INTRAGENIC VIRUS RESISTANCE IN POTATO

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INTRAGENIC VIRUS RESISTANCE IN *POTATO*

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The work detailed in this manuscript focuses on the eukaryotic translation initiation factor 4E (eIF4E), a protein involved in recruiting host messenger RNA to the ribosomal complex. eIF4E is also an important host factor that is utilized by invading plant viruses in order to complete their lifecycle. A number of virus resistant alleles that have evolved independently in diverse crop species are now known to correspond to *eIF4E*. Current work has focused on transgenesis of resistance alleles to gain a better understanding of how specific amino acid changes contribute to virus resistance. We present three studies that continue this line of research and apply this knowledge to address an economically important pathosystem.

The focus of the first chapter is on the evolutionary history of the *eIF4E* gene. We find that amino acids predicted to have been most strongly selected are those that are known to interact with a viral protein, suggesting that eIF4E resistance alleles have evolved in response to selective pressures exerted by phytopathogenic viruses. We then mutate the *eIF4E* gene from potato at specific amino acids in order to simulate natural evolutionary processes and disrupt eIF4E-viral interaction. When these mutated potato alleles are overexpressed in potato, they confer virus resistance. By using a potato gene to develop virus resistance, we hope to address the consumer and regulatory concerns that have thus far prevented commercialization of transgenic potato. Finally, we conduct a field experiment to study several resistant lines in more

detail. We find that virus resistance is not associated with a decrease in any of the yield or quality characteristics measured. We suggest that the technique described here may be applied to potato and other crops in order to develop virus resistant varieties that are more acceptable to consumers than other methods of genetic engineering.

BIOGRAPHICAL SKETCH

Jason R. Cavatorta was born on September 3, 1981 and grew up in the coastal town of Rowley, Massachusetts. He graduated from Triton Regional High School in 2000 and from Amherst College in 2004 where he majored in Biology and Geology. In 2006 he received a Masters of Science in Plant Breeding from Cornell University working with Dr. Molly Jahn.

To

my father, who taught by example that hard work is a virtue

and

my mother, who taught me to have confidence in myself

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Chapter I

Literature Review[1]

Virus infection and disease control

The global impact of plant viruses on crop productivity is difficult to estimate, because losses due to viruses frequently go unnoticed (Bos 1982). Viral infection may be inconspicuous, causing little or no obvious symptoms, while resulting in yield reduction (Waterworth and Hadidi 1998). Consequently, calculations of the monetary impact of viral pathogens on food production systems are chronically underreported and underestimated (Hull 2001). Regardless, observed economic losses to virus infection can be striking in magnitude. For instance, global annual reductions in yield are estimated at 7% in sugar beet and 8% in potato due to viruses (Oerke and Dehne 2004). Specific examples, of course, can be much more destructive than global averages. *Tomato spotted wilt virus* has been estimated to cause over US$1 billion in damage to cultivated plant species annually (Goldbach and Peters 1994). Another striking example is *Cocoa swollen shoot virus*, which resulted in the destruction of 200 million cocoa trees in Ghana alone (Lockhart and Sachey 2001) and caused annual losses of as many as 50,000 tons of cocoa pods in Africa (Bowers et al. 2001).

[1] This introduction was adapted from the book chapter "Biotechnology and the control of viral diseases of crops" in the book The Role of Biotechnology in a Sustainable Food Supply which is currently *in press* by Cambridge Press. My role in this chapter was as the primary author. I conducted the vast majority of the research and writing behind it, with help from my coauthors Dr. Stewart Gray and Dr. Molly Jahn.

Management options for viruses are relatively limited. They consist mainly of regulatory and cultural control methods (Agrios 2005). Regulatory control methods involve exclusion of the viral pathogen from a geographic area, typically involving i) controlling transportation of plant parts through legislation and international cooperation (Athey-Pollard et al. 2002) ii) eradication of infected hosts (Cambra et al. 2006) and iii) indexing and certification of virus free material for planting. Cultural control methods involve prevention of viral infection by keeping a pathogen which is already present in a given geographical area from contacting host plants by following proper sanitary procedures such as using disease-free seed (Tomlinson 1962), removing infected weedy species (Pineyro-Nelson et al. 2009), and controlling virus vectors (Satapathy 1998).

Unlike fungal pathogens, which may be controlled through the use of fungicides, there is no effective way to control viral pathogens directly. Chemical control of virus vectors is possible and has been widely adopted for controlling viral diseases despite limited success (Broadbent 1957; Perring et al. 1999). A partial literature search reveals that recommended rates of pesticide application can be very high and/or frequent and may still not effectively control virus spread (Table I.1). High pesticide rates are required because to effectively manage viral diseases, insect vectors must be controlled at threshold levels that are substantially lower than threshold values for controlling insects that cause direct plant damage (Satapathy 1998). Despite the potential environmental and health consequences incurred by this disease management strategy, viruses continue to pose problems in sprayed fields. Indirect chemical control of nonpersistent viruses, which can be

transmitted by a feeding insect in a matter of seconds, is particularly ineffective (Broadbent 1957).

One of the major goals of sustainable agriculture advocates is to reduce the amount of chemical inputs to farming systems. Chemical inputs have been shown to have a number of associated health risks including cancer, birth defects, immunological disorders, and even death (al-Saleh 1994; Coats 1994; Price et al. 2001; WHO/UNEP 1989). In addition, pesticides are associated with a number of environmental risks such as phytotoxicity, animal mortality, loss of soil microorganism diversity, eradication of pollinators and natural insect predators, and development of pesticide resistant organisms (Pimentel and Acquay 1992; Pimentel et al. 1997). In some cases, pesticides have been known to increase virus disease pressure by eliminating the natural predators of viral vectors (Roberts et al. 1993) or by increasing the movement of vectors resulting in more plants ultimately inoculated (Grilli and Holt 2000). Finally, chemical application can be quite expensive and can reduce profitability, especially for resource-limited farmers (Ecobichon 2001). Thus, management of virus diseases by control of viral vectors using pesticide applications is not only unreliable; it is also unsustainable and potentially dangerous.

Table I.1: Pesticide levels required for indirect control of virus diseases by targeting vector populations.

Pesticide Class (specific chemical)	Virus	Vector	Application Rate[2]	Reference
Carbamate (aldicarb)	*Potato leaf roll*	Aphid	1360 (g/acre)	(Powell and Mondor 1973)
Carbamate (aldicarb)	*Raspberry ring spot*	Nematode	3172 (g/acre)	(Trudgill and Alphey 1976)
Carbamate (carbofuran)	*Maize chlorotic dwrarf*	Leafhopper	907 (g/acre)	(Kuhn et al. 1975)
Neonicotinoid (Imidacloprid)	*Tomato spotted wilt*	Thrips	Seedling drench	(Coutts and Jones 2005)
Neonicotinoid (Imidacloprid)	*Barley yellow dwarf*	Aphid	Seed treatment	(Makkouk and Kumari 2007)
Organophosphate (demephion)	*Potato leaf roll*	Aphid	101 (g/acre)	(Woodford et al. 1983)
Organophosphate (demeton)	*Pea enation mosaic*	Aphid	227 (g/acre)	(Swenson et al. 1954)
Organophosphate (methamidophos)	*Tomato yellow leaf curl*	Whitefly	[1]111-437 (g/acre)	(Mazyad et al. 1986)
Organophosphate (phorate)	*Tomato spotted wilt*	Thrips	2770 (g/acre)	(Todd et al. 1996)
Organophosphate (phorate)	*Beet curly top*	Leafhopper	680 (g/acre)	(Hills et al. 1964)
Organophosphate (phosphamidon)	*Rice tungro*	Leafhopper	1214 (g/acre)	(Pathak et al. 1967)
phosphorodiamide (dimefox)	*Cocoa swollen shoot*	Mealybug	Trunk injections	(Moore et al. 2005)
Pyrethroid (deltamethrin)	*Barley yellow dwarf*	Aphid	[1]4-25 (g/acre)	(McGrath and Zitter 2009)
Pyridine azomethine (pymetrozine)	*Lilly mottle*	Aphid	401 (g/acre)	(Asjes and Blom-Barnhoorn 2002)
Pyrethroid (cypermethrin)	*Strawberry mild yellow edge*	Aphid	27 (g/acre)	(Converse and Aliniazee 1988)
Organophosphate (dimethoate)	*Pea leaf roll*	Aphid	77 (g/acre)	(Stoltz and Forster 1984)

[1] Application rate was not reported so the current recommended application rate by the producer is listed.

[2] Application rate refers to amount of active ingredient (not commercial product) per acre.

Host plant resistance

The most sustainable and effective method of virus disease control is the use of host plant resistance when available. In this approach, commercially acceptable cultivars are developed that have reduced disease severity or disease incidence. Development of resistant cultivars is time consuming and costly, but once completed and adopted by farmers, successful disease control can be achieved with little effort (Khetarpal et al. 1998). The major limitations of host resistance are the identification of appropriate and effective genes that confer resistance to a virus or group of viruses, the expense of introgressing the resistance trait into commercial genetic backgrounds and the risk of the virus(es) evolving to overcome genetic resistance (Kang et al. 2005b). The expense of resistance introgression may be mitigated by marker assisted selection or genetic engineering. Resistance durability has proven difficult to predict but may be maximized by the use of recessively inherited or horizontal disease resistance genes (Fraser 1990).

Host plant resistance through conventional breeding

Host plant resistance to viruses has traditionally been achieved by identifying economically important pathosystems, screening sexually compatible germplasm (often from wild sources) for appropriate virus resistance genes, studying the inheritance of resistance, and introgressing resistance into a

commercially acceptable genetic background (Khetarpal et al. 1998; Robinson and Provvidenti 1993). In some cases, further effort has been made to study the mode of action of resistance and to characterize the specificity of resistance alleles against multiple viral strains (Kang et al. 2005b). This method has proven successful against a number of phytopathogenic viruses. To date, numerous resistance genes have been described with a diversity of inheritance types and modes of action (Diaz-Pendon et al. 2004; Kang et al. 2005b; Maule et al. 2007; Provvidenti and Hampton 1992). These have been used to develop virus resistant cultivars in many crops (McGrath and Zitter 2009). For example, the *I* gene has been used effectively in bean breeding to develop bean varieties resistant to *Bean common mosaic virus* (Collmer et al. 2000; Kyle and Provvidenti 1994). Additionally, *Tobacco mosaic virus* has been successfully controlled in tobacco cultivars using the *N* gene (Holmes 1938).

Although conventional breeding is an effective method for managing many plant-virus pathosystems, it does have limitations. Conventional breeding is limited by virus species or type specificity of resistance genes and by the substantial effort required to introgress each gene from wild sources into varieties or hybrids with acceptable agronomic and end use characteristics (Ritzenthaler 2005). This process of backcrossing and selection to remove horticulturally undesirable traits may require many years of backcrossing and selection (Harlan 1976). It is also possible that virus resistance genes cannot be found within plant species that are sexually compatible with a given crop. Finally, breeding new virus-resistant cultivars, especially of clonally propagated crops such as potato, grape, and fruit trees, may not be accepted

by growers or consumers who are reluctant to adopt new cultivars, either because cultivar choice is important to the identity of their product or because other characteristics make existing cultivars preferable.

Host plant resistance through genetic engineering

Relatively recently, advances in plant biotechnology have provided additional methods of creating virus resistant plants. Genetic engineering of plants has allowed for the development of virus resistance utilizing genes from many diverse organisms (Grumet 1990). One of the first uses of this technology was to express viral genes in plants to see what effect this had on virus infection (Goldbach et al. 2003; Sanford and Johnston 1985; Wilson 1993). Tobacco plants expressing the coat protein from *Tobacco mosaic virus* were found to be resistant (Abel et al. 1986). Since that time other viral genes have been expressed in plants with similar results including RNA-dependent RNA polymerase (Braun and Hemenway 1992; Golemboski et al. 1990), dysfunctional movement proteins (Lapidot et al. 1993; Malyshenko et al. 1993), and viral auto-proteases (Germundsson and Valkonen 2006). Other viral sequences, such as interfering DNA and satellite RNA, have also been utilized to obtain virus resistance (Farnham and Baulcombe 2006; Harrison et al. 1987; Stanley et al. 1990). The mechanism of this resistance was originally thought to be protein-based (Sanford and Johnston 1985). The authors state that “the strategy involves deriving resistance genes from the genome of the parasite itself. Key gene products from the parasite, if present in a dysfunctional form, in excess, or at the wrong developmental stage, should disrupt the function of the parasite while having minimal affect on the host.”

Further work found that expression of viral genes may provide resistant individuals by activating gene-silencing mechanisms (Hull 2001). Current thinking is that nucleic acid-based protection is the primary mechanism of developing pathogen-derived resistant plants (Sudarshana et al. 2007).

A number of nonviral genes have been used to develop transgenic resistance as well (Prins 2003; Sudarshana et al. 2007). Resistance has been successfully achieved when some plant genes have been expressed transgenically. Several resistance genes, for example *N* from tobacco and *Tm-2*^{2} from tomato, have been shown to confer resistance when expressed in susceptible genotypes (Lanfermeijer et al. 2003; Lellis et al. 2002). A protease inhibitor found naturally in plants has been used successfully to confer transgenic resistance to multiple potyviruses (Gutierrez-Campos et al. 1999). Different ribosome-inactivating proteins are further examples of plant genes that have been used successfully to develop resistance in a number of viral pathosystems: resistance to *Potato virus Y* (PVY) and *Potato virus X* (PVX) in tobacco and potato (Lodge et al. 1993), resistance to *African cassava mosaic virus* in *N. benthamiana* (Hong et al. 1996), and resistance to *Cucumber mosaic virus* and *Tobacco mosaic virus* in tobacco (Krishnan et al. 2002). Finally, mammalian genes have also been used to confer virus resistance in plants including single chain antibodies (Tavladoraki et al. 1993) and 2-5A synthetase from rat (which is involved in activating RNase) (Truve et al. 1994).

Despite the enthusiasm generated by these early results, the potential for commercialization of virus-resistant plant material has not been fully realized. Only several genetically modified cultivars have been commercialized, all

relying on pathogen-derived resistance. Virus resistant transgenic squash (*Cucurbita pepo*), developed by Asgrow Seed Co., was been approved for commercialization (APHIS 1996a). Cultivars developed from lines 'ZW-20' and *'CZW-3'* contain coat-protein mediated protection against combinations of Watermelon mosaic virus 2, Zucchini yellow mosaic virus and Cucumber mosaic virus (APHIS 1996a; Tepfer 2002; Tricoll et al. 1995). The 'Rainbow' papaya cultivar, developed by Cornell University and the University of Hawaii, has resistance to *Papaya ringspot virus* (PRSV) (APHIS 1996b; Ferreira et al. 2002). Finally, the 'Newleaf' potato cultivar, which contained the *Bt* gene and resistance to either *Potato leafroll virus* (cultivar 'Newleaf Plus') or PVY (cultivar 'Newleaf Y'), was marketed briefly by the Monsanto subsidiary company Naturemark (APHIS 1995; Kaniewski and Thomas 2004; Tepfer 2002).

The few transgenic virus resistant crops that have been commercialized have only been planted on limited acreage. Nearly three-quarters of Hawaiian papaya acres are planted with transgenic trees, but this only accounts for less than 1000 acres and less than 0.1% of global papaya production (Anonymous 2005; USDA 2007). No transgenic trees are grown or fruit sold outside of North America and attempts to do so have met with considerable resistance (Davidson 2008; Gonsalves 2004). A similar story exists for virus resistant transgenic squash as only 12% of US summer squash acreage has been planted to genetically engineered squash, accounting for about 7000 acres (Fuchs 2008; Shankula 2006). Resistant transgenic squash has not been adopted anywhere outside of North America and accounts for a marginal percentage of global summer squash production (James 2007). Potato was the most widely planted crop with transgenic virus resistance, but was

discontinued after just a single field season. Although resistance to economically important viral diseases has been successfully obtained in many additional crops, regulatory approval and commercialization has not been achieved.

Dominantly-inherited virus resistance

Genomic research of virus resistance has led to the cloning and detailed study of an increasing number of virus resistance genes (Goldbach et al. 2003). Several dominantly inherited plant virus resistance genes have been sequenced and studied in detail. Nearly all belong to the nucleotide binding site plus leucine rich repeat (NB-LRR) class of resistance genes with one of the following at the N terminus: a leucine zipper, a coiled coil, or a region similar to the Toll and Interleukin 1 receptor (Jones and Dangl 2006). An exception to this is *RTM1* and *RTM2* against *Tobacco etch virus* (TEV) in *Arabidopsis*, which appear to be a lectin and a heat shock protein, respectively (Chisholm et al. 2000; Elke et al. 1997; Lellis et al. 2002). This detailed study of dominantly inherited resistance has facilitated the discovery of additional resistance genes and has offered an alternative to pathogen derived transgenic resistance, as already discussed.

Recessively-inherited virus resistance

Study of recessively-inherited resistance has lagged behind that of dominantly-inherited resistance and thus less is understood about this class of resistance genes (Kang et al. 2005b). There are notable differences between

recessively- and dominantly-inherited resistance mechanisms. Dominant resistance is often associated with a hypersensitive response after specifically recognizing a particular pathogen component (Goodman and Novacky 1994). Dominant resistance genes typically interact in some way with a specific effector molecule from an invading pathogen in a 'gene-for-gene' manner resulting in the activation of defense responses (Florr 1942; Jones and Dangl 2006). Recessive resistance genes, in contrast, are thought to be host genes that are utilized by the pathogen to complete its lifecycle and are often plant proteins involved in cellular processes. If this host protein is mutated in such a way as to prevent the virus from performing an essential function (such as replication or movement) the host plant may be resistant (Diaz-Pendon et al. 2004; Fraser 1990). There is no associated programmed cell death and no activation of defense responses within the plants, causing some to refer to recessive resistance as "passive resistance" (Fraser 1990). The virus is simply unable to complete its life cycle in the host and therefore infection does not occur. Although recessive resistance is not unique to virus resistance (Jørgensen 1992), it does appear to be a more common defense mechanism against viruses than against other pathogen types (Diaz-Pendon et al. 2004; Provvidenti and Hampton 1992). Furthermore, recessive resistance, in general, seems to be more durable and less strain specific than dominant forms of resistance (Kang et al. 2005b).

The virus life cycle may be disrupted at several different stages. Recessive resistance genes have been shown to disrupt viral replication (Deom et al. 1997; Murphy et al. 1998), cell-to-cell movement (Gao et al. 2004; Gibb et al. 1989), or long-distance movement within plants (Hamalainen et al. 2000;

Schaad et al. 1997). A better understanding of the identity and function of recessive resistance genes will provide additional tools for developing virus-resistant crop varieties. By coupling knowledge of virus infection strategies with new advances in plant genomics it is likely that novel methods of combating virus infection will arise. In the following sections we discuss the most well-studied recessive resistance gene to illustrate this point and describe successful utilization of this gene to engineer virus-resistant potato lines. Future prospects for developing resistance in other crops and for improving commercialization of biotechnology are also discussed.

Eukaryotic translation initiation factor 4E

The *eukaryotic translation initiation factor 4E* (*eIF4E*) is a host gene involved in binding to the 5' cap structure of messenger RNA (Sonenberg et al. 1978). A remarkable amount of information is known about this gene because translation initiation occurs in a similar manner among all eukaryotes, and because it is associated with one of the highly-studied steps in the cell cycle (De Benedetti and Graff 2004).

The eIF4E-cap interaction occurs during the initiation phase of translation and is required for recruitment of capped mRNA to the ribosomal complex (Browning 2004; Gingras et al. 1999). If eIF4E is not present, the translation efficiency of capped mRNA is greatly reduced (Svitkin et al. 1996). Sequence analysis and rescue of lethal *eIF4E* mutants has determined that eIF4E structure and function is highly conserved among all eukaryotes (Altmann et al. 1989; Charron et al. 2008; Marcotrigiano et al. 1997). The co-crystal

structure of eIF4E and 7-methyl-GDP is available (Marcotrigiano et al. 1997) as well as the predicted structure of eIF4E from *Capsicum* (Kang et al. 2005b).

Translation of the RNA genome of plant viruses in an infected host cell appears to share similarities to translation of host cellular mRNA. During plant virus infection, translation initiation factors function to circularize the RNA genome and bring it into contact with the ribosomal complex (Browning 2004). eIF4E interacts with viral RNA directly (Gazo et al. 2004) or through a viral protein bound to viral RNA (Ruffel et al. 2002). Potyviruses, for instance, utilize the genome-linked protein called VPg (Shahabuddin et al. 1988) that binds directly to eIF4E in a strain-specific manner (Kang et al. 2005b; Nicaise et al. 2003; Ruffel et al. 2002; Yeam et al. 2007). VPg-eIF4E binding is not observed in plant-virus combinations where systemic infection does not occur (Kang et al. 2005b).

Virus resistance alleles arise when natural eIF4E mutations disrupt the ability of the virus to interact with the modified eIF4E protein. As a result, the mutant form of eIF4E is thought to prevent the translation of the viral genome within plant cells while host mRNA continues to be translated. The evolution of mutant *eIF4E* genes that now function as virus resistance genes has occurred independently in multiple plant species. Because a single copy of the wildtype allele is sufficient to support viral infection in plants, this type of resistance is recessively inherited and has thus been difficult to work with and define. In recent years, a number of recessive resistance alleles from several plant species have been cloned and found to encode *eIF4E*. Initial work discovered that eIF4E from *Arabidopsis* was able to bind VPg from TEV strains able to

infect the plant, but were unable to bind VPg from TEV strains that were unable to cause an infection (Schaad et al. 2000). Subsequently, a candidate-gene approach identified differences in eIF4E between susceptible and resistant genotypes of other plants including pepper (*Capsicum*). In some instances eIF4E-mediated resistance is effective against multiple Potyviruses or even other families of viruses. Pathosystems identified so far include tomato-*Potyviridae* (Ruffel et al. 2005), pea-*Potyviridae* (Gao et al. 2004), lettuce-*Potyviridae* (Nicaise et al. 2003), barley-*Bymovirus* (Stein et al. 2005), and melon-*Carmovirus* (Nieto et al. 2006). In addition, knock-out mutants of eIF4E in *Arabidopsis* have gained resistance to *Cucumber mosaic virus* (*Cucumoviridae*) (Yoshii et al. 2004). A number of animal viruses have even been found to interact with the translation initiation factors of their hosts (Ohimann et al. 1995). Viral interaction with translation initiation factors appears to be a crucial step during the viral infection process of many diverse pathosystems.

In pepper (*Capsicum*), many natural recessive resistance alleles have been described at the *Potyvirus resistance* (*pvr1*) locus corresponding to the *eIf4E* gene (Charron et al. 2008; Kang et al. 2005a; Ruffel et al. 2002). Two of these alleles, *pvr1* and *pvr1*^{2}, have been intensively studied and found to each contain a different combination of 3 amino acid changes relative to wildtype. These mutations are responsible for the strain-specific resistance observed in host plants homozygous for one of these alleles (Kang et al. 2005b; Ruffel et al. 2002). Although the mutations in each allele are unique, they are clustered in the region of the three-dimensional eIF4E structure that is predicted to be involved in VPg binding (Kang et al. 2005a; Ruffel et al. 2002). Considerable

effort has been expended to investigate the contribution of individual amino acid changes in eIF4E (German-Retana et al. 2008; Yeam et al. 2007). It is remarkable that only a few polymorphisms are responsible for such a dramatic phenotypic change. Similarly, only 4 amino acid changes were required in VPg to alter virulence specificity (Ayme et al. 2007). A series of alleles conferring strain-specific resistance suggests that coevolution between host and pathogen is focused around changes in potyviral *VPg* and *Capsicum eIF4E*. Detection of positive selection in both of these genes appears to confirm this hypothesis (Cavatorta et al. 2008; Moury et al. 2004). The interaction between host and pathogen in this system is therefore understood on an individual amino acid level. The detailed knowledge of eIF4E and VPg makes this coevolutionary pathosystem among the best-understood in biology.

