild symptoms
17-1-2-13, 29-2-1-5, 18-1-1-2, 18-1-1-11, 21-1-1-7 and 7-1-3-3
Susceptible lines: Nine lines were fully susceptible and showed strong symptoms
17-1-2-14, 8-1-2-7, 8-1-2-4, 8-1-2-16, 11-2-1-10, 29-2-1-9, 1-2-10-2, 1-2-10-10, and 21-1-1-17

The Gilbertson lab agroinoculated four seedlings of each line with TYLCV. One uninfected plant was maintained as a control. Four TYCLV resistant sister lines were identified.

BC3F1 fruit, 7-1-3

BC3F1 fruit, 11-2-1

BC3F1 fruit, 9-2-2



BC3F1 fruit, 17-1-2, some off-type, extremely blocky

GemPride, Plant 2

GemPride, Plant 1

Appendix Figure 6. BC3F1 fruit final selections. BC3F1 fruit from lines which made the final selection are pictured below. Lines were selected based on superior horticultural characteristics, homozygousity for the *pvr1* transgene and TYLCV resistance. Other than 17-1-2 which produced off-type and overly blocky fruit, all selections produced fruit similar to the GemPride.

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APPENDIX 2

MOLECULAR BIOLOGY FOR AGRICULTURAL RESEARCH APPLICATIONS

August 27th – 31st, 2007 – Bamako, Mali

Introduction

This document outlines a curriculum for an intensive hands-on workshop on molecular biological theory and techniques to be held in Bamako, Mali August 27th – 31st, 2007. The workshop will introduce the ABSPII West African NARES partners, who have been involved in conducting trials of begomovirus-resistant tomatoes, to applications of molecular plant genetics and pathology through integrated lectures and laboratory exercises. The goal of the workshop is to build regional intellectual capacity in West Africa for the continuation of ABSPII-related activities, including modern plant breeding, germplasm screening, and pathogen detection by scientists and practitioners in the region. Structured to acknowledge the conditions of the research programs in the participants' countries while simultaneously building expertise for future capacity expansion, the workshop's laboratory exercises cover techniques with minimal equipment requirements alongside the state-of-the-art approaches to transgene detection and pathogen identification

Course Details

Instructors

Jeff Gordon, graduate student, Cornell University. jsg54@cornell.edu

Kari Perez, graduate student, Cornell University. kwp6@cornell.edu

Location

Biotechnology Laboratory, University of Bamako, Mali

Dean of the Faculty of Science and Technology: Saliku Sanogo

Director of the Biotechnology Laboratory: Ousmane Koita

Onsite Coordinators

Dr. Issoufou Kollo Abdourhamane, Project Coordinator, AVRDC

Dr. Ousmane Cisse, University of Bamako

Dr. Youssouf Sanogo, University of Bamako

Course Outline:

The course will be conducted over a period of five days, with all days having both lecture and laboratory components. The following is a breakdown of the topics covered by day:

Section Topics:

1. Basic plant molecular biology and genetics

   Associated laboratory exercise: getting acquainted with the lab

2. Basic techniques for detecting, manipulating and identifying nucleic acids

   Associated laboratory exercise: DNA extraction

3. Genetic engineering and plant transformation

   Associated laboratory exercise: DNA detection – PCR and gel electrophoresis

4. Basic molecular plant pathology and disease resistance

   Associated laboratory exercise: DNA detection – Squash blots

5. Detection of proteins

   Associated laboratory exercise: Protein detection – ELISA and immunostrips

Day 1: Basic plant molecular biology and genetics

This section will serve as an introduction to the biological concepts necessary for subsequent sections of the workshop. Of course, it is impossible to teach all of plant molecular biology in one day, and therefore this section will be carefully designed to emphasize the aspects of plant molecular biology that are most relevant to the techniques covered in the workshop.

Topics:

- Introduction to the plant cell
- From DNA to protein – transcription and translation
- Cell cycle: Mitosis and meiosis
- Molecular genetics: Mendel meets DNA

Laboratory Exercise: Getting acquainted with the lab

Workshop participants may be unfamiliar with the basic tools, facilities, and safety procedures of molecular biology labs. This laboratory section will be used to ensure that participants are ready to begin conducting experiments by day 2.

- Tour of laboratory equipment and facilities
- Overview of laboratory safety regulations and protocols
- Pipetting practice

Day 2: Basic techniques for detecting, manipulating and identifying nucleic acids

Detection and manipulation of nucleic acids serve as the foundation of molecular genetics. In this section, workshop participants will learn about the basic toolkit available to molecular geneticists from a practical standpoint.