Transgenesis of resistant alleles controls virus infection

Mutant forms of *eIF4E* that convey resistance to multiple plant viruses have been identified in several species, but there are many more plant species that are not known to have evolved such resistance alleles. Understanding the mechanisms of *eIF4E*-mediated resistance opens up opportunities to use biotechnology to express virus resistant alleles from one species in another. This provides the potential to transfer virus resistance to crops that lack known resistance alleles at the *eIF4E* locus. This approach has worked for other resistance genes such as the *N* gene from tobacco, which conferred virus resistance when expressed transgenically in tomato (Lellis et al. 2002). The major difference is that the *N* gene, a dominantly inherited resistance gene, confers the ability to recognize an invading pathogen and mount an

appropriate resistance response. The recessive nature of *eIF4E*-mediated resistance means that transgenic expression in a susceptible genotype would not make the recipient host resistant unless it performed in a “dominant negative” manner (described below).

Tomato plants (*Solanum lycopersicum*) were transformed with the *pvr1* resistance allele from *Capsicum chinense*. The transgene, overexpressed using the constitutive promoter CaMV 35S, was found to confer broad-spectrum potyvirus resistance in transgenic tomato lines (Kang et al. 2007). PVY and TEV isolates that fail to infect pepper plants with the *pvr1* gene also failed to accumulate in inoculated and non-inoculated leaves of transgenic tomato plants expressing the pepper *pvr1* allele (Kang et al. 2007). A single copy of the transgene was sufficient to confer resistance even in the presence of the susceptible endogenous tomato *eIF4E* gene.

A “dominant negative” model of protein interactions has been proposed to explain these observations and is continuing to be refined as our understanding of this mode of resistance increases (Kang et al. 2007). In the dominant negative model, virus resistance is a dominant trait when the mutant eIF4E is expressed transgenically using a constitutive promoter. Several models for the mechanism of eIF4E-mediated virus resistance have been proposed, all predicated on a direct interaction between eIF4E and VPg (Zhang et al. 2006). The dominant negative model should therefore be applicable whether this interaction is responsible for translation initiation, genome stability, intracellular trafficking, or any other potential mode of action of the *eIF4E* resistance gene. Presumably, both transgenic and endogenous

eIF4E proteins exist, but the population of eIF4E protein in transgenic plants consists predominantly of transgenic eIF4E. The mutant resistant allele from pepper is unable to interact with potyviral VPg, but may still perform translation of host plant mRNA. The limited endogenous susceptible tomato eIF4E peptides that are present will still be able to interact with potyviral VPg, but their relative scarcity makes this interaction unlikely. If any endogenous eIF4E-VPg complexes do form, infection may still be blocked because the majority of translation initiation complexes will already be bound to the more abundant transgenic eIF4E peptides.

In order to understand the precise amino acid changes responsible for conferring virus resistance, experimentally modified versions of pepper *eIF4E* were created and transformed into tomato. The amino acid sequence of the susceptible pepper allele *Pvr1*+ was modified at each of six amino acid residues, either singly or in various combinations. These six amino acids were known to be polymorphic in resistance alleles and thought to be involved in disease resistance. The specific amino acids responsible for conferring resistance phenotypes were from two known resistant alleles (Yeam et al. 2007). Whether resistant versions of *eIF4E* maintain the ability to bind to the 5' mRNA cap structure is uncertain. Several *in vitro* cap-binding experiments indicate some resistance alleles maintain cap binding while others do not (Kang et al. 2005a; Yeam et al. 2007). Recently, resistance alleles from pepper were shown to complement an eIF4E knockout mutant in yeast, suggesting resistance alleles maintain their function for the host (Charron et al. 2008). Regardless, alleles with and without the ability to bind to the 5' mRNA

cap *in vitro* conferred virus resistance to transgenic plants without any obvious pleiotropic consequences.

The experiments in tomato are proof-of-concept for *eIF4E* resistance alleles that conferred virus resistance across species. PVY is the most economically important virus disease in potato (*Solanum* tuberosum) production and is a practical target for virus resistance using this method. Although natural PVY resistance genes have been described in potato, conventional breeding in a tetraploid is difficult and commercially acceptable PVY-resistant potato cultivars are limited. This work describes development of PVY resistance in potato. First, it is shown that *eIF4E* is under positive selection by phytopathogenic viruses. Next, information on naturally-arising resistance alleles is used to mutate potato *eIF4E* to develop novel resistance alleles that confer virus resistance when expressed back in potato. Finally, virus resistance is verified in the field and tested for pleiotropic consequences.

REFERENCES

Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-43

Agrios GN (2005) Plant Pathology. Elsevier Academic Press, Burlington, MA

al-Saleh IA (1994) Pesticides: a review article. J Environ Pathol Toxicol Oncol 13:151-61

Altmann M, Muller PP, Pelletier J, Sonenberg N, Trachsel H (1989) A mammalian translation initiation factor can substitute for its yeast homologue in vivo. J. Biol. Chem. 264:12145-12147

Anonymous (2005) Papaya Market in EU. Agri Export Advantage 4:3-4. Available online at: http://www.eximbankindia.com/agri-exp-adv/agrisept.pdf

APHIS (1995) Monsanto Co.; Receipt of petition for determination of nonregulated status for potato lines genetically engineered for insect and virus resistance. In: Service AaPHI (ed). Federal Register Docket No. 96-002-2. http://www.aphis.usda.gov/brs/aphisdocs2/95_35201p_com.pdf.

APHIS (1996a) Asgro Seed Co.; Availability of determination of nonregulated status for squash line genetically engineered for virus resistance. In: Service AaPHI (ed). Federal Register Docket No. 96-002-2. http://www.aphis.usda.gov/brs/aphisdocs2/95_35201p_com.pdf.

APHIS (1996b) Cornell University and University of Hawaii; Availability of determination of nonregulated status for papaya lines genetically engineered for virus resistance. In: Service AaPHI (ed). Federal

Register Docket No. 96-024-2. http://www.aphis.usda.gov/brs/aphisdocs2/96_05101p_com.pdf.

Asjes CJ, Blom-Barnhoorn (2002) Control of Aphid Vector Spread of Lily Symptomless Virus and Lily Mottle Virus by Mineral Oil/Insecticide Sprays in Lilium. *Acta Hort. (ISHS)* 570:277-281

Bos L (1982) Crop losses caused by viruses. Crop Protection 1:263-282

Bowers JH, Bailey BA, Hebbar PK, Soumaila S, Lumsden RD (2001) The Impact of Plant Diseases on World Chocolate Production. Plant Health Progress

Braun CJ, Hemenway CL (1992) Expression of Amino-Terminal Portions or Full-Length Viral Replicase Genes in Transgenic Plants Confers Resistance to Potato Virus X Infection. Plant Cell 4:735-744

Broadbent L (1957) Insecticidal Control of the Spread of Plant Viruses. Annual Review of Entomology 2:339-354

Browning KS (2004) Plant translation initiation factors: it is not easy to be green. Biochem Soc Trans 32:589-91

Cambra M, Capote N, Myrta A, Ll, cer G (2006) Plum pox virus and the estimated costs associated with sharka disease. EPPO/OEPP Bulletin 36:202-204

Cavatorta JR, Perez K, Van Eck J, Austen-Phillips S, Yeam I, Gray SM, Jahn MM (*In Prep*) Precision-breeding for crop plants.

Charron C, Nicolai M, Gallois JL, Robaglia C, Moury B, Palloix A, Caranta C (2008) Natural variation and functional analyses provide evidence for co-evolution between plant eIF4E and potyviral VPg. Plant J 54:56-68

Chisholm ST, Mahajan SK, Whitham SA, Yamamoto ML, Carrington JC (2000) Cloning of the Arabidopsis RTM1 gene, which controls

restriction of long-distance movement of tobacco etch virus. Proceedings of the National Academy of Sciences of the United States of America 97:489-494

Coats JR (1994) Risks from natural versus synthetic insecticides. Annu Rev Entomol 39:489-515

Collmer CW, Marston MF, Taylor JC, Jahn M (2000) The I Gene of Bean: A Dosage-Dependent Allele Conferring Extreme Resistance, Hypersensitive Resistance, or Spreading Vascular Necrosis in Response to the Potyvirus Bean common mosaic virus. Molecular Plant-Microbe Interactions 13:1266-1270

Converse RH, Aliniazee MT (1988) Effects of insecticides applied in the field on incidence of aphid-borne viruses in cultivated strawberry. Plant Disease 72

Coutts BA, Jones RAC (2005) Suppressing spread of *Tomato spotted wilt virus* by drenching infected source or healthy recipient plants with neonicotinoid insecticides to control thrips vectors. Annals of Applied Biology 146:95-103

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826-833

Davidson SN (2008) Forbidden Fruit: Transgenic Papaya in Thailand. Plant Physiol. 147:487-493

De Benedetti A, Graff JR (2004) eIF-4E expression and its role in malignancies and metastases. Oncogene 23:3189-3199

Deom CM, Murphy JF, Paguio OR (1997) Resistance to Tobacco Etch Virus in Capsicum annuum: Inhibition of Virus RNA Accumulation. Molecular Plant-Microbe Interactions 10:917-921

Diaz-Pendon JA, Truniger V, Nieto C, Garcia-Mas J, Bendahmane A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Molecular Plant Pathology 5:223-233

Ecobichon DJ (2001) Pesticide use in developing countries. Toxicology 160:27-33

Elke D, Martin S, Lothar W, Jens K (1997) Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum* L.) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism. The Plant Journal 12:323-333

Farnham G, Baulcombe DC (2006) Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. Proc Natl Acad Sci U S A 103:18828-33

Ferreira SA, Pitz KY, Manshardt R, Zee F, Fitch M, Gonsalves D (2002) Virus Coat Protein Transgenic Papaya Provides Practical Control of Papaya ringspot virus in Hawaii. Plant Disease 86:101-105

Flor HH (1971) Current Status of the Gene-For-Gene Concept. Annual Review of Phytopathology 9:275-296

Foster JA, Hadidi A (1998) Exclusion of plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant virus disease control. APS Press, St. Paul, Minneapolis, p 208-229

Fraser RSS (1990) The Genetics of Resistance to Plant Viruses. Annual Review of Phytopathology 28:179-200

Fuchs M (2008) Why virus resistance in transgenic plants, and are there risks associated with the release of virus-resistant transgenic plants? Agricultural Biotechnology

Gao Z, Johansen E, Eyers S, Thomas CL, Noel Ellis TH, Maule AJ (2004) The potyvirus recessive resistance gene, sbm1, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. The Plant Journal 40:376-385

Gazo BM, Murphy P, Gatchel JR, Browning KS (2004) A novel interaction of Cap-binding protein complexes eukaryotic initiation factor (eIF) 4F and eIF(iso)4F with a region in the 3'-untranslated region of satellite tobacco necrosis virus. J Biol Chem 279:13584-92

German-Retana S, Walter J, Doublet B, Roudet-Tavert G, Nicaise V, Lecampion C, Houvenaghel M-C, Robaglia C, Michon T, Le Gall O (2008) Mutational analysis of a plant cap-binding protein eIF4E reveals key amino-acids involved in biochemical functions and potyvirus infection. J. Virol.:JVI.00209-08

Germundsson A, Valkonen JPT (2006) P1- and VPg-transgenic plants show similar resistance to Potato virus A and may compromise long distance movement of the virus in plant sections expressing RNA silencing-based resistance. Virus Research 116:208-213

Gibb KS, Hellmann GM, Pirone TP (1989) Nature of resistance of a tobacco cultivar to *Tobacco vein mottling virus*. MPMI 2:332-339

Gingras AC, Raught B, Sonenberg N (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68:913-63

Goldbach R, Bucher E, Prins M (2003) Resistance mechanisms to plant viruses: an overview. Virus Research 92:207-212

Goldbach R, Peters D (1994) Possible causes of the emergence of tospovirus diseases. Seminars in Virology 5:113-120

Golemboski DB, Lomonossoff GP, Zaitlin M (1990) Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. Proceedings of the National Academy of Sciences of the United States of America 87:6311-6315

Gonsalves D (2004) Transgenic papaya in Hawaii and beyond. AgBioForum 7:36-40. Available online at: http://www.agbioforum.org.

Goodman RN, Novacky AJ (1994) The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. APS Press, St. Paul

Grilli MP, Holt J (2000) Vector feeding period variability in epidemiological models of persistent plant viruses. Ecological Modelling 126:49-57

Grumet R (1990) Genetically Engineered Plant Virus Resistance. HortScience 25:508-513

Gutierrez-Campos R, Torres-Acosta JA, Saucedo-Arias LJ, Gomez-Lim MA (1999) The use of cysteine proteinase inhibitors to engineer resistance against potyviruses in transgenic tobacco plants. Nat Biotech 17:1223-1226

Hamalainen JH, Kekarainen T, Gebhardt C, Watanabe KN, Valkonen JPT (2000) Recessive and Dominant Genes Interfere with the Vascular Transport of Potato virus A in Diploid Potatoes. Molecular Plant-Microbe Interactions 13:402-412

Harlan JR (1976) Genetic Resources in Wild Relatives of Crops. Crop Sci 16:329-333

Harrison BD, Mayo MA, Baulcombe DC (1987) Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature 328:799-802

Hills OA, Coudriet DL, Brubaker RW (1964) Phorate treatments against the beet Leafhopper on cantaloupe for prevention of curly top. J Econ Entomol 57:85-89

Holmes FO (1938) Inheritance of resistance to tobacco-mosaic disease in tobacco. Phytopathology 28:553-561

Hong Y, Saunders K, Hartley MR, Stanley J (1996) Resistance to Geminivirus Infection by Virus-Induced Expression of Dianthin in Transgenic Plants. Virology 220:119-127

Hull R (2002) Mathews' Plant Virology. Academic Press, London, UK

James C (2007) ISAAA Report on Global Status of Biotech/GM Crops. Available online at: http://www.isaaa.org

Jørgensen IH (1992) Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. Euphytica 63:141-152

Kang BC, Yeam I, Jahn MM (2005) Genetics of plant virus resistance. Annu Rev Phytopathol 43:581-621

Kaniewski WK, Thomas PE (2004) The Potato Story. AgBioForum 7:41-46

Khetarpal RK, Maisonneuve B, Maury Y, Chalhoub B, Dinant S, Lecoq H, Varma A (1998) Breeding for resistance to plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant virus disease control. APS PRESS, St. Paul, Minnesota

Krishnan R, McDonald KA, Dandekar AM, Jackman AP, Falk B (2002) Expression of recombinant trichosanthin, a ribosome-inactivating protein, in transgenic tobacco. Journal of Biotechnology 97:69-88

Kuhn CW, Jellum MD, All JN (1975) Effect of carbofuran treatment on corn yield, maize chlorotic dwarf and maize dwarf mosaic virus diseases, and leafhopper populations. Phytopathology 65:1017-1020

Kyle MM, Provvidenti R (1994) Genetics of broad spectrum viral resistance in bean and pea. In: Kyle MM (ed) Resistance to viral diseases of vegetables. Timber Press, Inc., Portland, Oregon, p 153-166

Lanfermeijer FC, Dijkhuis J, Sturre MJG, Haan Pd, Hille J (2003) Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-22 from Lycopersicon esculentum. Plant Molecular Biology 52:1039-1051

Lapidot M, Gafny R, Ding B, Wolf S, Lucas WJ, Beachy RN (1993) A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. The Plant Journal 4:959-970

Lockhart BE, Sachey ST (2001) Cacao swollen shoot. In: Maloy OC, Murray TD (eds) Encyclopedia of Plant Pathology. John Wiley & Sons, Inc., New York, p 172-173

Lodge JK, Kaniewski WK, Tumer NE (1993) Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. Proceedings of the National Academy of Sciences of the United States of America 90:7089-7093

Makkouk KM, Kumari SG (2007) Epidemiology and integrated management of persistently aphid-transmitted legume and cereal viruses in West Asia and North Africa 10th International Plant Virus Epidemiology Symposium, Patancheru, India

Malyshenko SI, Kondakova OA, Nazarova JV, Kaplan IB, Taliansky ME, Atabekov JG (1993) Reduction of tobacco mosaic virus accumulation in transgenic plants producing non-functional viral transport proteins. J Gen Virol 74:1149-1156

Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK (1997) Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell 89:951-61

Maule AJ, Caranta C, Boulton MI (2007) Sources of natural resistance to plant viruses: status and prospects. Molecular Plant Pathology 8:223-231

Mazyad HM, Nakhla MK, El-Amrety AA, Doss SA (1986) Further studies on the epidemiology of tomato yellow leaf curl virus in Egypt. Acta Hort. (ISHS) 190:121-130

McGrath M, Zitter T (2009) Resistant Varieties Cornell University Vegetable MD Online

Moore S, Payton P, Wright M, Tanksley S, Giovannoni J (2005) Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. J. Exp. Bot. 56:2885-2895

Moury B, Morel C, Johansen E, Guilbaud L, Souche S, Ayme V, Caranta C, Palloix A, Jacquemond M (2004) Mutations in potato virus Y genome-linked protein determine virulence toward recessive resistances in Capsicum annuum and Lycopersicon hirsutum. Mol Plant Microbe Interact 17:322-9

Murphy JF, Blauth JR, Livingstone KD, Lackney VK, Jahn MK (1998) Genetic Mapping of the pvr1 Locus in Capsicum spp. and Evidence That Distinct Potyvirus Resistance Loci Control Responses That Differ at the Whole Plant and Cellular Levels. Molecular Plant-Microbe Interactions 11:943-951

Nicaise V, Gallois JL, Chafiai F, Allen LM, Schurdi-Levraud V, Browning KS, Candresse T, Caranta C, Le Gall O, German-Retana S (2007)

Coordinated and selective recruitment of eIF4E and eIF4G factors for potyvirus infection in Arabidopsis thaliana. FEBS Lett

Oerke EC, Dehne HW (2004) Safeguarding production--losses in major crops and the role of crop protection. Crop Protection 23:275-285

Ohimann T, Rau M, Morley SJ, Pain VM (1995) Proteolytic cleavage of initiation factor elF-4{gamma} in the reticulocyte lysate inhibits translation of capped mRNAs but enhances that of uncapped mRNAs. Nucl. Acids Res. 23:334-340

Pathak MD, Vea E, John VT (1967) Control if insect vectors to prevent virus infection of rice plants. J Econ Entomol 60:218-225

Perring TM, Gruenhagen NM, Farrar CA (1999) Management of plant viral diseases through chemical control of insect vectors. Annual Review of Entomology 44:457-481

Pidgeon NF, Poortinga W, Rowe G, Jones TH, Walls J, O'Riordan T (2005) Using surveys in public participation processes for risk decision making: the case of the 2003 British GM Nation? Public debate. Risk Anal 25:467-79

Pimentel D, Acquay H (1992) Environmental and economic costs of pesticide use. Bioscience 42:750-760

Pimentel D, Acquay H, Biltonen M, Rice P, Silva M, Nelson J, Lipner V, Giordano S, Horowitz A, D'Amore M (1997) Assessment of Environmental and Economic Impacts of Pesticide Use. In: Shrader-Frechette K, Westra L (eds) Technology and Values. Rowman and Littlefield, p 375-413

Pineyro-Nelson A, Van Heerwaarden J, Perales HR, Serratos-Hernandz JA, Rangel A, Hufford MB, Gepts P, Garay-Arroyo A, Rivera-Bustamante R,

Alvarez-Buylla E (2009) Transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. Molecular Ecology 18:750-761

Powell DM, Mondor TW (1973) Control of the green peach aphid and suppression of leaf roll on potatoes by systemic soil insecticides and multiple foliar sprays. J Econ Entomol 66:170-7

Price PS, Young JS, Chaisson CF (2001) Assessing aggregate and cumulative pesticide risks using a probabilistic model. Ann Occup Hyg 45:S131-142

Prins M (2003) Broad virus resistance in transgenic plants. Trends in Biotechnology 21:373-375

Provvidenti R, Hampton RO (1992) Sources of resistance to viruses in the Potyviridae. Arch Virol Suppl 5:189-211

Ritzenthaler C (2005) Resistance to plant viruses: old issue, news answers? Current Opinion in Biotechnology 16:118-122

Roberts JMF, Hodgson CJ, Jackai LEN, Thottappilly G, Singh SR (1993) Interaction between two synthetic pyrethroids and the spread of two non-persistent viruses in cowpea. Annals of Applied Biology 122:57-67

Robinson RW, Provvidenti R (1993) Breeding lettuce for viral resistance. In: Kyle MM (ed) Resistance to viral diseases of vegetables. Timber Press, Inc., Portland, Oregon, p 61-79

Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C (2002) A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). Plant J 32:1067-75

Ruffel S, Gallois JL, Lesage ML, Caranta C (2005) The recessive potyvirus resistance gene pot-1 is the tomato orthologue of the pepper pvr2-eIF4E gene. Mol Genet Genomics 274:346-53

Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance--Deriving resistance genes from the parasite's own genome. Journal of Theoretical Biology 113:395-405

Satapathy MK (1998) Chemical control of insect and nematode vectors of plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant Virus Disease Control. APS Press, St. Paul, Minnesota, p 188-195

Schaad MC, Anderberg RJ, Carrington JC (2000) Strain-specific interaction of the tobacco etch virus NIa protein with the translation initiation factor eIF4E in the yeast two-hybrid system. Virology 273:300-6

Schaad MC, Lellis AD, Carrington JC (1997) VPg of tobacco etch potyvirus is a host genotype-specific determinant for long-distance movement. J. Virol. 71:8624-8631

Shahabuddin M, Shaw JG, Rhoads RE (1988) Mapping of the tobacco vein mottling virus VPg cistron. Virology 163:635-7

Shankula S (2006) Quantification of the impacts on US agrigulture of biotechnology-derived crops planted in 2005. Available online at: http://www.ncfap.org

Sonenberg N, Morgan MA, Merrick WC, Shatkin AJ (1978) A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA. Proc Natl Acad Sci U S A 75:4843-7

Stanley J, Frischmuth T, Ellwood S (1990) Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. Proceedings of

the National Academy of Sciences of the United States of America 87:6291-6295

Stein N, Perovic D, Kumlehn J, Pellio B, Stracke S, Streng S, Ordon F, Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive Bymovirus resistance in Hordeum vulgare (L.). Plant J 42:912-22

Stoltz RL, Forster RL (1984) Reduction of pea leaf roll of peas (*Pisum sativum*) with systemic insecticides to control the pea aphid (Hopoptera: Aphididae) vector. J Econ Entomol 77:1537-41

Sudarshana MR, Roy G, Falk BW (2007) Methods for engineering resistance to plant viruses. Methods Mol Biol 354:183-95

Svitkin YV, Ovchinnikov LP, Dreyfuss G, Sonenberg N (1996) General RNA binding proteins render translation cap dependent. Embo J 15:7147-55

Swenson KG, Davis AC, Schroeder WT (1954) Reduction of pea virus spread by insecticide application. J Econ Entomol 47:490-493

Tavladoraki P, Benvenuto E, Trinca S, De Martinis D, Cattaneo A, Galeffi P (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature 366:469-472

Tepfer M (2002) Risk assessment of virus-resistant transgenic plants. Annu Rev Phytopathol 40:467-91

Todd JW, Culbreath AK, Brown SL (1996) Dynamics of vector populations and progress of spotted wilt disease relative to insecticide use in peanuts. *Acta Horticulturae* 431:483-490

Tomlinson JA (1962) Control of lettuce mosaic by the use of healthy seed. Plant Pathology 11:61-64

Tricoll DM, Carney KJ, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD (1995) Field Evaluation of Transgenic Squash Containing Single or Multiple Virus Coat Protein Gene Constructs for Resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus 2, and Zucchini Yellow Mosaic Virus. Nat Biotech 13:1458-1465

Trudgill DL, Alphey JW (1976) Chemical Control of the Virus-vector Nematode *Longidorus elongatus* and of *Pratylenchus creimtus* in Raspberry Plantations. Plant Pathology 25:15-20

Truve E, Kelve M, Aaspollu A, Kuusksalu A, Seppanen P, Saarma M (1994) Principles and background for the construction of transgenic plants displaying multiple virus resistance. Arch Virol Suppl 9:41-50

USDA (2007) 2007 Papaya Output Higher. Available online at: http://www.nass.usda.gov/hi/fruit/annpap.pdf

Waterworth HE, Hadidi A (1998) Economic Losses Due to Plant Viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant Virus Disease Control. APS Press, St. Paul, Minnesota, p 1-13

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene N: Similarity to toll and the interleukin-1 receptor. Cell 78:1101-1115

Whitham S, McCormick S, Baker B (1996) The N gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato. Proc Natl Acad Sci U S A 93:8776-81

Whitham SA, Anderberg RJ, Chisholm ST, Carrington JC (2000) Arabidopsis RTM2 Gene Is Necessary for Specific Restriction of Tobacco Etch Virus

and Encodes an Unusual Small Heat Shock-like Protein. Plant Cell 12:569-582

WHO/UNEP (1989) Public Health Impact of Pesticides Used in Agriculture. In: World Health Organization/United Nations Environment Programme. Geneva S (ed) World Health OrganizationAJnited Nations Environment Programme

Wilson TM (1993) Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. Proc Natl Acad Sci U S A 90:3134-41

Woodford JA, Harrison BD, Aveyard CS, Gordon SC (1983) Insecticidal control of aphids and the spread of potato leafroll virus in potato crops in eastern Scotland. Annals of Applied Biology 103:117-130

Yoshii M, Nishikiori M, Tomita K, Yoshioka N, Kozuka R, Naito S, Ishikawa M (2004) The Arabidopsis cucumovirus multiplication 1 and 2 loci encode translation initiation factors 4E and 4G. J Virol 78:6102-11

Zhang Y-Y, Li H-X, Ouyang B, Ye Z-B (2006) Regulation of Eukaryotic Initiation Factor 4E and Its Isoform: Implications for Antiviral Strategy in Plants. Journal of Integrative Plant Biology 48:1129-1139

Chapter II[2]

Positive Darwinian selection at single amino acid sites conferring plant virus resistance

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Explicit evaluation of the accuracy and power of Maximum Likelihood (ML) and Bayesian methods for detecting site-specific positive Darwinian selection presents a challenge because selective consequences of single amino acid changes are generally unknown. We exploit extensive molecular and functional characterization of amino acid substitutions in the plant gene *eIF4E* to evaluate the performance of these methods in detecting site-specific positive selection. We document for the first time a molecular signature of

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positive selection within the *eIF4E* coding sequence, and find that sites inferred with statistical significance to undergo positive selection are non-randomly distributed relative to sites that confer virus resistance. A Maximum Likelihood genealogy of genomic *eIF4E* sequences from pepper (*Capsicum*) demonstrates that extant resistance alleles are the result of both convergence and common ancestry. Our results indicate that ML and Bayesian site-specific inference of positive selection generates a correct yet conservative estimate of positive selection. This contradicts previous analyses of sequence data where the functional consequence of each amino acid polymorphism was unknown.

Introduction

Maximum Likelihood (ML) and Bayesian estimators have been a critical development for inferring positive Darwinian selection at the molecular level (Ford 2002; Nielsen and Yang 1998) because they enable identification of individual codon sites under positive selection and eliminate the requirement for population-representative allelic sampling associated with other statistical tests of selection (McDonald and Kreitman 1991; Tajima 1989). ML and Bayesian methods are frequently used to localize the action of positive selection to specific codons and to show tight association between these sites and functionally important regions of the protein they encode (Hughes and Nei 1988; Meyers et al. 1998; Savage and Miller 2006). However, positive selection acting directly on functionally significant amino acids is rarely demonstrated because the fitness-related phenotypic consequences of individual amino acids are usually unknown (Bishop 2005). Consequently, the accuracy of site-specific tests of selection remains fundamentally in question.

Several studies have criticized ML and Bayesian methods, claiming high false-positive rates and low power (Anisimova et al. 2002; Suzuki and Nei 2001). These criticisms compelled us to devise a strategy for unambiguous assessment of ML and Bayesian methods by utilizing the well-characterized plant virus-resistance gene, eukaryotic translation initiation factor 4E (*eIF4E)*. Successful Potyvirus infection of plant tissue requires an interaction with the cap-binding region of the wild-type eIF4E protein (Schaad et al. 2000). In several cultivars of pepper (Kang et al. 2005a; Ruffel et al. 2002), tomato (Ruffel et al. 2005), and pea (Gao et al. 2004), naturally-occurring amino acid substitutions in *eIF4E* have been identified that disrupt virus binding and confer strain-specific resistance to Potyviruses. The individual amino acid changes that define resistant and susceptible *eIF4E* alleles have been carefully detailed genetically (Kang et al. 2005b; Ruffel et al. 2002), biochemically (Kang et al. 2005a; Yeam et al. 2007) and by functional complementation in plants (Gao et al. 2004; Ruffel et al. 2002; Ruffel et al. 2005; Yeam et al. 2007). This precise knowledge of the effects on plant survival due to single substitutions in *eIF4E* provides an *a priori* expectation allowing us to assess the accuracy of Maximum Likelihood and Bayesian methods for inferring positive selection.

Methods

Sequence Data

All plant *eIF4E* coding sequences in GenBank were compiled. Sequences with sufficient homology were aligned using Seqman II version 5.07

(DNASTAR Inc.), including *eIF4E* from *Pisum sativum* (8), *Solanum lycopersicum* (2), *Solanum habrochaites* (formerly *Lycopersicon hirsutum*) (2), *Capsicum annuum* (10), and *Capsicum chinense* (3) (GenBank accession numbers DQ641470-DQ641474, AY423373, AY423374, AY611425, AY723733- AY723736, AY485127- AY485131, AF521963, AF521965, AY725849, AY723737- AY723741). Nine of the 25 sequences are known Potyvirus resistance alleles. Genomic *eIF4E* sequences from 18 genotypes across seven *Capsicum* species (*C. annuum*, *C. baccatum*, *C. Chacoense*, *C. chinense*, *C. frutescens*, *C. minutiflorum*, and *C. pubescens,*) were generated using two primer sets (available upon request) that amplified exon 1 through intron 3 excluding part of intron 1 (GenBank accession numbers EF589572 through EF589607). Amplification was performed by running 31 cycles (94°C for 45 sec, 55° for 45 sec, 72° for 1 min) on a PTC-225 Thermal Cycler, repeating at 50° if a band was not observed. Cloning and sequencing were performed as previously reported (Kang et al. 2005a).

Statistical analysis and genealogy construction

Average ω was measured in DnaSP (Rozas and Rozas 1995) using the modified Nei-Gojobori model with Jukes-Cantor correction. Site-specific ω was measured in PAML (Phylogenetic Analysis using Maximum Likelihood) version 3.13d (Yang 1997). Log-likelihood scores were generated in PAML for models of neutral (M1, M7) and positive (M2, M8) selection and compared using likelihood ratio tests. Bayes Empirical Bayes posterior probabilities were calculated for sites with $\omega > 1$ under M2 or M8. A genealogy of genomic pepper *eIF4E* was constructed in PAUP* (Swofford 2002) using *Capsicum*

minutiflorum as the outgroup. Model parameters were determined using the Akaike information criterion in Modeltest version 3.6 (Posada and Crandall 1998) . The best-fit model (HKY+I) was used with the heuristic search option, TBR branch swapping, MulTrees option in effect, and a neighbor-joining tree as the starting topology. Bootstrap values were determined from 500 full heuristic replicates with 10 random addition sequence replicates.

Results

Positive selection is detected statistically as a nonsynonymous to synonymous nucleotide substitution rate-ratio (ω) significantly greater than 1. Across our 231-nucleotide alignment of 25 complete pepper, tomato and pea *eIF4E* coding sequences, ω is 0.243. This low value is consistent with previous analyses of *eIF4E* across eukaryotes (Athey-Pollard et al. 2002; Gao et al. 2004; Marcotrigiano et al. 1997); an excess of synonymous substitutions is unsurprising because many coding sites experience purifying selection to maintain *eIF4E*'s pleiotropic function of translation initiation. Thus, we performed log-likelihood ratio tests (LRTs) to search for a molecular signature of positive selection acting on sites within the *eIF4E* coding region. Because no *a priori* expectation exists for the distribution of ω values for any given alignment, we compared likelihood values for 2 pairs of models with different assumed ω distributions: (1) M1 (a model of neutral evolution where all sites fall under $\omega < 1$ or $\omega = 1$) versus M2 (a model of positive selection allowing sites to have $\omega > 1$), which assume ω values are drawn from a normal distribution, and (2) M7 versus M8, models that mirror the evolutionary constraints of M1 and M2 but assume ω values are drawn from a beta distribution (Nielsen and Yang 1998). For each LRT, the model allowing sites

to be under positive selection (M2 or M8) fit the *eIF4E* data from potato, pepper, and pea significantly better than the neutral model (M1 or M7) ($P < 0.0001$; Table II.1). Under model M2, most sites within the *eIF4E* coding sequence experience purifying selection ($\omega < 73.5\%$) or neutral evolution ($\omega = 1$; 21.7%), while few codons have a signature of positive selection ($\omega > 1$; 4.85%). However, the mean ω value for sites under positive selection is 7.91 – nearly an 8-fold excess of non-synonymous to synonymous substitutions – indicating that *eIF4E* has a molecular signature of strong positive selection targeted to a small subset of sites. The proportion of sites falling into each ω class was similar under M8 (Table II.1).

Fourteen amino acid sites across the *eIF4E* coding sequence are known to confer or contribute to virus resistance in pepper, tomato or pea (Figure II.1A). The M8 model assigned 12 amino acid sites to the positive selection class, including 9 associated with resistance alleles. Likewise, the M2 model assigned 8 amino acid sites to this class, including 6 with known resistance effects (Figure II.1). Sites identified as positively selected are non-randomly distributed with respect to those involved in virus resistance (binomial probability < 0.001 for both models). When mapped on the predicted tertiary structure of the eIF4E molecule, the sites detected as positively selected cluster in the region of the protein that interacts with the virus (Yeam et al. 2007) (Figure II. 2).

Because ML models including positive selection (M2 and M8) fit our data significantly better than their corresponding neutral models (M1 and M2) (Table II.1), we performed Bayes Empirical Bayes (BEB) calculation of

posterior probabilities for each positively selected codon site to estimate the probability that those sites have true ω values significantly greater than 1 (Yang et al. 2005). This analysis further discriminated between amino acid sites that determine virus resistance and those that do not. Each of the 3 codon sites with BEB posterior probabilities greater than 0.95 (equivalent to $P < 0.05$; sites 70, 76 and 77) are resistance-determining amino acids (Figures II.1 and II.2). The valine-to-glutamate amino acid substitution at position 70 is the only nonsynonymous polymorphism present in the virus-resistant pepper allele *pvr1*^{3} (also known as *pvr2*^{3}) relative to wild-type *eIF4E (Ayme et al. 2007)*. Nonsynonymous mutations at codons 76 and 77 are present in the pea resistance allele *sbm1*^{1} and comprise 2 out of the 3 amino acid differences between resistant and wild-type *eIF4E*. Although the separate effect of each amino acid has yet to be demonstrated in laboratory experiments, they fall in the region of the pea eIF4E protein predicted to be involved in viral RNA binding (Gao et al. 2004). Similarly, site 77 is 1 of 4 amino acid substitutions in the tomato resistance allele *pot-1* relative to wildtype (Figure II.1) (Ruffel et al. 2005).

Alleles *pvr1, pvr1*^{1}, *pvr1*^{2} and *pvr1*^{3} are the 4 known naturally occurring *eIF4E* alleles from pepper that confer viral resistance with different specificities. While *pvr1* has a unique set of 3 substitutions, the remaining 3 alleles contain 1 (*pvr1*^{3}), 2 (*pvr1*^{1}) or 3 (*pvr1*^{2}) common amino acid substitutions relative to wild-type *eIF4E*. These similarities prompted us to investigate the origins of *Capsicum* virus-resistant *eIF4E* alleles in the context of 2 possible evolutionary scenarios: (1) allelic convergence, where the same amino acid substitutions occurred in multiple evolutionary lineages and persisted due to the conferred

selective advantage, or (2) identity by descent, meaning the shared amino acid substitutions arose in an ancestral allele, followed by unique substitutions that gave rise to the set of extant alleles. To differentiate between these scenarios, we constructed an ML genealogy of *eIF4E* alleles from pepper using partial genomic sequences (Figure II.3) (Swofford 2002). Inclusion of intronic *eIF4E* regions to infer evolutionary history confirms that *pvr1*^{1} and *pvr1*^{2} share a common evolutionary origin and form a well-supported, monophyletic clade. Non-coding sequence for *pvr1*^{3} could not be obtained, but the *pvr1*^{3} eIF4E coding region is identical to wild-type *eIF4E* except for a single amino acid substitution (site 70) that is shared with *pvr1*^{1} and *pvr1*^{2} (Ayme et al. 2007) and is therefore likely to be the sister taxon to this clade. In contrast, allele *pvr1* appears to have arisen independently from, and earlier than, the other 2 resistance alleles (Figure II.3). This indicates that phenotypic similarities with the other resistance alleles are the result of convergent evolution.

Discussion

In this study we have shown that the plant virus resistance gene encoding the eIF4E protein appears to be under positive selection based on statistical analyses that consider the rate of accumulation of synonymous and nonsynonymous nucleotide substitutions. This is particularly interesting considering that the viral protein VPg, which interacts directly with eIF4E in susceptible host-pathogen combinations (Schaad et al. 2000), is also positively selected, and the specific viral amino acids under positive selection are found in regions of the protein known to be virulence determinants (Moury

et al. 2004). The evidence we present for positive selection acting within eIF4E as well as VPg therefore suggests a co-evolutionary “arms race” is occuring between host and pathogen in this system (Dawkins and Krebs 1979). Several important implications can be drawn from these results. First, we provide additional support for the theory that molecular evolutionary arms races occur between plant hosts and viral pathogens. Second, we suggest that the statistical methods used for identifying positive selection accurately and precisely pinpoint single amino acids with biological relevance to the fitness of the host.

As expected under an ‘arms race’ scenario, a number of viral strains with different infectivity spectra are known to exist (Kyle and Palloix 1997). In this study we examined the corresponding plant resistance alleles in *Capsicum*. Two of these alleles belong to a lineage found in *Capsicum annuum* that has presumably arisen and diverged to compensate for the development of resistance-breaking viral strains. A third resistance allele, *pvr1* known from *C. chinense*, arose independently from the alleles in *C. annuum* but has a similar phenotype, demonstrating the importance of particular amino acid regions in conferring resistance.

Interaction with translation initiation factors appears to be a common infection strategy for viruses infecting both plants (Schaad et al. 2000) and animals (Schneider and Shenk 1987). Amino acid changes at the eIF4E locus resulting in resistance to a number of viral families have evolved independently in a variety of plant taxa (Yeam et al. 2007). A better understanding of the selective forces driving disease evolution will, we hope,

provide insight into viral infection strategies and offer opportunities to engineer viral resistance in susceptible plant genotypes.

Integrating applied studies of disease-resistant *eIF4E* alleles with statistical and phylogenetic methodologies traditionally reserved for analyzing variation in natural populations has allowed us to assess the performance of a widely used technique in molecular evolutionary biology. Our results support the use of ML and Bayesian methods for detecting site-specific positive selection, particularly in natural populations where the fitness consequences of single amino acid changes cannot experimentally be demonstrated. Further, because ML and Bayesian methods accurately identify amino acids with dramatic biological consequences, such as disease resistance, they provide a powerful predictive tool for targets of genetic crop improvement studies.

Table II.1. Likelihood ratio tests comparing models of neutral evolution (M1, M7) and positive selection (M2, M8) across eIF4E.

Model of selection	ℓ	$2\Delta\ell$[a] ***(df, P-value)***	***Estimated parameters***[b]	***Position of positively selected site (BEB posterior probability)***
M1: nearly neutral	-2123.0		p_0 = 0.731; p_1 = 0.269 ω_0 =0.064; ω_1 = 1(fixed)	
M2: positive selection	-2112.8	20.4 (2, <0.0001)	p_0 = 0.735; p_1 = 0.217; p_2=0.0485 ω_0 = 0.070; ω_1 = 1.00; ω_2 = 7.91	70 (P>0.95) 76 (P>0.99) 77 (P>0.95)
Model 7: beta (10 site classes)	-2124.6		Each of 10 p_0 = 0.1 ω_0 = 0, 0.00022, 0.0030, 0.017, 0.062, 0.16, 0.35, 0.60, 0.85, 0.99	
Model 8: beta&w>1 (11 site classes)	-2111.8	25.6 (2, <0.0001)	Each of 10 p_0 = 0.093; p_1 =0.07 ω_0 = 0.00006, 0.002, 0.010, 0.029, 0.065, 0.12, 0.21, 0.34, 0.52, 0.787; ω_1 = 6.38	70 (P>0.99) 76 (P>0.99) 77 (P>0.95)

[a] Log-likelihood scores (ℓ) are compared for each pair of models (M1 versus M2, M7 versus M8) using the test statistic $2\Delta\ell$**,** with significance evaluated from a Chi-square distribution.

[b] eIF4E coding sequences from *Pisum sativum*, *Solanum lycopersicum*, *Solanum habrochaites*, *Capsicum annuum*, and *Capsicum chinense* are compared. The proportion of amino acid sites (p_n) falling into each selection class (ω_n) is estimated from the data.

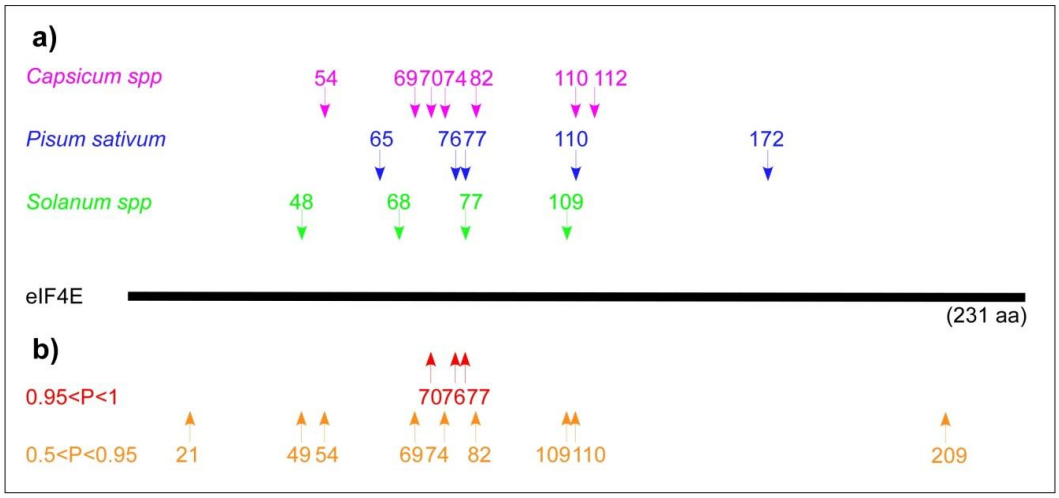

Figure II.1: Spatial relationship between a) eIF4E amino acid substitutions contributing to virus resistance in pepper (purple), pea (blue), and tomato (green) and b) Maximum Likelihood/Bayesian-inferred positively selected amino acid sites ($\omega > 1$; yellow and red). Model M8 detected all positively selected sites depicted. M2 detected the same sites, excluding amino acids 49, 74, 82, and 109. Under both models, sites 70, 76, and 77 had $\omega > 1$ with posterior probability (P) > 0.95 (red).

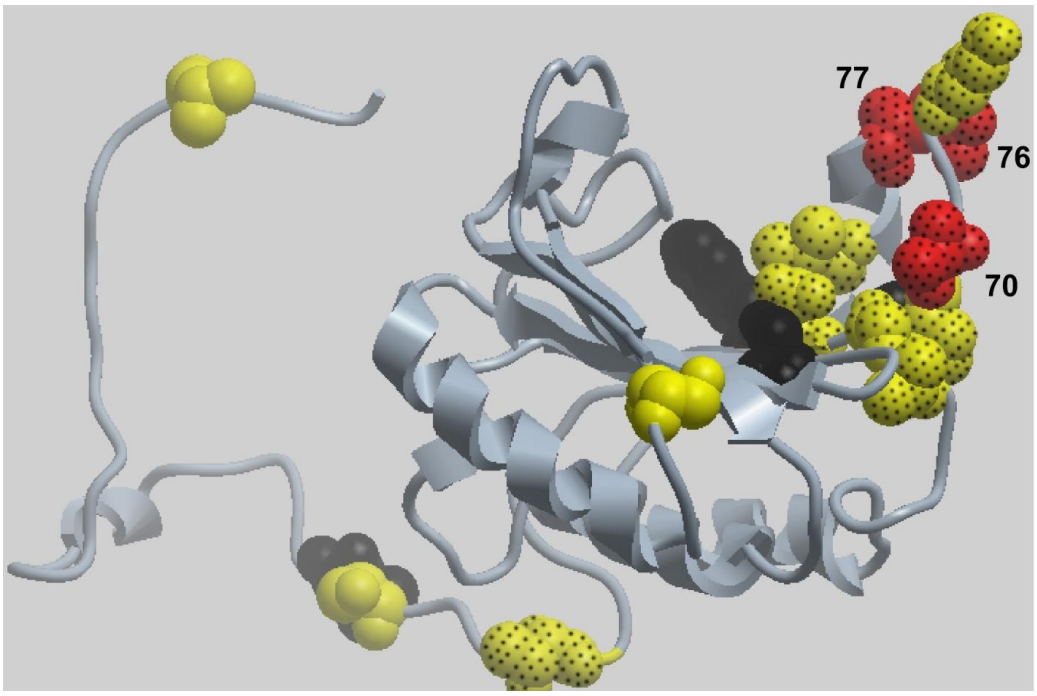

Figure II.2: Positively selected codons identified by M2 and M8 mapped onto the predicted eIF4E 3-dimensional crystal structure (Yeam et al. 2007). Red indicates sites where $\omega > 1$ with posterior probability (P) > 0.95 and yellow indicates sites where $\omega > 1$ with $0.5 < P < 0.95$. All sites were identified equivalently by M2 and M8, except sites 49, 74, 82, and 109, which were only detected as positively selected using M8. Resistance-associated amino acids from pepper, pea, and tomato are depicted in black. Stippling denotes overlap of positively selected sites and sites with known contribution to virus resistance.

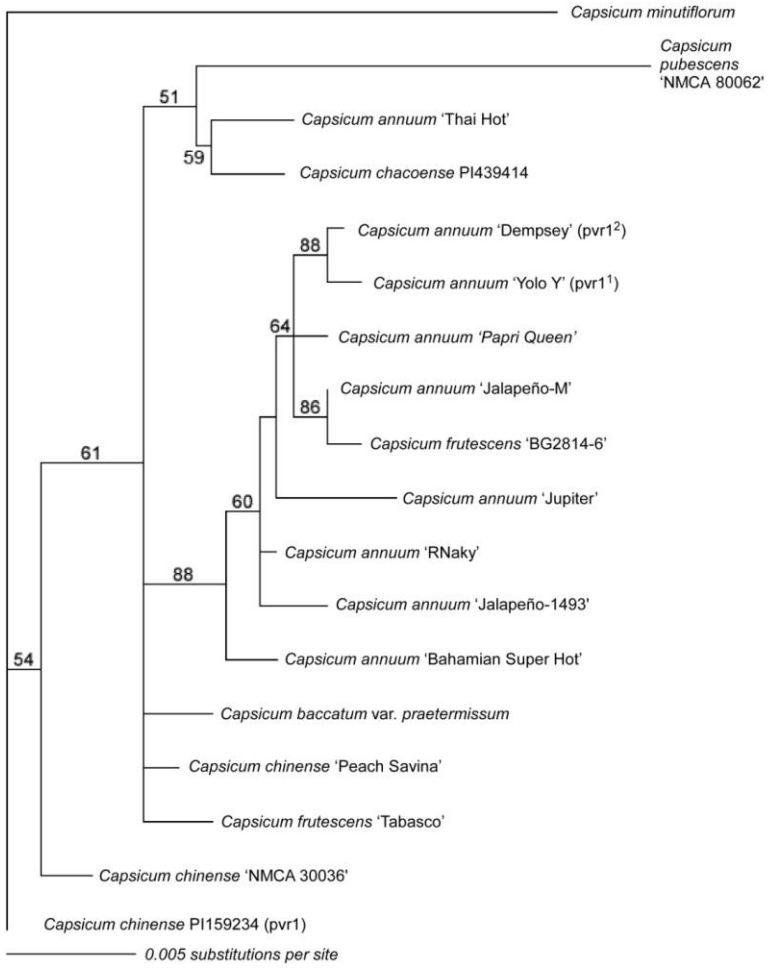

Figure II.3: Maximum Likelihood genealogy of 18 *eIF4E* partial genomic sequences from pepper. The naturally occurring Potyvirus-resistance conferring alleles *pvr1*, *pvr1*^{1} and *pvr1*^{2} are identified in parentheses. Bootstrap values > 50% are indicated.