Topics:

- Theory and practice of DNA extraction
- Hybridization – using complementarity for specific detection
- Cutting and pasting – restriction enzymes and ligases
- Separation – gel electrophoresis
- Amplification – PCR, RT, and bacterial amplification
- Combining techniques for in-depth analysis – Mapping, cloning, and sequencing

Laboratory Exercise: DNA Extraction

Laboratory exercises for days 2 will focus on three different methods for extracting DNA from plant materials. Each method meets different scientific needs and is appropriate for different analyses:

- CTAB method – used to purify DNA from fresh leaf tissues in the laboratory. Is appropriate for most downstream applications.

- FTA cards – used for collecting samples in the field, and for long-term storage of DNA or RNA at room temperature. Can be used as templates for DNA or RNA amplification.

- Squash blots on nylon membranes – also used for collecting samples in the field. They are appropriate for detection of specific DNA sequences by hybridization.

Day 3: Genetic engineering and plant transformation

The lectures on day 3 will focus on how and why transgenics are made. Genetic engineering of plants can potentially offer solutions to agricultural problems in the developing world, from virus resistance to drought tolerance. It is also a major tool in modern molecular biology, and a capacity that is relevant to modern laboratory research even when no agricultural product is intended. This section will elucidate the basic processes involved in generating transgenic plants.

Topics:

- Why transgenics – crossability barriers and the linkage problem
- Genetic engineering – design and assembly of a transgene cassette

- Transformation – Agrobacterium and gene guns

Laboratory Exercise: DNA Detection

Laboratory exercises for day 3 will introduce two different approaches for detecting specific sequences in DNA samples. For these exercises participants will use the DNA samples they extracted the previous day.

- Transgene detection by PCR: Polymerase chain reaction (PCR) is a powerful technique used for amplifying short, specific sequences of DNA for detection or further manipulation. Participants will use PCR to amplify the sequences for both a transgene and a housekeeping gene from both transgenic and wild-type DNA samples. Agarose gel electrophoresis will be used to visualize the PCR results.

- Geminivirus detection by squash blot: Squash blots allow for the direct detection of high-copy sequences, such as viral genomes, in tissue samples collected in the field. Participants will be use squash blots to search for geminiviruses in plants collected in Bamako. On day 3 participants will set up the squash blot hybridizations.

Day 4: Basic molecular plant pathology and disease resistance

This section will mark a change of focus from the theory and practice of molecular biology to the more applied topic of plant pathology. Pathogens are a serious constraint to production, and appropriate control depends on proper pathogen identification. A special session on day 4, led by Dr. Issoufou Kollo Abdourhamane, will introduce participants to many of the pathogens endemic to West Africa. Lectures will address the different types of pathogens, their impacts on plant function, and plant defense responses, with a focus on the translation of molecular processes into visible symptoms.

Topics:

- Fungi, nematodes, bacteria and viruses – an overview of plant pathogens
- Local plant diseases – an introduction to West African pathogens
- Molecular disease – pathogen effects on cell and molecular processes
- Resistance genes – dominant vs. recessive resistance

Laboratory Exercise: Squash Blot Detection of Geminiviruses (cont'd)

Workshop participants will continue the squash blot protocol started on day 3 by conducting various washes of the blots, and setting up the color development reaction.

Day 5: Detection of proteins

The detection and analysis of proteins is vital to molecular biology. While the tools of genetics offer scientists many opportunities to study protein function indirectly, it is often necessary to more directly detect, isolate, and manipulate proteins to better understand their functions. The session will begin with a study of antibodies and their use in diagnostic aspects of molecular plant pathology, and will continue to cover more advanced protein analysis techniques and their uses in modern proteomics studies.

Topics:

- Antibodies – what are they?
- Antibody production and purification
- Protein detection methods using antibodies
- Advanced protein analysis

Laboratory Exercise: *Ralstonia* detection in tomato

Ralstonia solanacearum is a bacterial pathogen that causes bacterial speck disease in tomato. It can be detected using DNA or protein methods – in this session we will be using two different protein methods to detect *Ralstonia* in samples collected from

around Bamako.

- Enzyme-Linked ImmunoSorbent Assay (ELISA) is a sensitive laboratory technique for detecting proteins with specific antibodies. Antibodies are linked to an enzyme that generates a colored precipitate when exposed to a colorless buffer, allowing detection of very low levels of the protein being sought.
- Immunostrips are modern protein detection kits designed for use directly in the field. Though based on similar chemistry to ELISA, they sacrifice the flexibility and semi-quantitative nature of ELISA for extremely high speed and ease of use.