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REFERENCES

Anisimova M, Bielawski JP, Yang Z (2002) Accuracy and power of bayes prediction of amino acid sites under positive selection. Mol Biol Evol 19:950-8

Athey-Pollard AL, Kirby M, Potter S, Stringer C, Mills PR, Foster GD (2002) Comparison of partial sequence of the cap binding protein (eIF4E) isolated from Agaricus bisporus and its pathogen Verticillium fungicola. Mycopathologia 156:19-23

Ayme V, Petit-Pierre J, Souche S, Palloix A, Moury B (2007) Molecular dissection of the potato virus Y VPg virulence factor reveals complex adaptations to the pvr2 resistance allelic series in pepper. J Gen Virol 88:1594-1601

Bishop JG (2005) Directed mutagenesis confirms the functional importance of positively selected sites in polygalacturonase inhibitor protein. Mol Biol Evol 22:1531-4

Dawkins R, Krebs JR (1979) Arms races between and within species. Proc R Soc Lond B Biol Sci 205:489-511

Ford MJ (2002) Applications of selective neutrality tests to molecular ecology. Mol Ecol 11:1245-62

Gao Z, Johansen E, Eyers S, Thomas CL, Noel Ellis TH, Maule AJ (2004) The potyvirus recessive resistance gene, sbm1, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. The Plant Journal 40:376-385

Hughes AL, Nei M (1988) Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. Nature 335:167-70

Kang BC, Yeam I, Frantz JD, Murphy JF, Jahn MM (2005a) The pvr1 locus in Capsicum encodes a translation initiation factor eIF4E that interacts with Tobacco etch virus VPg. Plant J 42:392-405

Kang BC, Yeam I, Jahn MM (2005b) Genetics of plant virus resistance. Annu Rev Phytopathol 43:581-621

Kyle MM, Palloix A (1997) Proposed revision of nomenclature for potyvirusresistance genes in Capsicum. Euphytica 97:183-188

Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK (1997) Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell 89:951-61

McDonald JH, Kreitman M (1991) Adaptive protein evolution at the Adh locus in Drosophila. Nature 351:652-654

Meyers BC, Shen KA, Rohani P, Gaut BS, Michelmore RW (1998) Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. Plant Cell 10:1833-46

Moury B, Morel C, Johansen E, Guilbaud L, Souche S, Ayme V, Caranta C, Palloix A, Jacquemond M (2004) Mutations in potato virus Y genome-linked protein determine virulence toward recessive resistances in Capsicum annuum and Lycopersicon hirsutum. Mol Plant Microbe Interact 17:322-9

Nielsen R, Yang Z (1998) Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. Genetics 148:929-36

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817-8

Rozas J, Rozas R (1995) DnaSP, DNA sequence polymorphism: an interactive program for estimating population genetics parameters from DNA sequence data. Comput Appl Biosci 11:621-5

Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C (2002) A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). Plant J 32:1067-75

Ruffel S, Gallois JL, Lesage ML, Caranta C (2005) The recessive potyvirus resistance gene pot-1 is the tomato orthologue of the pepper pvr2-eIF4E gene. Mol Genet Genomics 274:346-53

Savage AE, Miller JS (2006) Gametophytic self-incompatibility in Lycium parishii (Solanaceae): allelic diversity, genealogical structure, and patterns of molecular evolution at the S-RNase locus. Heredity 96:434-44

Schaad MC, Anderberg RJ, Carrington JC (2000) Strain-specific interaction of the tobacco etch virus NIa protein with the translation initiation factor eIF4E in the yeast two-hybrid system. Virology 273:300-6

Schneider RJ, Shenk T (1987) Impact of virus infection on host cell protein synthesis. Annu Rev Biochem 56:317-32

Suzuki Y, Nei M (2001) Reliabilities of parsimony-based and likelihood-based methods for detecting positive selection at single amino acid sites. Mol Biol Evol 18:2179-85

Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, MA

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-95

Yang Z (1997) PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci 13:555-6

Yang Z, Wong WS, Nielsen R (2005) Bayes empirical bayes inference of amino acid sites under positive selection. Mol Biol Evol 22:1107-18

Yeam I, Cavatorta JR, Ripoll DR, Kang BC, Jahn MM (2007) Functional Dissection of Naturally Occurring Amino Acid Substitutions in eIF4E That Confers Recessive Potyvirus Resistance in Plants. Plant Cell 9:2913-2928

Chapter III[3]

"Intragenic" Virus Resistance in Potato

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Abstract

Transgenesis enables the creation of novel plant or animal genotypes expressing sequence from almost any organism. This technique has been widely implemented in global agriculture, resulting in dramatic gains in crop yield and yield stability (Lemaux 2008; Lemaux 2009). The engineered expression of genes derived from within sexually compatible barriers is also a useful approach that permits precision breeding by introducing targeted

[3] This work is currently being prepared for publication. My contribution was in doing the majority of the research involving the lines expressing the potato mutant alleles. I developed the constructs, carried out the transgenesis and regeneration of potato plantlets, and tested the transgenic plants for virus resistance. I also was the major contributor for the writing up of results.

mutations to the gene in question (Nielsen 2003). In this study we demonstrate that transgenic expression of a virus resistance gene from pepper confers resistance to *Potato virus Y* (PVY) in potato. We then use this information to convert the susceptible potato ortholog into a *de novo* allele for resistance through precision breeding. Potato plants overexpressing the mutated potato allele are immune to virus infection. The 'intragenic' nature of our approach, whereby the transgene is derived from the target crop itself, avoids many of the concerns surrounding creation of transgenic crops (Rommens 2007). This technique disrupts a key step in the viral infection process and may potentially be used to engineer virus resistance in a number of economically important pathosystems.

Introduction

Viruses are especially problematic in clonally propagated crops such as potato because they have a high chance of being transmitted between generations. Despite a costly nationwide seed certification effort to reduce virus incidence, *Potato virus Y (*PVY) continues to be a major challenge for seed producers with roughly 20% of North American certified seed lots infected (Crosslin et al. 2006). Yield of PVY-infected fields may be reduced by 80% when disease pressure is high (de Bokx and Huttinga 1981), and some strains of PVY make tubers unmarketable due to the development of necrotic lesions (Beczner et al. 1984). Controlling PVY through expensive certification programs will continue to have limited success given the prevalence of cultivars that do not exhibit mosaic symptoms and are therefore difficult to eliminate based on visual inspection (Crosslin et al. 2006).

Host plant resistance is one of the most successful strategies to control viral diseases in crop species (Khetarpal et al. 1998). Plant breeders have therefore utilized dominant resistance genes to develop PVY resistant potato cultivars (Solomon-Blackburn and Barker 2001). Although these cultivars are effectively virus resistant, they have seen little market acceptance due to growers' strong preference for the horticultural characteristics of existing varieties (Douches et al. 1996). This makes genetic engineering an attractive alternative because disease resistance can be added to a cultivar while maintaining all other desirable traits. Widespread commercialization of genetic engineering technology, however, has been hindered in potato by consumer concerns (Borlaug 2000; Kaniewski and Thomas 2004). In this study we have attempted to address both of these issues by engineering PVY resistance using a modified potato gene.

The highly conserved eukaryotic translation initiation factor 4E (eIF4E) has been widely studied because of its essential role in protein translation and its secondary role in phytopathogenic virus infection. Resistance alleles encoding variant forms of *eIF4E* have been found and deployed in breeding of cereals, diverse vegetables, legumes and other crops since the 1950s (Kang et al. 2005b). Although genes at this locus have been used to develop resistant vegetable varieties since the 1950s (Greenleaf 1986), we have only been able to understand the mechanism of virus resistance as current genomic approaches have become available (Kang et al. 2005b).

The eIF4E protein is a host factor that binds to the 5' cap of mRNA and aids in recruitment to the host ribosomal complex (Gingras et al. 1999). A number of plant viruses with single-stranded RNA genomes also interact with eIF4E, often through a virus genome-linked protein (VPg) covalently bound at the 5' terminus of the viral genome (Leonard et al. 2000; Schaad et al. 2000). This interaction is necessary for successful virus infection and is thought to facilitate translation, replication, and/or cell-to-cell movement of the virus genome (Gao et al. 2004; Kang et al. 2005a; Robaglia and Caranta 2006).

Mutations at the *eIF4E* locus have evolved in some plants that disrupt the interaction with VPg, resulting in virus resistant individuals (Cavatorta et al. 2008). The *pvr1* and *pot-1* loci of pepper and tomato, respectively, provide virus resistance against several potyvirus species and have been shown to encode orthologous copies of *eIF4E* (Charron et al. 2008; Kang et al. 2005a; Ruffel et al. 2002; Ruffel et al. 2005). In addition to pepper and tomato, recessive resistance alleles at the *eIF4E* locus have been identified in barley (Stein et al. 2005), lettuce (Nicaise et al. 2003), melon (Nieto et al. 2006), and pea (Gao et al. 2004). Resistant versions of eIF4E differ from the susceptible wildtype form of the protein by between just 1 to 5 amino acid changes. Many of these polymorphisms tend to be clustered in the region of the protein that is predicted to be bound by VPg based on the crystallized structure of murine eIF4E (Gao et al. 2004; Kang et al. 2005a; Marcotrigiano et al. 1997; Nicaise et al. 2003; Ruffel et al. 2005).

Detailed understanding of this system has provided novel means of controlling PVY and other viruses in crop species, such as marker-assisted selection

(Yeam et al. 2005) and transgenesis (Kang et al. 2007). Overexpression of a resistance allele from pepper has been shown to confer virus resistance in tomato, despite the presence of the susceptible endogenous tomato gene (Kang et al. 2007). This “dominant negative” method of transferring virus resistance between plant species may be able to address virus disease problems in crops that have not developed eIF4E-mediated resistance naturally, such as potato.

Building off of previous studies that showed transgenic expression of resistance alleles from pepper conferred virus-resistance in tomato (Kang et al. 2007), this work describes the development of transgenic virus resistance in potato. We demonstrate that transgenic expression of a virus resistance allele from pepper confers resistance to PVY in potato. We then sequence the previously-uncharacterized potato *eIF4E* gene and produce a series of novel alleles by mutating codons implicated in disease resistance. These mutant potato alleles are then expressed ‘intragenically’ in potato, and the resulting plants are challenged with several viral isolates. Our results show that precision breeding can be used to engineer virus resistance in potato, a species that is not known to have naturally evolved resistance alleles at the *eIF4E* locus. Resistant plants contain the trait of interest derived from sequences obtained from within the potato genome. This may have improved consumer acceptance over other transgenic techniques and has the potential to control many plant viruses that are known to interact with eIF4E (Robaglia and Caranta 2006).

Results

Potato plants expressing the pepper allele pvr1^2 *are resistant to PVY*

To establish the potential relevance of eIF4E to the potato-PVY pathosystem, the PVY-susceptible *S*olanum *tuberosum* cultivar '*Russet Burbank'* was transformed with either the beta-glucuronidase (GUS) reporter gene, the wild type pepper gene *Pvr1*+ isolated from a susceptible pepper cultivar¸ or one of the pepper resistance genes *pvr1* or *pvr1*^{2}. Infectivity was monitored by the appearance of visible symptoms and ELISA. Transgenic potato plants expressing pepper alleles *pvr1* or *pvr1*^{2} were tested for resistance to three agriculturally significant potato PVY strains: PVY^O, PVY$^{N:O}$ and PVY^{NTN} (Baldauf et al. 2006). Lines were considered resistant if all three or more plants challenged with each strain of virus remained virus free. Non-transformed plants and positive control plants expressing *Pvr1*+ or *GUS* behaved as expected, displaying strong viral symptoms with high ELISA values (Table III.1A). Transgenic potato plants expressing the pepper gene *pvr1* were also virus-susceptible. Lines expressing *pvr1*^{2}, in contrast, were resistant to all 3 PVY strains (Table III.1A). To verify that resistance precludes virus transmission between generations, tubers were harvested from a subset of inoculated plants. In all cases, next generation plants from tubers of plants expressing *pvr1*^{2} were virus free (data not shown).

Table III.1: Testing of transgenic lines for virus resistance against 3 strains of *Potato virus Y*. 1A) Resistance phenotype of potato plants expressing different eIF4E from pepper or potato. 1B) Amino acid modifications made to potato eIF4E to develop several novel alleles.

a)

Transgene		***# Lines tested***	***PVY^{O}***	***PVY$^{N:O}$***	***PVY^{NTN}***
Negative controls	**Non-transgenic**	1	S	S	S
	GUS	4	S	S	S
Pepper alleles	**Pepper4E:wild type**	5	S	S	S
	Pepper4E:pvr1	5	S	S	S
	Pepper4E:pvr1^{2}	**23[a]**	**R**	**R**	**R**
Potato alleles	**Potato4E:wild type**	1	S	S	S
	Potato4E:G110R	17	S	S	S
	Potato4E:pvr1^{2}	**30[b]**	**R**	**R**	**R**
	Potato4E:pvr1+pvr1^{2}	27	S	S	S
	Potato4E:pot-1	12	S	S	S

[a]out of 23 lines tested, 13 lines were resistant. [b]out of 30 lines tested, 25 were resistant.

b)

Peppere Allele	***Amino Acid Location***								
	48	68	69	70	77	82	109	110	112
Potato4E:wild type	L	S	P	I	A	L	M	G	D
Potato4E:G110R	-	-	-	-	-	-	-	R	-
Potato4E:pvr1^{2}	-	-	-	N	-	R	-	-	N
Potato4E:pvr1+pvr1^{2}	-	-	T	N	-	R	-	R	N
Potato4E:pot-1	F	K	-	-	D	-	I	-	-

Potato eIF4E is similar to its tomato and pepper orthologs

To determine whether we could achieve the same outcome via precision breeding at the *eIF4E* allele in potato, the gene was amplified using primers designed from the 5' and 3' sequence of pepper *eIF4E* and cloned into pCR 2.1 TOPO (Invitrogen). Eighteen separate cDNA molecules of potato *eIf4E* were cloned and sequenced. The consensus potato *eIF4E* cDNA is 696 nucleotides long and contains 231 amino acids with a stop codon at the 3' terminus. Three similar but unique alleles of endogenous potato *eIF4E* were identified (Figure III.3). Each allele was identified independently multiple times. Potato Allele 1 was chosen arbitrarily and used for manipulation to generate novel, potentially resistant, potato *eIF4E* alleles. Potato *eIF4E* is similar to the tomato ortholog *pot-1* and the pepper ortholog *pvr1* (Figure III.1) (Kang et al. 2005a; Ruffel et al. 2002; Ruffel et al. 2005). Potato *eIF4E* shares 97% nucleotide identity and 95% amino acid identity with the susceptible tomato allele *Pot-1*$^{+}$, and 88% nucleotide identity and 86% amino acid identity with the susceptible pepper allele *Pvr1*$^{+}$. The first 19 and last 15 nucleotides of the alignment were not used for these calculations because these nucleotides in the potato sequence were dictated by the primers used in the initial amplification. Like tomato, potato *eIF4E* has 3 more amino acids than the pepper ortholog.

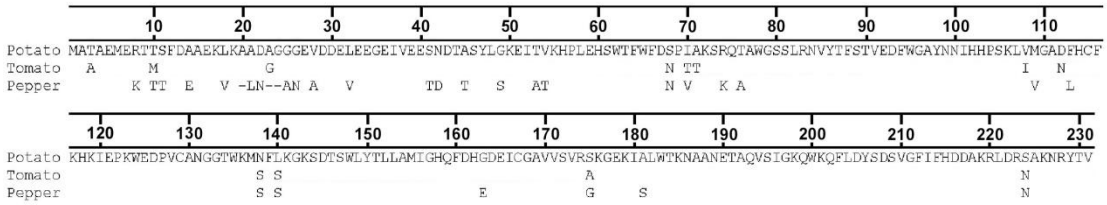

Figure III.1: Amino acid alignment of eIF4E orthologs from potato, tomato, and pepper. Amino acids are identical where not otherwise indicated. Dashes are used to represent delettions in the pepper sequence relative to tomato and potato. The first 7 and last 5 amino acids of the potato sequence were dictated by the primers used for amplification.

Novel potato alleles were generated by site-directed mutagenesis

Four unique potato *eIF4E* alleles were generated by site-directed mutagenesis (Table III.1B). First, novel allele Potato4E:G110R was created by introducing mutation G110R. This mutation is homologous to the G107R mutation in the pepper allele *pvr1* and is known to be responsible for *Tobacco etch virus* (TEV) resistance in transgenic tomato plants expressing eIF4E from pepper (Yeam et al. 2007). Second, novel allele Potato4E:pvr1^2 was created by introducing mutations I70N, L82R, and D112N to mimic the amino acid substitutions in the pepper resistance allele *pvr1*^{2}, which conferred resistance when expressed in potato (Table III.1A). Third, novel allele Potato4E:pvr1+pvr1^2 was created by introducing mutations P69T, I70N, L82R, G110R, and D112N. This allele was generated to mimic all the nucleotide polymorphisms in pepper alleles *pvr1* and *pvr1*^{2} combined into a single allele. When homologous mutations were made to the pepper *eIF4E*, they conferred

TEV resistance in transgenic tomato plants (Yeam et al. 2007). In this case, however, we did not mutate V54 since valine is biochemically similar to alanine, the amino acid found at the homologous position in the resistant pepper allele. Finally, allele Potato4E:pot-1 was created by introducing mutations L48F, S68K, A77D, and M109I to mimic the amino acid substitutions in the tomato resistance allele *pot-1*.

'Intragenic' expression of the Potato4E:pvr1^2 allele results in virus resistant plants

Of the four alleles tested, only potato plants expressing Potato4E:pvr1^2 resulted in a gain of virus resistance (Table III.1A). Combining all substitutions from pvr1 and pvr1^2 resistance alleles (i.e. Potato4E:pvr1+pvr1^2) did not result in virus resistance, but introducing 3 of those 5 changes did result in resistance. It appears that only particular amino acid polymorphisms in the eIF4E protein confer virus resistance.

Eight of the 25 resistant lines expressing Potato4E:pvr1^2 were selected for more in-depth analyses. All 8 lines failed to accumulate detectable levels of viral protein. They remained asymptomatic following inoculation by each of the three PVY strains and no virus was detected by DAS-ELISA (Figure III.2). Copy number of the transferred allele was determined by Southern blot and found to vary between one and four copies for all resistant lines (Figure III.4A). Thus, single or low-copy transformation events appear sufficient for generating virus resistance. RNA expression level of eIF4E was analyzed by Northern blotting and found to be higher in transgenic individuals (Figure III.4B). This

analysis quantified the entire eIF4E mRNA pool and, because of near sequence identity, could not distinguish between endogenous and transgenic transcripts. To better understand the composition of the transcript pool, eIF4E mRNA was amplified from virus resistant plants using *eIF4E*-specific primers. A total of 31 transcripts were cloned and sequenced. All represented transgenic mRNA (data not shown), suggesting that the majority of the eIF4E transcript pool was derived from the transgene rather than the endogenous gene.

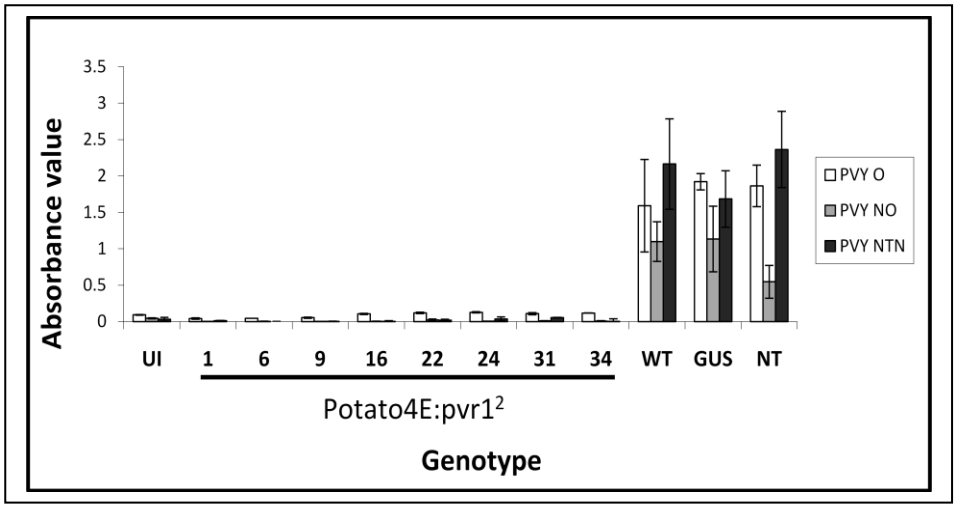

Figure III.2: ELISA results for Potato4E: pvr1^2. ELISA value averages are shown with standard deviation. Four individuals per line were challenged with either PVY strain NTN, NO, or O. ELISA results indicate the 8 transgenic lines tested had significantly less virus accumulation than the susceptible controls. UI = Uninoculated, WT = transgenic line expressing wild type potato eIF4E allele 1. GUS = transgenic line expressing GUS gene. NT = nontransgenic potato cultivar 'Russet Burbank' that was regenerated from callus tissue in culture.

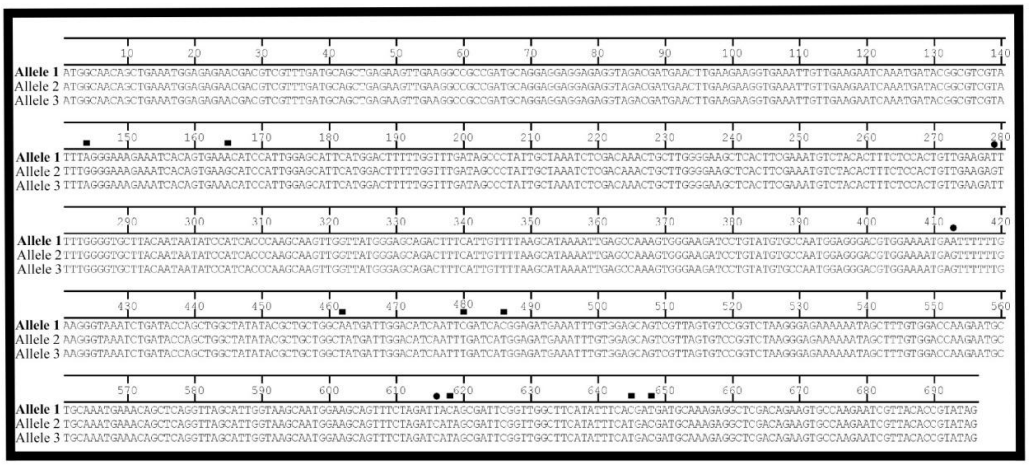

Figure III.3: Nucleotide alignment of the 3 potato *eIF4E* alleles sequenced from cultivar 'Russet Burbank' clone 'Ida'. Solid squares identify nucleotides that have a synonymous polymorphism between alleles. Solid circles identify nucleotides that have a nonsynonymous polymorphism between alleles. Relative to Allele 1, only one nonsynonymous nucleotide polymorphism exists in Allele 2, and only 3 nonsynonymous polymorphisms were found in Allele 3. None of the amino acid polymorphisms between the potato alleles are at sites known to be involved in virus resistance in other Solanaceous species. Relative to Allele 1, there are two synonymous nucleotide polymorphisms in Allele 2, and 8 synonymous nucleotide polymorphisms in Allele 3. The first 19 and last 15 nucleotides of the alignment were dictated by the primers used in the initial amplification.

Despite multiple attempts with several anti-eIF4E antibodies, the potato eIF4E protein could not be visualized. To improve detection of the transgenic protein, an HA tag was added to allele Potato4E:pvr1[2] y PCR. This construct was used to generate transgenic potato plants which were screened for virus resistance. A single resistant line was chosen based on criteria previously

described (data not shown). Western blotting revealed a strong band in transgenic plants, which indicates transgenic protein expression in virus resistant lines (Figure III.4C).

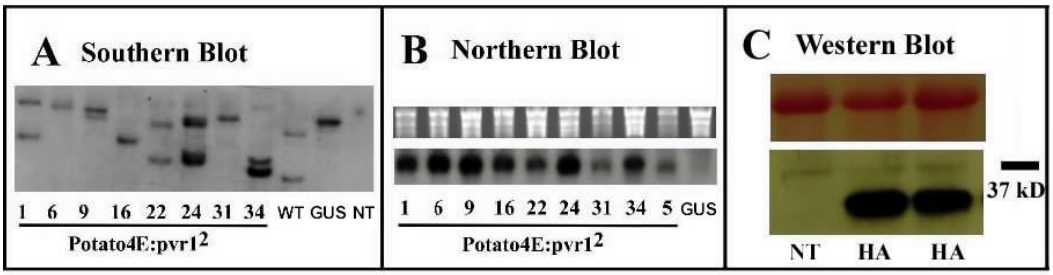

Figure III.4: Characterization of intragenic resistant plants. A) Copy number of 8 lines expressing potato4E:pvr1^2, a control line expressing the wild type potato gene (WT), an empty vector control expressing the GUS gene (GUS), and a nontransgenic control (NT). B) eIF4E mRNA expression level of plants transformed with potato4E:pvr1^2 compared to a plant transformed with GUS. A gel image of the relative amount of RNA loaded (above) is included with a Northern blot (below). C) Western blot of HA-tagged transgenic potato4E:pvr1^2 plants (HA). The endogenous eIF4E (represented by the nontransformed control) does not contain an HA tag and is not detected. An image of the stained membrane is provided to demonstrate equal loading (above). The Western blot (below) was performed using an anti-HA antibody.

Discussion

Previous work has shown that transgenic expression of virus resistance alleles at the *pvr1* locus in pepper confer resistance in tomato (Kang et al. 2007). In this study we demonstrate that potato transgenic lines overexpressing the pepper resistance allele *pvr1*^{2} are resistant to the three predominant strains of PVY (Table III.1A). Resistance appears to persist through generations since all resistant plants yielded tubers with virus-free sprouts. We believe that this virus resistance behaves in a 'dominant negative' manner whereby the transgenic pepper protein is much more abundant than endogenous potato protein and thereby monopolizes translation initiation machinery. This makes ribosomal entry unavailable for endogenous potato eIF4E that may be complexed with an invading virus (Chandler and Werr 2003; Kang et al. 2007). This proposed mechanism is supported by *eIF4E* cDNA cloning, which recovered only *pvr1*^{2}-*eIF4E* sequence from resistant transgenic lines.

eIF4E from a number of plant species have been described in relation to their connection with virus infection including *Arabidopsis (Yoshii et al. 2004)*, barley (Stein et al. 2005), corn (Manjunath et al. 1999), lettuce (Nicaise et al. 2003), melon (Nieto et al. 2006), pea (Gao et al. 2004), pepper (Kang et al. 2005a; Ruffel et al. 2002), tomato (Ruffel et al. 2005), watermelon (Manjunath et al. 1999), and wheat (Monzingo et al. 2007). As part of this work, the *eIF4E* gene in potato is described (Figure III.1). Reverse transcription polymerase chain reaction of potato *eIF4E* yielded a single band. Sequencing of individual cDNA molecules identified three similar alleles, one apparently a result of an intergenic recombination event between the other two. Presumably, cultivar

'Russet Burbank', a tetraploid, is duplex for one of the three alleles. Although this gene is part of a multigene family (Browning 2004; Robaglia and Caranta 2006), the close resemblance of the three alleles identified implies that the primers used resulted in amplification of a single gene copy. The high degree of similarity to the potato ortholog *pot-1* and the pepper ortholog *pvr1* strongly suggests that the gene isolated from potato in this study is the direct ortholog of both of these resistance genes. This was important to establish since PVY has been shown to utilize *pot-1* and *pvr1* but apparently not other members of the multi-gene family such as eIF(iso)4E (Kang et al. 2005a; Ruffel et al. 2002; Ruffel et al. 2005).

The similarity of the potato gene to homologous virus resistance genes in other related plant species made it possible to align with confidence the potato sequence with that of tomato and pepper (Figure III.2). Differences between susceptible and resistant forms of *pot-1* and *pvr1* from these species provided predictions of which amino acids in the potato eIF4E protein are involved in the interaction with the viral protein VPg. Although no natural resistance alleles at this locus in potato are known, we hypothesized that they could be constructed in the laboratory and expressed transgenically to confer resistance in potato. Novel alleles Potato4E:G110R, Potato4E:pvr1+pvr1^2, and Potato4E:pot-1did not confer virus resistance when expressed transgenically in potato plants. Allele Potato4E:pvr1^2 contains 2 fewer amino acid changes than Potato4E:pvr1+pvr1^2 and successfully confers virus resistance when expressed in potato plants. The position in the tertiary structure and the effect on virus resistance is not easily predicted. It appears that making only specific amino acid polymorphisms in the eIF4E protein

confers virus resistance when it is expressed transgenically back in potato. Too few or unsuitable mutations may not modify the protein sufficiently or appropriately to disrupt viral interaction. Too many mutations may cause improper folding and cause the transgenic protein to be degraded, or else the additional mutations may have a compensatory effect to restore wild type function.

Transgenesis of a gene native to a crop genome, categorized by a growing body of researchers as “intragenic” (Nielsen 2003; Rommens 2007; Rommens 2008), may improve market acceptance and reduce regulatory hurdles. Previous reports of genetically engineered disease-resistant potatoes have relied upon pathogen-derived resistance. Concerns over the expression of viral and bacterial genes was a contributing factor to the decision of major potato producers and food companies to cease sales of genetically engineered potato (Kaniewski and Thomas 2004). However, some evidence suggests that consumers would look more favorably upon genetic engineering of vegetables modified with genes from within sexually compatible germplasm sources (Lusk and Sullivan 2002). The work described here uses foreign DNA for selectable markers and regulatory elements, and serves only as proof-of-concept. Recent technology exists to replicate this work using only potato sequences (Rommens 2004) or even to replace the endogenous *eIF4E* gene with the mutant version by zinc finger nuclease-mediated homologous recombination (Wright et al. 2005). By genetically engineering potato to be virus resistant using consumer acceptable methods we hope to develop a useful plant variety with a maximized likelihood of consumer acceptance and commercialization.

Precision-breeding approach may be broadly applicable

All eukaryotes use eIF4E to recruit mRNA to the ribosomal complex (Gingras et al. 1999). A large number of potyviruses are known to require eIF4E for infection, as well as cucumoviruses (Yoshii et al. 2004), carmoviruses (Nieto et al. 2006), and bymoviruses (Stein et al. 2005). We have shown that modified eIF4E from potato, re-introduced and expressed in a susceptible genotype, provides PVY immunity, despite the fact that no known resistance alleles at this locus have ever been described and no viral interaction with potato eIF4E has ever been observed. This methodology may therefore be applicable to other pathosystems, particularly ones involving potyviruses such as plum-*Plum pox virus*, beet-*Beet mosaic virus*, and squash-*Zucchini yellow mosaic virus*. This paper provides the first demonstration of disease resistance developed using precision breeding, whereby an endogenous host gene is isolated, mutated at sites predicted to be important in a host-pathogen interaction, and re-introduced to produce the desired trait. This strategy could potentially be used in any pathosystem where a pathogen relies on a known host factor to carry out its life cycle if that host factor can be manipulated to exclude the pathogen but leaves the host unaffected.

Experimental Procedures

Plant material used

All plant material used in this study consists of potato cultivar ‘Russet Burbank’ strain ‘Ida’. Multiple-node *in vitro* plants were obtained from the North Dakota State Seed Department (http://www.nd.gov/seed/index.aspx). Plants were screened using ELISA to ensure they were virus free. Plants were maintained on Murashige and Skoog media supplemented with 2% sucrose (Murashige and Skoog 1962) with subculturing approximately every 90 days.

Sequencing of potato eIF4E

RNA was extracted from leaves of the ‘Russet Burbank’ clone ‘Ida’ with an RNeasy Plant Mini Kit (Qiagen Inc.). Reverse transcriptase PCR was performed by incubating the resulting RNA with eIF4ESac1R primer (TCCGAGCTCCTATACGGTGTAACG), nucleotides (8 mM each), 5X MMLV buffer, RNase inhibitor (Ambion, Inc), and reverse transcriptase (Promega) for 1 hour at 42ºC. The resulting cDNA was then amplified by PCR using eIF4ESmaIF (TCCCCCGGGATGGCAACAGCTGAAATGG) and eIF4ESac1R primers (see above) (amplification cycle of 94 for 45 sec, 60 degrees for 45 sec, 72 degrees for 45 sec). The PCR product was resolved on a 1% agarose gel. The single resulting band was cut, purified, ligated into the pCR 2.1-TOPO cloning vector (Invitrogen), transformed into the accompanying TOP10 competent *E.coli*, and grown overnight. The following day, colony PCR confirmed 18 independent insertions using M13 primers and the PCR product

was sequenced in both the forward and reverse direction on an automated 3730 DNA Analyzer (Applied Biosystems). Sequences were examined using Seqman software (DNASTAR Inc.).

Generation of novel alleles

Nucleotide polymorphisms were introduced by subjecting potato *eIF4E*, which was cloned into the pCR 2.1-TOPO cloning vector, to site-directed mutagenesis. This was performed using either the Quickchange Site-Directed Mutagensis Kit or the Quickchange Multi Site-directed Mutagenesis Kit (both from Stratagene) following the manufacturer's instructions. Primers were designed to introduce amino acid mutations to produce novel potato alleles with polymorphisms similar to those found in resistant alleles in other species (Table III.2).

Transformation of potato

Potato *eIF4E* wild type and mutant forms were cloned into the plant transformation vector pBI121 using the *Sma1* and *Sac1* restriction sites introduced by PCR. Proper ligation was verified by sequencing and vectors were transformed into the *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium*-mediated transformation was performed on potato stem internode segments from *in vitro*-grown plants (Van Eck et al. 2007). Two to 4 weeks after transfer to rooting medium, DNA was extracted from leaf tissue of putative transformants (Edwards et al. 1991), and PCR was performed using the 35S F primer (GCTCCTACAAATGCCATCATTGCG) and eIF4ESac1R

primer (see above) (amplification cycle of 94 degrees for 45 seconds, 60 degrees for 45 seconds, 72 degrees for 45 seconds). PCR products were resolved on a 1% agarose gel.

Table III.2: Primer sequences used to generate novel potato eIF4E alleles.

Primer Name	Primer Sequence	Mutation
PotatoM2F	GGTTTGATAGCACTATTGCTAAATCTCG	P69T
PotatoM2R	CGAGATTTAGCAATAGTGCTATCAAACC	P69T
PotatoM3F	CCCAAGCAAGTTGGTTATGAGAGCAGACTTTC	G110R
PotatoM3R	GAAAGTCTGCTCTCATAACCAACTTGCTTGGG	G110R
PotatoM4F	GGTTTGATAGCCCTAATGCTAAATCTCGAC	I70N
PotatoM4R	GTCGAGATTTAGCATTAGGGCTATCAAACC	I70N
PotatoM5F	GGGGAAGCTCACGTCGAAATGTCTACAC	L82R
PotatoM5R	GTGTAGACATTTCGACGTGAGCTTCCCC	L82R
PotatoM6F	GGTTATGGGAGCAAACTTTCATTG	D112N
PotatoM6R	CAATGAAAGTTTGCTCCCATAACC	D112N
Pot-1 M1F	CAAATGATACGGCGTCGTATTTTGGGAAAGAAATCACAGT	L48F
Pot-1 M1R	ACTGTGATTTCTTTCCCAAAATACGACGCCGTATCATTTG	L48F
Pot-1 M2F	GGAGCATTCATGGACTTTTTGGTTTGATAAACCTATTGCTAAATCTCG	S68K
Pot-1 M2R	CGAGATTTAGCAATAGGTTTATCAAACCAAAAAGTCCATGAATGCTCC	S68K
Pot-1 M3F	CTAAATCTCGACAAACTGATTGGGGAAGCTCACTTCG	A77D
Pot-1 M3R	CGAAGTGAGCTTCCCCAATCAGTTTGTCGAGATTTAG	A77D
Pot-1 M4F	CATCACCCAAGCAAGTTGGTTATCGGAGCAGACTTT	M109I
Pot-1 M4R	AAAGTCTGCTCCGATAACCAACTTGCTTGGGTGATG	M109I

Screening transgenic plants for virus resistance

Three PVY isolates (PVY^{O}-Oz, $PVY^{N:O}$-PB209 and PVY^{NTN}-PB312), which represent the three strains predominant in the U.S. potato crop, were described previously (Baldauf et al. 2006). The virus isolates were maintained in fresh or frozen *Nicotiana tabacum* 'Samsun NN' tissue that was used to mechanically inoculate young potato plants three to four weeks after propagation from cuttings**.** Three weeks after inoculation, potato leaves were

harvested and tested for virus susceptibility by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as previously described (Baldauf et al. 2006) using the commercial antibodies IF5 to detect PVY^{NTN} and 4C3 to detect PVY^{O} and $PVY^{N:O}$ (Agdia Inc.). Equal amounts of fresh leaf tissue were sampled from multiple locations on the plants and used in ELISA detection the same day it was harvested. When possible, 4 plants per transformation event were tested. All plants with absorbance values significantly greater than uninoculated controls were considered susceptible.

Genomic DNA extraction and Southern Blot

Genomic DNA from 3 susceptible control lines and 8 transgenic resistant lines expressing the Potato4E:$pvr1^{2}$ allele was extracted from whole leaf tissue (Prince et al. 1993). Southern blotting was performed using 10 micrograms of EcoRI-digested potato genomic DNA and probed with 100 ng of *NPTII*-specific sequence (Moore et al. 2005).

Northern Blot

RNA from approximately 100 mg of transgenic potato leaf tissue was extracted using an RNeasy Plant Mini Kit (Qiagen Inc.). RNA was resolved on a 1.2 g agarose gel (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 0.7% formaldehyde) and transferred to an Amersham Hybond-XL membrane (GE Healthcare) overnight using upward capillary transfer. The membrane was UV crosslinked and probed overnight with approximately 75 nanograms of potato *eIF4E* cDNA radioactively labeled using Ready-To-Go DNA Labeling Beads

(GE Healthcare) in ULTRAhyb hybridization buffer (Ambion). The membrane was washed with decreasing concentrations of SSC (0.1% w/v SDS) and exposed to film at -80 degrees C.

Hemagglutinin Tag

A Hemagglutinin (HA) Tag was added to the N-terminus of allele Potato4E:M456 consisting of the following amino acid sequence: YPYDVPDYA. Three PCRs were run sequentially (amplification cycle of 94 degrees for 45 seconds, 60 degrees for 45 seconds, 72 degrees for 45 seconds). After each PCR the product was cloned and sequenced. Three different forward primers were used to add the HA Tag: 5'HA1F (GACTATGCCGCAACAGCTGAAATGGAG), 5'HA2F (TGACGTGCCTGACTATGCCGCAACAGC), and 5'HA3F (ATGTATCCTTATGACGTGCCTGACTATGC).

Western Blot

Approximately 100 mg of potato leaf tissue was ground in liquid nitrogen and resuspended in 100 µL GTEN protein extraction buffer (10% glycerol, 25 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) and 2X SDS loading buffer with 10 mM dithiothreitol and 1% plant protease inhibitor added just before resuspension. The sample was boiled for 10 minutes, centrifuged at max speed for 8 minutes in a microcentrifuge, and run on a 12% PAGE gel. Protein was transferred to a Nitrocellulose membrane and detected using an anti-HA High Affinity antibody (Roche) with a goat anti-rat HRP secondary

antibody (Santa Cruz Biotechnology) and an ECL Plus detection kit (GE Healthcare). Antibodies were incubated with the membrane for approximately 1 hour at a 1:2000 (primary) or 1:5000 (secondary) dilution in TBS-T and washed with TBS-T 3 times for 5 minutes between incubations.

REFERENCES

Baldauf P, Gray S, Perry K (2006) Biological and Serological Properties of Potato virus Y Isolates in Northeastern United States Potato. Plant Disease 90:559-566

Beczner L, Horváth J, Romhányi I, Förster H (1984) Studies on the etiology of tuber necrotic ringspot disease in potato. Potato Research 27:339-352

Borlaug NE (2000) Ending world hunger. The promise of biotechnology and the threat of antiscience zealotry. Plant Physiol 124:487-90

Browning KS (2004) Plant translation initiation factors: it is not easy to be green. Biochem Soc Trans 32:589-91

Cavatorta JR, Savage AE, Yeam I, Gray SM, Jahn MM (2008) Positive darwinian selection at single amino Acid sites conferring plant virus resistance. J Mol Evol 67:551-9

Chandler JW, Werr W (2003) When negative is positive in functional genomics. Trends in Plant Science 8:279-285

Charron C, Nicolai M, Gallois JL, Robaglia C, Moury B, Palloix A, Caranta C (2008) Natural variation and functional analyses provide evidence for co-evolution between plant eIF4E and potyviral VPg. Plant J 54:56-68

Crosslin JM, Hamm PB, Hane DC, Jaeger J, Brown CR, Shiel PJ, Berger PH, Thornton RE (2006) The Occurrence of PVY^{O}, PVY^{N}, and $PVY^{N:O}$ Strains of *Potato virus Y* in Certified Potato Seed Lot Trials in Washington and Oregon. Plant Dis 90:1102-1105

de Bokx JA, Huttinga H (1981) Potato Virus Y. Descriptions of plant viruses. Association of applied biosciences. Available online at http://www.dpvweb.net/dpv/showdpv.php?dpvno=242. 242

Douches DS, Maas D, Jastrzebski K, Chase RW (1996) Assessment of Potato Breeding Progress in the USA over the Last Century. Crop Sci 36:1544-1552

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19:1349

Gao Z, Johansen E, Eyers S, Thomas CL, Noel Ellis TH, Maule AJ (2004) The potyvirus recessive resistance gene, sbm1, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. The Plant Journal 40:376-385

Gingras AC, Raught B, Sonenberg N (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68:913-63

Greenleaf WH (1986) Pepper Breeding *In* Breeding Vegetable Crops ed. M. Bassett. The AVI Publishing Company, Inc.:67 - 134

Kang BC, Yeam I, Frantz JD, Murphy JF, Jahn MM (2005a) The pvr1 locus in Capsicum encodes a translation initiation factor eIF4E that interacts with Tobacco etch virus VPg. Plant J 42:392-405

Kang BC, Yeam I, Jahn MM (2005b) Genetics of plant virus resistance. Annu Rev Phytopathol 43:581-621

Kang BC, Yeam I, Li H, Perez KW, Jahn MM (2007) Ectopic expression of a recessive resistance gene generates dominant potyvirus resistance in plants. Plant Biotechnol J:1-11

Kaniewski WK, Thomas PE (2004) The Potato Story. AgBioForum 7:41-46

Khetarpal RK, Maisonneuve B, Maury Y, Chalhoub B, Dinant S, Lecoq H, Varma A (1998) Breeding for resistance to plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant virus disease control. APS PRESS, St. Paul, Minnesota

Lemaux PG (2008) Genetically Engineered Plants and Foods: A Scientist's Analysis of the Issues (Part I). Annu Rev Plant Biol 59:771-812

Lemaux PG (2009) Genetically Engineered Plants and Foods: A Scientist's Analysis of the Issues (Part II). Annual Review of Plant Biology 60

Leonard S, Plante D, Wittmann S, Daigneault N, Fortin MG, Laliberte JF (2000) Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. J Virol 74:7730-7

Lusk J, Sullivan P (2002) Consumer Acceptance of Genetically Modified Foods. FoodTechnology 56:32-37

Manjunath S, Williams AJ, Bailey-Serres J (1999) Oxygen deprivation stimulates Ca2+-mediated phosphorylation of mRNA cap-binding protein eIF4E in maize roots. The Plant Journal 19:21-30

Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK (1997) Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell 89:951-61

Monzingo AF, Dhaliwal S, Dutt-Chaudhuri A, Lyon A, Sadow JH, Hoffman DW, Robertus JD, Browning KS (2007) The Structure of Translation Initiation Factor eIF4E from Wheat Reveals a Novel Disulfide Bond. Plant Physiol

Moore S, Payton P, Wright M, Tanksley S, Giovannoni J (2005) Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. J. Exp. Bot. 56:2885-2895

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15:473-497

Nicaise V, German-Retana S, Sanjuan R, Dubrana MP, Mazier M, Maisonneuve B, Candresse T, Caranta C, LeGall O (2003) The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the Potyvirus Lettuce mosaic virus. Plant Physiol 132:1272-82

Nielsen KM (2003) Transgenic organisms--time for conceptual diversification? Nat Biotechnol 21:227-8

Nieto C, Morales M, Orjeda G, Clepet C, Monfort A, Sturbois B, Puigdomenech P, Pitrat M, Caboche M, Dogimont C, Garcia-Mas J, Aranda MA, Bendahmane A (2006) An eIF4E allele confers resistance to an uncapped and non-polyadenylated RNA virus in melon. Plant J 48:452-62

Prince JP, Pochard E, Tanksley SD (1993) Construction of a molecular linkage map of pepper and a comparison of synteny with tomato. Genome 36:404-17

Robaglia C, Caranta C (2006) Translation initiation factors: a weak link in plant RNA virus infection. Trends Plant Sci 11:40-5

Rommens CM (2004) All-native DNA transformation: a new approach to plant genetic engineering. Trends Plant Sci 9:457-64

Rommens CM (2007) Intragenic crop improvement: combining the benefits of traditional breeding and genetic engineering. J Agric Food Chem 55:4281-8

Rommens CM (2008) The need for professional guidelines in plant breeding. Trends in Plant Science 13:261-263

Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C (2002) A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). Plant J 32:1067-75

Ruffel S, Gallois JL, Lesage ML, Caranta C (2005) The recessive potyvirus resistance gene pot-1 is the tomato orthologue of the pepper pvr2-eIF4E gene. Mol Genet Genomics 274:346-53

Schaad MC, Anderberg RJ, Carrington JC (2000) Strain-specific interaction of the tobacco etch virus NIa protein with the translation initiation factor eIF4E in the yeast two-hybrid system. Virology 273:300-6

Solomon-Blackburn RM, Barker H (2001) Breeding virus resistant potatoes (Solanum tuberosum): a review of traditional and molecular approaches. Heredity 86:17-35

Stein N, Perovic D, Kumlehn J, Pellio B, Stracke S, Streng S, Ordon F, Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive Bymovirus resistance in Hordeum vulgare (L.). Plant J 42:912-22

Van Eck J, Conlin B, Garvin D, Mason H, Navarre D, Brown C (2007) Enhancing beta-carotene content in potato by rnai-mediated silencing of the beta-carotene hydroxylase gene. American Journal of Potato Research 84:331-342

Wright DA, Townsend JA, Winfrey RJ, Jr., Irwin PA, Rajagopal J, Lonosky PM, Hall BD, Jondle MD, Voytas DF (2005) High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J 44:693-705

Yeam I, Cavatorta JR, Ripoll DR, Kang BC, Jahn MM (2007) Functional Dissection of Naturally Occurring Amino Acid Substitutions in eIF4E That Confers Recessive Potyvirus Resistance in Plants. Plant Cell 9:2913-2928

Yeam I, Kang BC, Lindeman W, Frantz JD, Faber N, Jahn MM (2005) Allele-specific CAPS markers based on point mutations in resistance alleles at the pvr1 locus encoding eIF4E in Capsicum. Theor Appl Genet 112:178-86

Yoshii M, Nishikiori M, Tomita K, Yoshioka N, Kozuka R, Naito S, Ishikawa M (2004) The Arabidopsis cucumovirus multiplication 1 and 2 loci encode translation initiation factors 4E and 4G. J Virol 78:6102-11

Chapter IV[4]

Field Validation of Intragenic Virus Resistance in Potato

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The previous chapter outlines a technique used to engineer virus resistance in potato. The potato *eIF4E* gene was isolated and characterized. Based on homology to orthologs in other species it was mutated at precise codon sites predicted to be involved in disease resistance. The resulting mutant alleles were cloned into a plant transformation vector and expressed transgenically (or "intragenically") in potato. Transgenic plants were inoculated with *Potato virus Y* using mechanical infection in the greenhouse. A number of resistant lines were identified and characterized in detail. However, several outstanding questions remain. First, does virus resistance hold up in the field? Second, is there a yield penalty associated with virus resistance? Finally, will tubers from virus resistant plants produce sprouts that are virus free?

[4] My contribution to this work was in planning and carrying out the field experiment and collecting the majority of the data from the field. I also was responsible for virus indexing in the field and in the greenhouse, analyzing all data collected and writing up this report.

Although transgenic plants are resistant to mechanical virus infection in the greenhouse, it is not clear whether virus resistance will hold up under agricultural conditions. A greenhouse environment differs from that on a grower's field in many ways such as soil type, spacing, fluctuations in temperature, irregular moisture availability, and increased pressure from weeds and disease. Perhaps more importantly, virus transmission in the field occurs through aphid vectors as opposed to mechanical inoculation. Resistance, even immunity under one set of conditions does not necessarily mean that resistance is effective under other conditions. For instance, we have found in this work that grafting of transgenic potato scions onto nontransgenic infected rootstock resulted in virus accumulation in tissues that are immune to mechanical infection (see results). Other work has found that pathogen-derived resistance to *Potato virus Y* (PVY) in potato was effective in the greenhouse but failed in the field (Schubert et al. 2004).

Many forms of disease resistance appear to be associated with a yield penalty. In conventional breeding using resistance genes from wild relatives it can be difficult to distinguish whether this yield cost is due to linkage with undesirable traits or pleiotropy (Duprat et al. 2002). Transgenic disease resistance avoids yield costs associated with linkage, but may still experience losses due to pleiotropy (Bergelson and Purrington 1996). In this work we mutate the potato gene *eIF4E*, which is involved in translation initiation of host messenger RNA, and overexpress it in potato (Gingras et al. 1999). It is therefore conceivable that manipulating expression of this gene, particularly overexpression of a mutated version, may alter translation of host messenger RNA. This could have major effects on many aspects of normal plant

development including such agronomically important traits as tuber yield and specific gravity.

A single potato plant produces only a few tubers that can be used the following season for seed (Elke et al. 1997). Potato production relies on multiple generations of vegetative propagation in order to produce commercial quantities of seed tubers. It is therefore not sufficient for the mother potato plant to be virus resistant. The sprouts obtained from tubers harvested must be virus-free for planting the next season. Early work performed by the Monsanto subsidiary company Naturemark towards producing their transgenic potato cultivar Newleaf Plus, which was resistant to *PLRV*, resulted in potato lines that yielded virus-infected sprouts the following generation. Although 47 transgenic lines were obtained that had desirable agronomic traits, only 31 had virus-free sprouts (Kaniewski and Thomas 2004).

The purpose of this work is to develop potato plants that are agronomically useful. While the existence of plants resistant to mechanical infection in the greenhouse is useful for scientific study, further work is needed to confirm the commercial utility of developing virus resistant crops using this method. In order to answer these questions and validate the functionality of intragenic virus resistance, field experiments were conducted during the summer of 2009.

Materials and Methods

Grafting

Grafting experiments were conducted by first inoculating nontransgenic 'Russet Burbank' potatoes with PVY strain NTN. Two weeks after inoculation, transgenic scions overexpressing the mutant allele Potato4E:pvr1^2 line 6 and nontransgenic control scions were grafted onto infected scions. Grafting was performed by making a longitudinal incision in both the rootstock and scion, inserting the scion along the two incisions, and wrapping the graft in parafilm. After one month, disease was analyzed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as previously described (Baldauf et al. 2006) using the commercial antibody IF5 (Agdia Inc.).

Cuttings were then made from graft-inoculated scions and the plants were grown for 1 month. ELISA was performed on leaf tissue to determine whether virus was present in transgenic tissue. This was performed in order to determine whether virus was able to replicate in transgenic tissue following graft-inoculation.

Production of minitubers

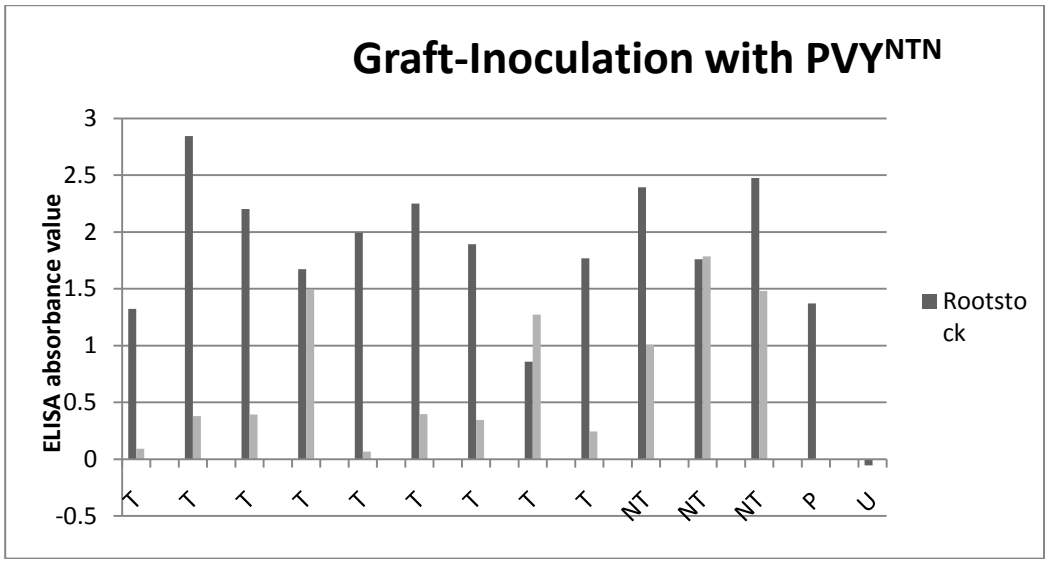
Minitubers were produced in the greenhouse. Cuttings were made from month-old plants in the fall of 2008 between October 21 and November 20. At least 75 plants for each of 8 genotypes were grown. Four lines overexpressed the mutated allele potato4E:pvr1^2, one line was not transformed, and the

remaining lines overexpressed either the pepper allele pvr1^2, wildtype potato *eIF4E*, or the GUS reporter gene (Table IV.1). Plants were tied to wooden stakes and grown for several months. Tops were cut off in early March and the tubers were allowed to dry out in pots for 2 weeks. Tubers were harvested and sorted between March 23 and 26, 2009. They were then placed at 40 degrees C for 1 week followed by warming at room temperature for one day. This sequence of cooling and heating was repeated twice. Because this was not enough time to fully break dormancy, tubers were treated for 3 days with Rindite vapor in a sealed container.

Table IV.1: Treatments assigned to 8 different transgenic lines that were tested in the field in the summer of 2009.

Treatment	Transgene	Line #
1	Nontransgenic	3
2	Potato4E:pvr1^2	1
3	Potato4E:Wildtype	3
4	GUS	23
5	Pepper4E:pvr1^2	21
6	Potato4E:pvr1^2	6
7	Potato4E: pvr1^2	16
8	Potato4E: pvr1^2	34

A



B

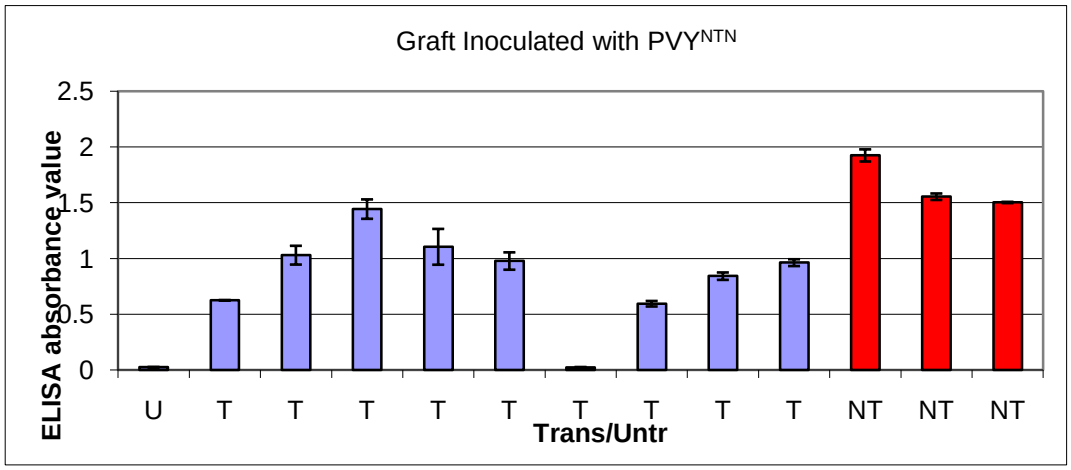


Figure IV.1: ELISA data for graft-inoculated transgenic potato scions overexpressing allele Potato4E:M456. A) Transgenic scions expressing mutant allele grafted onto infected rootstock. Adjacent bars correspond to samples taken from the rootstock and the scion grafted onto that rootstock, respectively. Rootstock consisted of Russet Burbank that had been inoculated with PVY strain NTN. Scions were either nontransformed or were taken from transgenic line 6 overexpressing allele Potato4E:M456. Each bar corresponds

to an average of samples taken from the same plant. B) ELISA values corresponding to presence of virus in plants grown from cuttings of graft-inoculated scions. T = transgenic scion, NT = nontransgenic scion, P = virus infected positive control, U = Uninoculated negative conrol.

Field design and planting

Tubers were planted in two locations: Ithaca, NY (42 27' 40.40N x 76 26'09.93W) and Kimberly, Idaho (42 32'54.665N x 114 20'11.210W) in a complete randomized block design (Figure IV.2). Two 24-plant rows per genotype per replicate were planted in Ithaca. One 24-plant row per genotype per replicate was planted in Idaho. Plants were grown according to standard conditions for each of the two areas. Tubers were planted with 10.6 inch in-row spacing and 3 feet between-row spacing. In New York, insecticide (Admire 6 fl oz/acre) was applied as an in-furrow drench. Fungicides (Quadris or Ridamil 6 fl oz/acre) were applied as an in-furrow drench and intermittently throughout the season to control for late blight. Irrigation was used in Idaho but not in New York. A Notification was received from the United States Department of Agriculture Animal and Plant Health Inspection Service Biotechnology Regulatory Service department permitting planting of transgenic material (notification # 08329102n). Rindite-treated minitubers were planted in the field using a 4-row planter on May 26, 2009 (Ithaca) and June 1, 2009 (Idaho). The cultivar Shepody was used as the border (‘Marcy’ and ‘Snowden’ were used in Ithaca once ‘Shepody’ seed ran out) because, being asymptomatic for PVY infection, it typically has high levels of infection. Based

on ELISA testing, this turned out to be true in our field. Four rows surrounding the experimental lines were inoculated mechanically on July 1, 2009 and again on July 6, 2009 with PVY^{O} isolate Oz, PVY^{NO} isolate PB 209, and PVY^{NTN} isolate PB 312 in order to provide higher levels of virus inoculum (yellow-shaded rows in Figure IV.2). Inoculation of 'Shepody' rows was performed by putting infected tobacco leaves in a blender with phosphate buffer (Phosphate Buffer pH 7.5 (0.1M K_2HPO_4, 0.025M KH_2PO_4) using 1.5 to 5.0 mL buffer per 1 to 1.5 g leaf tissue, screening through cheesecloth, mixing the inoculum with carborundum (6 milligrams/milliliter), and spraying onto plants in the field using an automotive paint sprayer connected to an air compressor.

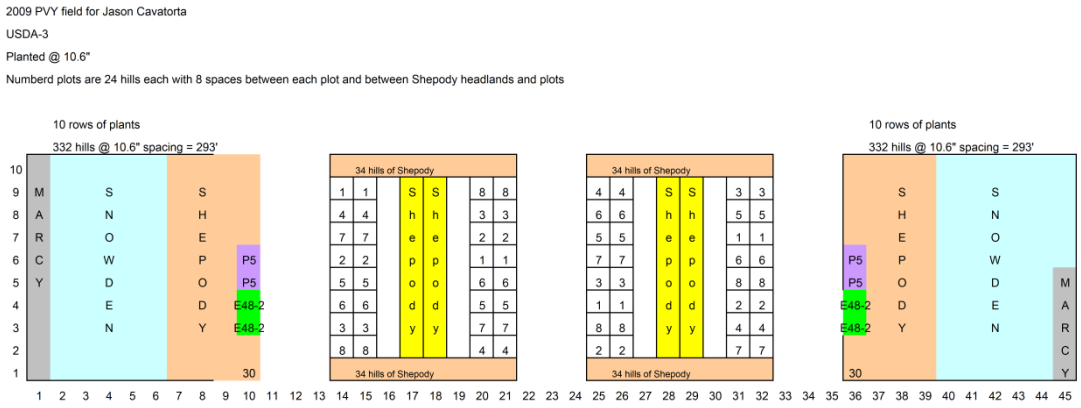


Figure IV.2: Planting plan for Ithaca, NY. Numbers 1-8 refer to the experimental genotypes listed in Table IV.1. 'Shepody', 'Snowden', and 'Marcy' are nontransgenic cultivars used as border plants. Yellow-shaded regions were inoculated with a mixture of PVY strains O, NO, and NTN. Cultivars 'Purple 5' (P5) and 'E48-2' were included as a separate experiment to test for disease resistance.

Virus Indexing in the Field

Virus was analyzed in the field by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as previously described (Baldauf et al. 2006) using the commercial antibody 4C3 (Agdia Inc.). Eight 5-leaf samples were collected randomly per genotype per replicate.

Harvesting and yield measurements

Vines were killed with herbicide in Ithaca on September 16, 2009. In Idaho, vines were 'beaten' and killed mechanically. Tubers were dug mechanically and collected by hand on October 5, 2009 (Ithaca) and October 13, 2009 (Idaho). The entire plot was harvested. Number of plants were not recorded. They were brought to the field house and sorted into size categories of less than 4 ounces, 4-6 ounces, 6-12 ounces, greater than 12 ounces, and malformed. For each category, the total weight and number of tubers were recorded. Specific gravity was measured using a hydrometer.

Winter Growout Disease Indexing

A random sample of 48 tubers per genotype per replicate were selected from the fall harvest. Tubers were collected randomly from all size classes. These were treated with Rindite and planted in November (Ithaca tubers) or stored in the cold (40 degrees) until late February (Idaho tubers). Tubers were sprouted in the greenhouse and assayed for visual symptoms. ELISA readings were

also taken on four 5-leaf composite samples per genotype per replicate. Several symptomatic plants were assayed further to determine the strain of PVY in the infected plant.

Characterization of PVY Isolates

PVY isolates were characterized using a reverse transcription Multiplex PCR Assay as previously described (Lorenzen et al. 2006).

Results

Graft-inoculated tissue accumulates virus

Nontransgenic potato plants were infected with PVY^{NTN} and used as rootstock for grafting experiments. Transgenic scions overexpressing allele Potato4E:$pvr1^2$ were grafted onto infected plants along with nontransgenic susceptible control scions. ELISA results confirmed the presence of virus in the rootstock and in all scions tested (Figure IV.1A). This results suggests that although transgenic plants are resistant to mechanical inoculation in the greenhouse, virus can move into transgenic tissue. Cuttings were made from graft-inoculated scions to determine whether transgenic tissue would be able to support viral replication. With the exception of 1 cutting, all transgenic plantlets appeared to support virus replication following graft inoculation (Figure IV.1B). Virus levels were appreciable, but slightly lower than nontransformed controls.

Intragenic virus resistance is effective in the field

The Ithaca location experienced high virus disease pressure in the 2009 growing season due to infected border plants. Left over border 'Shepody' seed was planted in the greenhouse in the fall of 2009 and the resulting sprouts were indexed for PVY using ELISA. Of the 52 plants that produced foliage, 10 (19%) were found to be infected with PVY. Nine of the 10 PVY-infected plants were further analyzed and found to be infected with PVY strain O (strain of one sample could not be determined due to multiplex failure) (Table IV.2). High levels of PVS were also present in the border rows (Table IV.2). However, all transgenic and nontransgenic treatments were observed to become infected (data not shown). Transgenic lines to not appear to be resistant to PVS.

The three susceptible check lines became infected with virus. This was obvious from visual symptoms in the field and was confirmed with ELISA. Eight 5-leaf samples were tested for each genotype in each replicate for a total of 32 samples. All check lines had heavy virus infection. Treatment 1 (nontransgenic) had 30/32 (94%) positive samples, Treatment 3 (overexpressing allele Potato4E:Wildtype) had 29/32 (91%) positive samples, and Treatment 4 (overexpressing GUS) had 27/32 (84%) positive samples. In contrast, no virus was detected in Treatments 2, 6, 7, and 8 (overexpressing Potato4E:pvr1^2) and Treatment 5 (overexpressing Pepper4E:pvr1^2) (Table IV.3).

Table IV.2: Virus indexing of remnant ‘Shepody’ seed. This information was used to assess inoculum levels in the Ithaca field location in the summer of 2009. Fifty-two sprouted seed tubers that were left over after planting were analyzed for virus infection. Only data from PVS or PVY positive tubers is reported.

Plant Number (out of 52 total)	PVS	PVY
1	+	+ strain O
4	+	-
5	+	-
14	-	+ strain NA
15	+	+ strain O
20	-	+ strain O
25	+	-
26	+	-
28	+	+ strain O
29	+	-
32	-	+ strain O
33	-	+ strain O
40	-	+ strain O
41	-	+ strain O
42	+	-
43	+	-
47	-	+ strain O
50	+	-
51	+	-

Table IV.3: Number of PVY positive samples detected in the field in New York in 2009. Each sample consists of 5-leaf composites taken on August 19 and tested for PVY using ELISA. *Treatments 1, 3, and 4 represent susceptible check lines.

	Number of PVY Positive Samples				
Treatment	Replicate I	Replicate II	Replicate IV	Replicate IV	Total
1*	6/8	8/8	8/8	8/8	30/32
2	0/8	0/8	0/8	0/8	0/32
3*	8/8	6/8	7/8	8/8	29/32
4*	5/8	8/8	8/8	6/8	27/32
5	0/8	0/8	0/8	0/8	0/32
6	0/8	0/8	0/8	0/8	0/32
7	0/8	0/8	0/8	0/8	0/32
8	0/8	0/8	0/8	0/8	0/32

No yield cost appears to be associated with intragenic virus resistance

In order to analyze whether yield differences existed between transgenic virus resistant potato lines and susceptible controls, we collected information on two yield components: tuber weight and tuber number. These measurements are reported as averages of the four blocks at each site, and tuber sizes were separated into several established market classes (less than 4 ounces, 4-6 ounces, 6-12 ounces, greater than 12 ounces, and misformed) (Tables IV.4, IV.5, IV.6, and IV.7). Because the number of individual plants in the field differed between locations, we first analyzed this information at each site separately and then used a plot size factor to combine the data and analyze them together. A one-way Analysis Of VAriance (ANOVA) was run on the

total weight and total tuber number per genotype (identified by treatment). When a Tukey range test (Tukey 1994) was performed to identify which lines were significantly different, resistant lines performed comparably to the nontransgenic control.

These tests assume independence of samples, that the distribution of the residuals are normal, and that their variances are roughly equivalent. Distributions of all data sets appeared to have relatively equal variances (Figure IV.3). A Levene's test (Levene 1960) was run for each comparison. In no case was the null hypothesis of homoscedasticity rejected (data not shown).

In Idaho, genotype had a strong effect on yield. Significant differences in the means existed for total tuber yield ($F = 0.02$; Table IV.4) and total tuber number ($F = 0.03$; Table IV.5). A Tukey range test was performed to identify which genotypes were significantly different from each other. Treatment 8 (a transgenic virus resistant line) had a higher total tuber weight and total tuber than treatment 3 (overexpressing Potato4E:wildtype) (Tables IV.4 and IV.5). Treatment 1, the nontransgenic control, was comparable to resistant lines.

In New York, tuber weight differed between genotypes ($F < 0.0001$), but genotype did not have a significant effect on tuber number ($F = 0.094$). A Tukey range test showed that Treatment 3 had significantly lower total tuber weight than treatments 1, 2, 6, and 8. The nontransgenic control, treatment 1, had higher tuber weight than 3, 4, 5, and 7, but was comparable to lines 2, 6, and 8, which are transgenic and virus resistant.

Looking at the data from the two field locations separately did not indicate any differences in yield between transgenic virus resistant lines and the nontransformed control line.

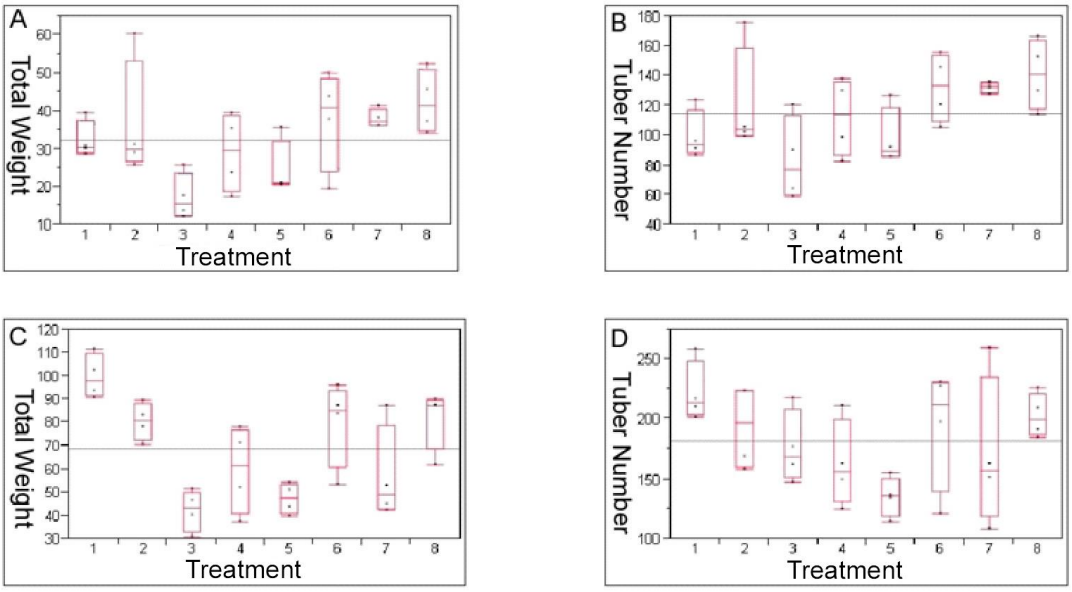


Figure IV.3: Box plots for 2 yield measurements at 2 locations. Total tuber weight and total tuber number per genotype is averaged across the 4 replicates per site. Boxplots show the first, second, and third quantiles as well as the highest and lowest data point. Treatments 1, 3, and 4 represent susceptible check lines.

Table IV.4: Idaho yield results showing the average weight (in pounds) of the 5 different tuber size categories. Averages and standard deviation are reported based on a single 24-foot row in each of 4 blocks. Genotypes not connected by the same letter are significantly different. Treatments 1, 3, and 4 represent susceptible check lines.

Treatment	< 4 Oz	4-6 Oz	6-12 Oz	> 12 Oz	Malformed	Total
1	6.5 ± 2.2	5.2 ± 1.5	6.5 ± 2.8	0.2 ± 0.4	13.9 ± 4.8	32.3 ± 4.9 AB
2	8.3 ± 1.6	7.4 ± 1.3	9.5 ± 7.4	0.6 ± 0.9	10.7 ± 7.1	36.6 ± 16.0 AB
3	9.0 ± 2.7	4.7 ± 2.4	1.7 ± 0.9	0.2 ± 0.4	1.8 ± 1.5	17.3 ± 6.1 B
4	9.6 ± 0.9	9.0 ± 3.7	7.1 ± 4.9	0.3 ± 0.5	3.1 ± 3.2	29.0 ± 10.3 AB
5	8.4 ± 1.5	7.5 ± 2.2	3.3 ± 2.6	0.8 ± 1.5	4.6 ± 1.0	24.5 ± 7.4 AB
6	9.6 ± 1.3	7.4 ± 2.1	7.9 ± 4.7	3.1 ± 3.2	9.8 ± 6.8	37.8 ± 13.1 AB
7	10.1 ± 1.3	7.3 ± 2.4	8.2 ± 2.3	0.0 ± 0	12.5 ± 5.8	38.1 ± 2.4 AB
8	10.2 ± 1.7	9.4 ± 2.5	11.4 ± 1.8	0.2 ± 0.5	11.2 ± 3.4	42.4 ± 8.4 A

Table IV.5: Idaho yield results showing the average number of tubers in the 5 different tuber size categories. Averages and standard deviation are reported based on a single 24-foot row in each of 4 blocks. Genotypes not connected by the same letter are significantly different. Treatments 1, 3, and 4 represent susceptible check lines.

Treatment	< 4 Oz	4-6 Oz	6-12 Oz	> 12 Oz	Malformed	Total
1	42.8 ± 9.4	17.0 ± 4.7	13.5 ± 5.8	0.3 ± 0.5	26.0 ± 9.1	99.5 ± 16.7 AB
2	55.3 ± 12.0	25.3 ± 4.5	20.5 ± 14.8	0.8 ± 1.0	18.8 ± 11.1	120.5 ± 37.0 AB
3	60.0 ± 18.0	15.8 ± 7.8	3.8 ± 2.1	0.3 ± 0.5	3.8 ± 3.5	83.5 ± 28.5 B
4	61.8 ± 4.3	30.5 ± 11.3	15.5 ± 10.7	0.3 ± 0.5	4.3 ± 3.2	112.3 ± 26.1 AB
5	56.3 ± 8.6	24.8 ± 7.0	7.0 ± 5.1	0.8 ± 1.5	9.0 ± 1.2	97.8 ± 19.7 AB
6	69.8 ± 11.6	24.5 ± 7.5	16.5 ± 10.0	3.5 ± 3.9	17.8 ± 12.0	132.0 ± 23.3 AB
7	66.3 ± 4.0	25.8 ± 8.7	17.8 ± 4.9	0.0 ± 0.0	22.5 ± 10.5	132.3 ± 3.3 AB
8	67.5 ± 10.3	31.3 ± 8.4	23.3 ± 2.9	0.3 ± 0.5	18.8 ± 3.8	141.0 ± 23.6 A

Table IV.6: New York yield results showing the average weight (in pounds) of the 5 different tuber size categories. Averages and standard deviation are reported based on double 24-foot rows in each of 4 blocks. Genotypes not connected by the same letter are significantly different. Treatments 1, 3, and 4 represent susceptible check lines.

Treatment	< 4 Oz	4-6 Oz	6-12 Oz	> 12 Oz	Malformed	Total
1	9.2 ± 1.8	10.1 ± 2.2	23.5 ± 7.1	11.1 ± 6.5	45.7 ± 8.7	99.6 ± 9.5 A
2	8.6 ± 1.4	9.0 ± 3.6	14.2 ± 2.7	6.8 ± 2.0	41.7 ± 3.0	80.2 ± 8.1 AB
3	13.7 ± 3.9	12.2 ± 1.6	9.5 ± 4.4	0.7 ± 0.9	6.2 ± 3.1	42.2 ± 8.8 D
4	8.7 ± 2.5	8.6 ± 2.2	14.3 ± 5.8	4.4 ± 3.0	23.6 ± 7.9	59.6 ± 18.4 BCD
5	7.5 ± 2.4	4.9 ± 0.7	6.5 ± 3.2	3.0 ± 1.9	25.6 ± 3.4	47.5 ± 6.6 CD
6	9.3 ± 2.2	12.2 ± 3.9	16.1 ± 6.0	6.9 ± 4.6	35.6 ± 10.6	80.0 ± 18.4 ABC
7	9.4 ± 3.5	8.2 ± 2.4	11.2 ± 8.4	3.1 ± 2.5	25.1 ± 7.7	56.9 ± 20.6 BCD
8	9.0 ± 1.1	10.8 ± 2.5	21.2 ± 4.6	8.3 ± 4.6	32.4 ± 8.2	81.7 ± 13.1 AB

Table IV.7: New York yield results showing the average number of tubers in the 5 different tuber size categories. Averages and standard deviation are reported based on a double 24-foot row in each of 4 blocks. Genotypes not connected by the same letter are significantly different. Treatments 1, 3, and 4 represent susceptible check lines.

Treatment	< 4 Oz	4-6 Oz	6-12 Oz	> 12 Oz	Malformed	Total
1	67.5 ± 13.6	33.3 ± 5.7	45.0 ± 15.8	12.0 ± 7.1	63.8 ± 10.1	221.5 ± 25.2 A
2	67.0 ± 16.7	31.8 ± 17.6	27.0 ± 4.3	7.8 ± 2.9	59.8 ± 7.6	193.3 ± 34.6 A
3	102.3 ± 19.5	42.0 ± 8.3	18.5 ± 9.0	0.8 ± 1.0	12.8 ± 5.7	176.3 ± 30.2 A
4	66.8 ± 19.3	29.0 ± 6.5	27.8 ± 10.2	5.3 ± 3.0	33.5 ± 8.7	162.3 ± 36.1 A
5	62.0 ± 15.6	16.5 ± 2.6	12.0 ± 5.4	3.5 ± 2.4	41.3 ± 7.9	135.3 ± 16.4 A
6	60.8 ± 23.4	37.0 ± 14.5	30.0 ± 12.1	7.3 ± 5.1	59.0 ± 19.6	194.0 ± 51.0 A
7	75.8 ± 30.3	28.3 ± 10.3	21.3 ± 15.0	3.3 ± 3.0	42.3 ± 14.1	170.8 ± 63.3 A
8	67.0 ± 18.9	36.8 ± 7.8	39.8 ± 9.6	9.0 ± 5.0	50.0 ± 8.2	202.5 ± 18.8 A

The yield data from the New York location is markedly higher than the data from Idaho because two 24-plant rows were grown per genotype per replicate in New York, compared to a single row in Idaho. In order to combine the data, therefore, the total yield by weight and tuber number from New York was divided by two. The total yield, as well as the specific gravity, for all genotypes across locations was calculated (Table IV.8).

In order to analyze this data we used the following linear model:

$$Y_{ijm} = \mu + g_i + l_j + gl_{ij} + r_{m(j)}$$

Where

μ is the grand mean

g_i is the fixed genotype effect (treatments 1 through 8)

l_j is the random location effect (1 = Ithaca, NY, 2 = Kimberly, ID)

gl_{ij} is the fixed genotype by location effect

$r_{m(j)}$ is the random replicate effect nested within location (blocks 1 through 4 in both locations)

A One-Way ANOVA was run to test for differences in yield and quality components between the 8 different genotypes (treatments). An ANOVA was run separately on three continuous variables: adjusted weight (the total weight in pounds per 21 foot row of potato plants), adjusted tuber number (total number of tubers per 21 foot row of potato plants), and specific gravity.

For adjusted total weight, a significant genotypic effect was detected ($P < 0.0001$). The R^2 value was equivalent to 0.67, indicating that a moderate amount of the variability in total tuber weight can be explained by differences in genotype (Figure IV.4A). A Tukey range test indicated that several genotypes yielded higher than others, but resistant lines performed comparable to the nontransformed control (Table IV.9). A significant difference was not observed in the total tuber weight between the two field locations (Student's t test $P > 0.05$).

A similar trend was seen for total tuber number. A significant effect was detected for genotype ($P = 0.0058$). The R^2 value was 0.59, indicating that genotype explains a moderate amount of the variability seen in total tuber

number (Figure IV.4B). A Tukey range test indicated that, although differences were observed between genotype, transgenic virus resistant lines produced as many tubers as the nontransgenic control (Table IV.9). A significant effect was seen for location. Significantly more tubers were produced in New York than in Idaho (Student's t test; $P < 0.05$).
A significant difference exists between genotypes for average specific gravity ($P < 0.0001$). Genotype controls a moderate portion of the variance observed, as is indicated by an R^2 value of 0.69 (Figure IV.4C). However, the differences between genotypes do not distinguish transgenic virus resistant lines from the nontransformed control (Table IV.9). Tubers grown in New York had significantly lower specific gravity (Student's t test; $P < 0.05$).

Resistant plants yield virus-free sprouts

Tubers harvested from 'Shepody' plants that were inoculated in the field were sprouted in the greenhouse. Of 71 sprouts tested, 68 (96%) were PVY positive. Sprouts from 'Shepody' seed left over from the spring planting had only 19% infection, indicating that the incidence of PVY infection increased considerably during the growing season. Two major sources of inoculum were present in the field: the infected seed in the border rows and the inoculation that was performed on 4 rows of 'Shepody'. The border rows were only observed to contain PVY strain O (Table IV.2), but strains O, NO, and NTN were used in the inoculation. In order to determine the effectiveness of mechanical inoculation of 4 'Shepody' rows in the field, twenty-four PVY-positive plants were tested using Multiplex PCR to determine PVY strain. Four samples (16%) contained PVY^O only, and 18 samples (75%) were infected

with both PVY^{O} and PVY^{NTN} (data not shown). The presence of PVY^{NO} could not be assayed in the latter group since the multiplex procedure cannot detect it in mixed PVY^{O} and PVY^{NTN} infections. The strain of two samples could not be determined due to multiplex failure. Mechanical inoculation therefore appears to be responsible, at least in part, for the high PVY infection rates in the inoculated 'Shepody' rows.

A subsample of 48 tubers harvested in New York was planted out for each genotype per replicate grown in the field. Sprouts were analyzed visually for PVY symptoms and a random subsample was confirmed with ELISA. In all cases, transgenic lines overexpressing the mutant alleles Potato4e:$pvr1^{2}$ (treatments 2, 6, 7, and 8) and Pepper4E:$pvr1^{2}$ (treatment 5) did not have detectable levels of virus (Table IV.10). In contrast, all three susceptible check lines had detectable levels of virus infection in all four replicates. The nontransgenic control (treatment 1) had 93 out of 97 (96%) symptomatic sprouts. Eighty-nine out of 91 (98%) of potato sprouts overexpressing the wildtype potato allele and 95 out of 97 (98%) of sprouts overexpressing the GUS reporter gene showed virus symptoms (Table IV.10). Multiplex PCR was run to identify the strain of PVY infecting 42 PVY-positive plants from the susceptible controls. In all instances, they were infected by strain O (data not shown). The fact that susceptible checks had high virus incidence, but putatively resistant lines did not indicates that the virus resistance developed by expressing mutated potato eIF4E appears to be effective under standard agronomic conditions.

Table IV.8: Raw yield data combined from the New York and Idaho field locations. New York data was divided by two since there were twice as many plants compared to Idaho. Adjusted weight refers to the total weight (in pounds) per 21 foot row. Adjusted tuber number refers to the total number of tubers per 21 foot row. Replicates correspond to the 4 replicates in the field. Location refers to two field sites. 1 = Ithaca, NY, 2 = Kimberly, ID.

Adj Weight	Adj Tuber Number	Specific Gravity	Treatment	Replicate	Location
39.5	124	78	1	1	2
31	99	80	2	1	2
13.6	59	82	3	1	2
17.4	83	82	4	1	2
20.6	92	77	5	1	2
19.5	105	82	6	1	2
38.2	136	82	7	1	2
37.1	130	79	8	1	2
28.6	87	81	1	2	2
29	105	86	2	2	2
12.1	64	84	3	2	2
23.7	98	84	4	2	2
21	86	74	5	2	2
37.8	146	76	6	2	2
41.5	132	79	7	2	2
34.1	114	83	8	2	2
30.1	96	87	1	3	2
25.9	102	88	2	3	2
25.8	121	88	3	3	2
39.7	138	85	4	3	2
35.6	127	82	5	3	2
43.8	121	75	6	3	2
36.4	133	73	7	3	2

Table IV.8 (Continued)

45.8	153	88	8	3	2
30.9	91	75	1	4	2
60.4	176	83	2	4	2
17.6	90	80	3	4	2
35.3	130	93	4	4	2
20.8	86	78	5	4	2
50	156	76	6	4	2
36.3	128	78	7	4	2
52.5	167	82	8	4	2
55.75	129	77	1	1	1
38.9	84.5	81	2	1	1
20.25	88.5	78	3	1	1
39.1	105.5	78	4	1	1
20	67	74	5	1	1
26.75	60.5	73	6	1	1
43.55	129.5	75	7	1	1
43.45	95	78	8	1	1
46.85	100.5	75	1	2	1
41.4	111.5	77	2	2	1
15.4	74	78	3	2	1
18.85	62.5	77	4	2	1
22.1	57.5	71	5	2	1
43.45	113.5	73	6	2	1
21.15	76	74	7	2	1
31.1	104.5	74	8	2	1
45.25	108.5	76	1	3	1
44.8	111.5	80	2	3	1
23.15	81	75	3	3	1
25.9	75	68	4	3	1
25.55	77.5	69	5	3	1
41.75	98.5	72	6	3	1
26.45	81.5	74	7	3	1
45.05	113	81	8	3	1
51.35	105	78	1	4	1
35.25	79	82	2	4	1
25.65	109	76	3	4	1
35.4	81.5	79	4	4	1
27.25	68.5	72	5	4	1
48	115.5	77	6	4	1
22.6	54.5	76	7	4	1
43.75	92.5	81	8	4	1

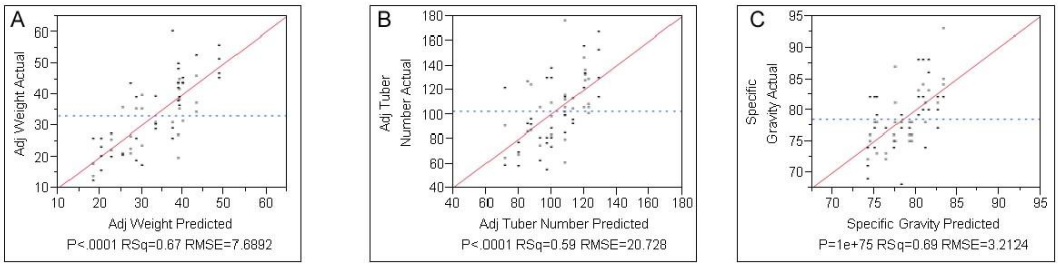


Figure IV.4: Regression plots for tuber yield and quality characteristics. For the three traits considered, the genotypic effect was significant and controlled a moderate amount of the observed variance. A) Adjusted total weight, B) Adjusted total tuber number, C) specific gravity.

Table IV.9: Average yield and specific gravity of the 8 genotypes considered in this study using data combined from the two field locations. All values are the average of 8 samples (4 replicates in each of 2 locations). Adjusted average yield is calculated as the average total tuber weight and total tuber number of a 21-foot row of potato plants. Genotypes not connected by the same letter are significantly different. Treatments 1, 3, and 4 represent susceptible check lines.

Treatment	Adjusted Average Yield (weight)		Adjusted Average Yield (# Tubers)		Average Specific Gravity	
1	41.0 ± 10.4	A	105.1 ± 15.0	A	78.4 ± 4.0	ABCD
2	38.3 ± 11.0	A	108.6 ± 29.7	AB	82.1 ± 3.5	A

Table IV.9: Average yield and specific gravity of the 8 genotypes considered in this study using data combined from the two field locations. All values are the average of 8 samples (4 replicates in each of 2 locations). Adjusted average yield is calculated as the average total tuber weight and total tuber number of a 21-foot row of potato plants. Genotypes not connected by the same letter are significantly different. Treatments 1, 3, and 4 represent susceptible check lines.

Treatment	Adjusted Average Yield (weight)		Adjusted Average Yield (# Tubers)		Average Specific Gravity	
1	41.0 ± 10.4	A	105.1 ± 15.0	A	78.4 ± 4.0	ABCD
2	38.3 ± 11.0	A	108.6 ± 29.7	AB	82.1 ± 3.5	A
3	19.2 ± 5.4	C	85.8 ± 21.2	B	80.1 ± 4.4	ABC
4	29.4 ± 9.0	ABC	96.7 ± 26.6	AB	80.8 ± 7.2	AB
5	24.1 ± 5.3	BC	82.7 ± 21.3	B	74.6 ± 4.2	D
6	38.9 ± 10.6	A	114.5 ± 29.3	AB	75.5 ± 3.2	CD
7	33.3 ± 8.7	AB	108.8 ± 32.6	AB	76.4 ± 3.1	BCD
8	41.6 ± 7.0	A	121.1 ± 27.0	A	80.8 ± 4.1	AB

Discussion

Virus resistance developed by mutating and overexpressing the eIF4E gene is an exciting method of disease control that offers the possibility of addressing a number of economically important pathosystems. We have been successful at developing resistance in potato against *Potato virus Y* when plants are grown in the greenhouse and infected mechanically. The purpose of this work has been to determine whether transgenic virus resistance holds up under agricultural conditions. In order to test this hypothesis, we grew potatoes in two environments and attempted to create conditions favorable for virus infection. We then carefully monitored disease spread and measured yield and quality characteristics of harvested tubers.

Considerable efforts were made during the planning of this field experiment to ensure that conditions were favorable for virus infection. The potato cultivar Shepody was used in the border rows because it expresses PVY symptoms very weakly and is consequently notorious for having a high percentage of infected tubers (Crosslin et al. 2006). In addition, three viral isolates were inoculated onto 4 rows of 'Shepody' plants. These efforts, combined with virus spread through aphid vectors, increased the incidence of virus infection considerably and provided environmental conditions that were favorable for a successful disease screen.

Table IV.10: Number of PVY positive samples in the winter growout for tubers collected in New York. A 48-tuber sample per genotype per replicate was treated with Rindite to break dormancy and sprouted in the greenhouse. Samples were rated visually for disease symptoms. Subsamples were validated using ELISA. Each data point corresponds to a single sprouted tuber. 48 tubers per treatment per replicate were planted but not all grew sprouts in the time allotted.

Number of PVY Positive Samples					
Treatment	**Replicate I**	**Replicate II**	**Replicate IV**	**Replicate IV**	**Percent Total**
1*	18/19	16/16	26/28	33/34	96 %
2	0/17	0/16	0/36	0/22	0 %
3*	24/25	14/15	28/28	23/23	98 %
4*	13/14	19/20	19/19	44/44	98 %
5	0/25	0/19	0/46	0/19	0 %
6	0/28	0/25	0/24	0/20	0 %
7	0/26	0/28	0/24	0/16	0 %
8	0/26	0/12	0/19	0/30	0 %

*Treatments 1, 3, and 4 represent susceptible check lines.

The three susceptible control lines accumulated high levels of virus during the growing season. These plants were not mechanically inoculated, and were confirmed to be disease-free by ELISA testing upon first emergence. In New York, 84% or more of the composite leaf samples tested for each of three susceptible control lines were virus positive after growing for several months in the field (Table IV.3). Despite high levels of virus inoculum and high infection rates in susceptible lines, all composite leaf samples were virus negative for the 5 putatively virus resistant lines tested (Table IV.3). The experimental lines were not mechanically inoculated in this field experiment. Rather, they were surrounded by virus-infected plants and disease was vectored by aphids as would happen in a commercial production setting. This indicates that the transgenic virus resistance developed during this study is effective under agricultural conditions.

Potato virus Y resistance is a desirable trait for potato growers and seed producers. However, further characterization of resistant plants was necessary in order to identify any pleiotropic effects with agronomic consequences. Two yield characteristics, total tuber weight and total tuber number, were examined in two separate locations. Specific gravity, a quality trait associated with starch content, was also examined. In all cases there was a significant genotypic effect that controlled a moderate amount of the variability associated with the yield or quality trait being examined (Figure IV.4). However, when further statistical analysis was conducted to identify which genotypes differed from each other, transgenic resistant plants behaved comparably to or better than the susceptible controls (Table IV.9).

One genotype, treatment 3 overexpressing the Potato4E:wildtype allele, had the lowest total tuber weight (Tables IV.4, IV.6, and IV.9) . It was observed in the greenhouse and the field that these plants were spindly and appeared weaker than other lines. It is possible that transgenic expression of the wildtype allele has a negative effect on tuber weight, particularly given that eIF4E is involved with translation initiation and may conceivably impact the protein profile of the plant. However, these results are inconclusive given that only a single line was examined. Additional lines representing other insertion events ar required to distinguish between pleiotropy and other "position effects" caused by where the gene is inserted.

This study measures yield and quality characteristics under disease pressure. Testing at additional locations that were maintained virus-free was considered but abandoned due to additional costs and the difficulty of ensuring virus exclusion. Now that disease resistance has been shown to be effective, further work may focus on addressing this issue.

Viruses are problematic for potato production because they are clonally propagated and require several generations of production to increase enough seed for commercial sale. Virus susceptible cultivars quickly accumulate high levels of virus even if a low infection rate per generation is maintained. This was seen very dramatically in our experiment, where susceptible cultivars produced nearly 100% infected sprouts the following generation (Table IV.10). Infected sprouts are problematic for growers, who see reduced yield and poor tuber quality, and seed producers, who receive a premium for tubers certified

as virus free. This is one reason for the proliferation of ‘Shepody’ potatoes and other symptomless carriers since they are more likely to pass certification (which is done by visual inspection) than cultivars such as ‘Russet Burbank’ which shows obvious symptoms. Ironically, it is the continued use of these cultivars that keeps national virus levels high. It is not enough, therefore, to produce plants that are virus resistant. They must produce sprouts the following year that are disease-free as well. Tubers were harvested at the end of the summer season and sprouted in the greenhouse. Dramatic differences were observed between resistant and susceptible lines. Sprouts from resistant mother plants had 0% infection rates, compared to nearly 100% for susceptible controls (Table IV.10). Thus, it appears that not only are the transgenic plants considered in this experiment resistant in the field, they produce tubers that are virus free as well.

A dominant negative model has been proposed to explain why transgenic plants are virus resistant. In this scenario, it is thought that endogenous susceptible eIF4E is present at low levels relative to transgenic resistant eIF4E. This low level of endogenous eIF4E is not thought to be sufficient to sustain virus infection since two rare events must occur: the virus genome must bind to endogenous eIF4E and those coupled proteins must bind to other proteins in the translation initiation complex. However, cuttings that are graft-inoculated with high levels of virus appear to sustain virus replication, albeit at levels that may be lower than nontransgenic tissue. In this scenario the binding of the prevalent viral genome to endogenous eIF4E occurs with a high enough frequency to sustain virus infection.

Transgenic virus resistance developed by mutating eIF4E appears to be relevant under agricultural conditions. Resistance is effective in the field and does not appear to negatively impact tuber yield or starch content. Furthermore, when tubers from resistant plants were harvested and used for seed, the resulting sprouts were also disease free. Additional work will be needed to confirm these claims over multiple years. However, intragenesis in potato is an effective means of controlling *Potato virus Y* that relies on modifications from within the plants own genome. This resistance mechanism is novel and may be more easily marketed to consumers than other forms of transgenic virus resistance

REFERENCES

Baldauf P, Gray S, Perry K (2006) Biological and Serological Properties of Potato virus Y Isolates in Northeastern United States Potato. Plant Disease 90:559-566

Bergelson J, Purrington CB (1996) Surveying Patterns in the Cost of Resistance in Plants. The American Naturalist 148:536-558

Brown JKM (2002) Yield penalties of disease resistance in crops. Current Opinion in Plant Biology 5:339-344

Crosslin JM, Hamm PB, Hane DC, Jaeger J, Brown CR, Shiel PJ, Berger PH, Thornton RE (2006) The Occurrence of PVY^{O}, PVY^{N}, and $PVY^{N:O}$ Strains of *Potato virus Y* in Certified Potato Seed Lot Trials in Washington and Oregon. Plant Dis 90:1102-1105

Elke D, Martin S, Lothar W, Jens K (1997) Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum* L.) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism. The Plant Journal 12:323-333

Gingras AC, Raught B, Sonenberg N (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68:913-63

Kaniewski WK, Thomas PE (2004) The Potato Story. AgBioForum 7:41-46

Levene H (1960) Robust tests for equality of variances. In: Olkam I, Hotelling H (eds) Contributions to probability and statisticsa: essays in honor of Harold Hotelling. Standford University Press, p 278-292

Lorenzen JH, Piche LM, Gudmestad NC, Meacham T, Shiel P (2006) A Multiplex PCR Assay to Characterize Potato virus Y Isolates and Identify Strain Mixtures. Plant Disease 90:935-940

Schubert J, Matousek J, Mattern D (2004) Pathogen-derived resistance in potato to Potato virus Y-aspects of stability and biosafety under field conditions. Virus Res 100:41-50

Tukey J (1994) The Collected Works of John W. Tukey Multiple Comparisons: 1948-1983. Chapman and Hall, New York, NY

Conclusion

My doctoral work has focused on the *eIF4E* virus resistance gene. In Chapter II we have shown that this gene is under strong selective pressure in crop plants. Chapter III describes how we have been able to use the detailed knowledge of the contribution of particular amino acid changes to generate virus resistant intragenic lines of potato, a species having no known natural resistance at this locus. Chapter IV validates this resistance in the field and shows that no obvious yield penalties are associated with transgenic resistance.

Rather than rely on expression of resistance alleles from other species, virus resistance is accomplished by working within the target crop's own genome. Resistance using this technology appears to be extremely effective. No virus accumulation was detected in inoculated tissue and plants were protected from multiple PVY strains simultaneously. The strategy described in this chapter could be used to generate resistance in any pathosystem involving viral infection reliant upon an interaction with eIF4E. For instance, another obvious crop that could benefit from this technique is Plum (*Prunus)*. Plum production in the United States is threatened by the recent introduction of *Plum pox virus* (PPV). Other possible targets for generation of host plant resistance by transgenic expression of *eIF4E* include papaya-*Papaya ringspot virus*, beet-*Beet mosaic virus*, and squash-*Zucchini yellow mosaic virus*. Genetic modification of eIF4E effectively conferred resistance to potyviruses, the largest genera of plant pathogenic viruses. The effectiveness for

conferring resistance to other viral genera cannot be predicted but is promising based on successes achieved thus far.

Another advantage to transgenic virus resistance using the *eIF4E* system is that it addresses some of the misgivings associated with using biotechnology to protect crops from virus infection. Because *eIF4E* from a susceptible host may be modified and used to generate virus resistance, the donor gene utilized is derived from the target crop. This so-called "intragenic" technology is predicted to be more acceptable to consumers (Rommens 2007). Empirical evidence suggests consumers are less concerned about transgenic plants expressing plant genes, as compared to genes derived of viral origin. In one study, consumer willingness to consume a transgenic vegetable with a viral transgene was only 14.3%, but increased to 81.3% when the transgene was from within the same species (Lusk and Sullivan 2002). Other researchers have commented on these trends (Nielsen 2003), and there is an increasing push to develop "intragenic" plants expressing donor DNA from the same species (Rommens 2007; Rommens 2008). This area of research is particularly active for crops such as potato that have met opposition to previous attempts at commercialization of genetically engineered varieties (Rommens 2004).

Consumer acceptance issues, more so than the lack of scientific progress, have limited commercialization of virus resistance in transgenic crops. Lack of market acceptance prevented 'Newleaf' potatoes from reaching its commercial potential despite positive responses from consumers initially and successful adoption by a number of potato growers. Activists carried out a successful

antibiotechnology campaign that ultimately convinced food service companies, potato vendors, and producers to adopt a genetically modified organism-free policy (Kaniewski and Thomas 2004). This resulted in the dissolution of Naturemark and cessation of 'Newleaf' potato sales. In addition, several promising areas of transgenic research in potato were abandoned due to the inhospitable marketing climate.

The high adoption rate of transgenic virus resistant crops by American farmers testifies to their importance in crop production. The failure of other countries to continue this trend underscores the uncertainty many people still have with pathogen-derived virus resistance. Development of transgenic virus resistance has proven to be an extremely effective and attainable method of developing host plant resistance in a large number of crops against a wide range of virus genera (Fuchs 2008; Goldbach et al. 2003; Sudarshana et al. 2007; Tepfer 2002; Wilson 1993). It is not scientific progress, however, that has prevented widespread adoption of virus-resistance technology by growers. Rather, it is an unreceptive market environment caused by lingering concerns held by consumers (Collinge et al. 2008). The work of scientists is not complete once resistant plants have been developed. In order to maximize the likelihood of commercialization, scientists must be sensitive to the concerns of consumers and possibly even attempt to address some of those concerns by performing appropriate techniques in the laboratory.

Although the scientific community largely agrees that most consumer concerns are not scientifically valid (EuropeanUnion 2008; Fuchs 2008), these concerns must nonetheless be addressed if transgenic products will be

successfully commercialized in food crops. Consumer concerns are an impediment to commercialization of genetically engineered virus resistant crops; this in itself makes attending to these concerns a legitimate exercise. History has proven that a failure to do so renders the transfer of technology from the researcher's bench to the farmer's field unlikely.

REFERENCES

Collinge D, Lund O, Thordal-Christensen H (2008) What are the prospects for genetically engineered, disease resistant plants? European Journal of Plant Pathology 121:217-231

EuropeanUnion (2008) Scientific Opinion of the Panel on Genetically Modified Organisms. The EFSA Journal 756:1-18

Fuchs M (2008) Why virus resistance in transgenic plants, and are there risks associated with the release of virus-resistant transgenic plants? Agricultural Biotechnology

Goldbach R, Bucher E, Prins M (2003) Resistance mechanisms to plant viruses: an overview. Virus Research 92:207-212

Kaniewski WK, Thomas PE (2004) The Potato Story. AgBioForum 7:41-46

Lusk J, Sullivan P (2002) Consumer Acceptance of Genetically Modified Foods. FoodTechnology 56:32-37

Nielsen KM (2003) Transgenic organisms--time for conceptual diversification? Nat Biotechnol 21:227-8

Rommens CM (2004) All-native DNA transformation: a new approach to plant genetic engineering. Trends Plant Sci 9:457-64

Rommens CM (2007) Intragenic crop improvement: combining the benefits of traditional breeding and genetic engineering. J Agric Food Chem 55:4281-8

Rommens CM (2008) The need for professional guidelines in plant breeding. Trends in Plant Science 13:261-263

Sudarshana MR, Roy G, Falk BW (2007) Methods for engineering resistance to plant viruses. Methods Mol Biol 354:183-95

Tepfer M (2002) Risk assessment of virus-resistant transgenic plants. Annu Rev Phytopathol 40:467-91

Wilson TM (1993) Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. Proc Natl Acad Sci U S A 90:3134-41

APPENDIX I

‘Salt and Pepper’: a disease-resistant heirloom type cucumber inbred

Cavatorta, J., Moriarty, G., Glos, M., Henning, M., Kreitinger, M., Mazourek, M., Munger, H., and Jahn, M.

The ‘Marketmore’ series has long been a standard for disease-resistance in cucumber. ‘Boothby’s Blonde’ is a cucumber heirloom praised by growers for its novelty, earliness, and eating qualities but is highly susceptible to fungal diseases. Here we report the development of the monoecious open-pollinated cucumber ‘Salt and Pepper’ that combines the desirable qualities of both of these cultivars. The fruit of ‘Salt and Pepper’ is white with black spines like that of ‘Boothby’s Blonde’, but has foliar powdery mildew resistance and downy mildew tolerance. Selection of ‘Salt and Pepper’ was performed on USDA certified organic ground, making it one of the first commercially-available modern vegetable cultivars adapted specifically (but not exclusively) for organic production.

Introduction

Cucumber, probably originating from India, has been domesticated for over 3000 years (Bates and Robinson 1995). In modern times plant breeders have selected for a diversity of fruit shapes and colors, sex expression, growth

habits, and disease resistances (Wehner and Robinson 1991). A long history of breeding at Cornell University and elsewhere has resulted in the development of cucumber cultivars that are resistant to a number of plant diseases (Peterson 1975; Cavatorta et al. 2007). Resistance breeding began in the 1920s to address *Cucumber mosaic virus* (Porter 1929) and now includes a number of virus, fungal, and bacterial diseases (McGrath and Zitter 2009). However, many farmer-developed heirloom cultivars continue to be grown that have novel appearances or high quality fresh market characteristics such as taste but lack disease resistance. Recognizing that private companies typically do not pursue improvement of such cultivars because they are grown on small acreage, the USDA-funded Public Seed Initiative (www.plbr.cornell.edu/psi) and subsequently the Organic Seed Partnership (www.organicseedpartnership.com) were set up at Cornell to address these underserved markets. As part of the work funded by these projects we have used Cornell germplasm to develop 'Salt and Pepper', a monoecious cucumber inbred that is adapted to Northeast growing conditions and combines disease resistance with the eating qualities of the heirloom cultivar 'Boothby's Blonde'.

Origin

During the summer growing season of 2005 an F_2 population of a cross between 'Marketmore 97' and 'Boothby's Blonde' was planted on an organic research plot at Cornell University's Freeville Organic Research Farm in Freeville, NY. A selection was made that had white-skinned fruit with black spines similar to 'Boothby's Blonde', but appeared to be resistant to powdery

mildew. Several generations of selection for mildew resistance, alternating between the greenhouse and field, were made to generate an F_6 line which was named ‘Salt and Pepper’. Seed from this generation was increased by allowing open pollination at an isolated location.

Description and Performance

The fruit of ‘Salt and Pepper’ has white skin with black spines (Figure AI.1A). It has small fruit with an average weight of 105 grams, a length of 10.8 cm, and a width of 4.1 cm (Table AI.1). The cucumber fruit is visually similar to the heirloom cultivar ‘Boothby’s Blonde’ and is comparable in weight and width but is slightly greater in length (Table AI.1). In blind taste tests, ‘Salt and Pepper’ routinely scores higher and has been described as being very sweet and having a hint of a black pepper flavor.

‘Salt and Pepper’ is resistant to two foliar pathogens that pose important problems for cucumber production in the Northeast. Under high disease pressure, no powdery mildew symptoms were observed, whereas ‘Boothby’s Blonde’ and even the resistant cultivar ‘Poinsett 97’ became infected (Table AI.2 and Figure AI.1B). ‘Salt and Pepper’ also appears to contain moderate tolerance to endemic strains of downy mildew relative to ‘Boothby’s Blonde’ (Table AI.2 and Figure AI.1B).

The disease resistance incorporated into ‘Salt and Pepper’ appears to be agronomically meaningful. In a field trial conducted in Ithaca, NY during the

2009 growing season, ‘Salt and Pepper’ remained alive and productive long after susceptible checks had been killed by disease. The additional harvest time resulted in much higher yields (Figure AI.2).

Availability

‘Salt and Pepper’ will be available as organic seed from Johnny’s Selected Seeds (http://www.johnnyseeds.com/) pending a successful 2010 seed production.

Acknowledgements

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Table AI.1: Descriptive measurements comparing the two cultivars 'Boothby's Blonde' and 'Salt and Pepper'. The two fruit are similar in size but 'Salt and Pepper' is slightly longer (Student's T-test $P < 0.01$). P values for weight and width are nonsignificant. Standard deviations are reported based on measurements made on 20 individual fruit.

Cultivar	***Weight (g)***	***Length (cm)***	***Width (cm)***
'Boothby's Blonde'	101 ± 20	9.6 ± 0.6	4.2 ± 0.4
'Salt and Pepper'	105 ± 17	10.8 ± 0.7	4.1 ± 0.3

Table AI.2: Resistance Index for powdery mildew (*Sphaerotheca fuliginea* Schl. ex Fr.) and downy mildew (*Pseudoperonospora cubensis* Berk and Curtis). Ratings were taken on August 11, 2009 (56 days after transplanting). Ratings were on a scale of 0 to 5 where 0 = no disease, 1 = 1-20% leaf area infected, 2 = 21-40%, 3 = 41-50%, 4 = 61-80%, and 5 = greater than 80% leaf area infected or plant dead. The value for each replicate is an average score of 8 plants except for 'Poinsett 97' Rep I (7 plants), Rep II (6 plants) and Rep III (7 plants). Standard deviation is shown.

Cultivar	**Powdery Mildew**			**Downy Mildew**		
	Rep I	Rep II	Rep III	Rep I	Rep II	Rep III
'Poinsett 97'	0.7 ± 0.5	0.7 ± 0.5	0.4 ± 0.4	1.3 ± 0.5	3.2 + 0.4	2.4 ± 0.5
'Boothby's Blonde'	2.4 ± 0.5	2.9 ± 0.6	2.3 ± 0.7	3.8 ± 0.5	4.3 ± 0.5	4.6 ± 0.5
'Salt and Pepper'	0	0	0	1.3 ± 0.5	1.8 ± 0.5	1.6 ± 0.5

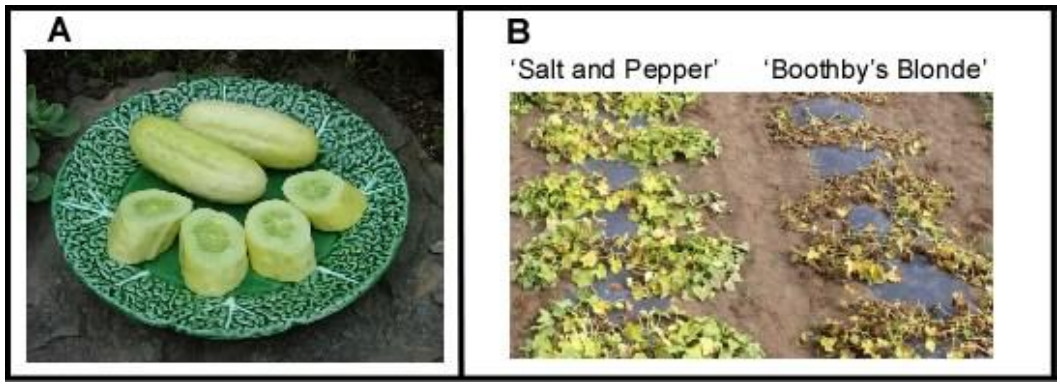

Figure AI.1: 'Salt and Pepper' cucumber photographs. 1A) shows the white-skinned cucumber with black spines. 1B) shows the foliar comparison of 'Salt and Pepper' (left) with 'Boothby's Blonde' (right) under heavy powdery and downy mildew disease pressure.

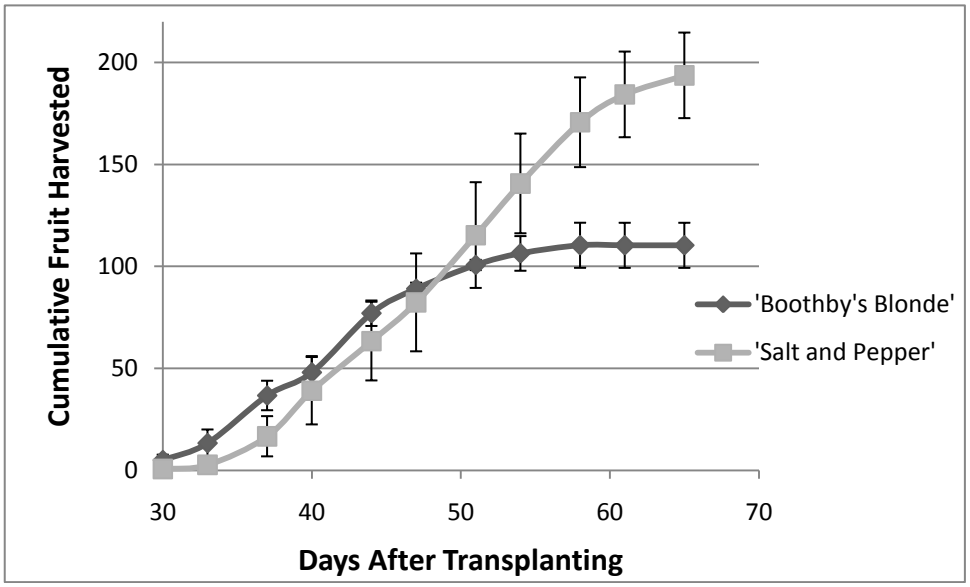

Figure AI.2: Yield comparison between 'Boothby's Blonde' and 'Salt and Pepper'. Measurements are averages of the cumulative number of fruit per plant for each of three replicates. Standard deviation is shown. Fruit was harvested and counted twice a week from 8 plants per genotype per replicate. 'Salt and Pepper' continued to yield marketable fruit after 'Boothby's Blonde' plants had succumbed to disease.

REFERENCES

Bates, D., and Robinson, R. Cucumber, melons and water-melons. In Evolution of Crop Plants. 2nd ed. Pgs. 89-96. John Wiley and Sons, Inc. New York, NY.

Cavatorta, J., Moriarty, G., Henning, M., Glos, M., Kreitinger, M., and Jahn, M. 2007. 'Marketmore 97': A monoecious slicing cucumber inbred with multiple disease and insect resistances. *Hortscience* 42: 707-709.

McGrath, M. and Zitter, T. 2009. Resistant Varieties. Cornell University Vegetable MD Online, http://vegetablemdonline.ppath.cornell.edu/Tables/TableList.htm

Peterson, C. 1975. Plant Introductions in the Improvement of Vegetable Cultivars. *Hortscience* 10: 575-579.

Porter, R. 1929. Reaction of Chinese cucumbers to mosaic. *Phytopathology* 19:85 (abstr.).

Wehner, T., and Robinson, R. 1991. A brief history on the development of cucumber cultivars in the U.S. **Cucurbit** *Genet* **Coop** *Rpt* 14: 1-4. Available on-line at http://cuke.hort.ncsu.edu/cgc/cgc14/cgc14-1.html

APPENDIX II

The double recessive mutant *pvr1pvr1/pvr6pvr6* is viable

Jason Cavatorta, Sarah Collier, Inhwa Yeam, Molly Jahn

Introduction

Pvr1 is the locus corresponding to *eIF4E* in pepper (Kang et al. 2005a; Ruffel et al. 2002). *Pvr6* has been shown to correspond to its paralog – *eIF(iso)4E* (Ruffel et al. 2006). Although these genes share little nucleotide or amino acid identity (58% and 48%, respectively), they have overlapping function in cap-binding and virus association.

Many viruses interact with *Pvr1*, and modifications to *Pvr1* confer recessive virus resistance (Charron et al. 2008; Kang et al. 2005a; Ruffel et al. 2002). Interestingly, some viruses (i.e. *Pepper veinal mottle virus*) can interact with either *Pvr1* or its paralog *Pvr6* (Caranta et al. 1996; Hwang et al. 2009; Ruffel et al. 2006). In order to obtain host plant resistance against these viruses, therefore, both loci must be homozygous for the recessive resistance allele. This has been known for some time, and varieties with the genotype *pvr1-^{2}pvr1^{2}/pvr6pvr6* have been developed.

The *pvr6* recessive resistance allele contains an 82 bp deletion that causes a premature stop codon in *eIF(iso)4E* (Ruffel et al. 2006). Presumably, in *pvr1-^{2}pvr1^{2}/pvr6pvr6* plants eIF4E is the sole protein involved in interaction with the mRNA 5' cap. This is possible because pvr1^{2} has been shown to retain some

cap binding ability (Kang et al. 2005a). The major driving question for this project is what would happen in *pvr1pvr1*/*pvr6pvr6* plants. In contrast to pvr1^2, pvr1 has been shown to have no or very little cap binding ability (Kang et al. 2005a). We therefore wanted to know whether *pvr1pvr1*/*pvr6pvr6* double homozygous recessive plants would be viable. If no, this suggests that i) *pvr1pvr1* plants truly do have no cap-binding ability whereas *pvr1^{2}pvr1^2* plants do and ii) there are no other genes (such as a 3rd paralog) that can take over the cap-binding function. If yes, are there any obvious physiological or morphological consequences to a plant of that genotype?

Materials and Methods

To answer this question, Sarah Collier made crosses between a *Pvr1+Pvr1+*/*pvr6pvr6* plant and two *pvr1pvr1* plants (Table AII.1). The F_1 progeny were selfed and F_2 seed collected. We grew out 90 plants and genotyped them to look for *pvr1pvr1*/*pvr6pvr6* plants as per (Yeam et al. 2005). An additional cross with a *pvr1^{2}pvr1^2* plant serves as a control since *pvr1^{2}pvr1^2*/*pvr6pvr6* plants are known to be viable. If we are able to identify around 4 double recessive homozygotes using 90 plants (which statistics predict we will be able to) from the *pvr1^2* cross, but 0 from either of the *pvr1* crosses, this is good evidence that *pvr1pvr1*/*pvr6pvr6* plants are not viable.

Table AII.1: Genotype of parental cultivars at 2 loci.

Cultivar	**Genotype**	
Perennial	Pvr1+Pvr1+	pvr6pvr6
5502	pvr1pvr1	Pvr6+Pvr6+
Ca4	pvr1pvr1	Pvr6+Pvr6+
Dempsey	$pvr1^2pvr1^2$	Pvr6+Pvr6+

Pvr1 genotyping follows the protocols of Yeam et al. (2005) where primer sets have been developed to amplify a segment of the Pvr1 gene that contains allele-specific restriction sites (Table AII.2). However, because *Pvr1*+ from 'Perennial' contains the M4 mutation, it behaves as if it were $pvr1^2pvr1^2$ when genotyped with this method. Therefore, the Pvr1-R2 F&R primers were used for all pvr1 genotyping (Table AII.2). Pvr6 genotyping will be performed using Polymerase Chain Reaction (amplification cycle of 94 for 45 sec, 60 degrees for 45 sec, 72 degrees for 45 sec) with the following primers: Pvr6 F 5'-GGTGAAACAGCCACATAAGC-3' and Pvr6 R 5'-GCCACCATTAGCGCACTCAGG-3' (Figure AII.1). Sequencing of parental cultivars and F1 are shown in Figure AII.2.

Results

Genotypes at the *Pvr6* locus were determined for 90 F_2 plants resulting from the cross '5502' by 'Perennial'. One genotype was unable to be determined so a total of 89 plants were genotyped. The genotypic ratio 16 *Pvr6+Pvr6+* /

43 *Pvr6+pvr6*/ 30 *pvr6pvr6* was obtained, which did not differ significantly from the expected ratio of 22.25/44.5/22.25 (Chi Square Goodness of Fit two-tailed P value = 0.1051) (Table AIII.3). Sequence could be obtained for 26 of the 30 *pvr6pvr6* individuals. The observed genotypic ratio 6 Pvr1+Pvr1+ / 14 Pvr1+pvr1 / 6 pvr1pvr did not differ significantly from the expected ratio of 6.5/13/6.5 (Chi Square Goodness of Fit two-tailed P value = 0.9260) (Table AIII.3).

Table AII.2: Pvr1 Genotyping. Primers, restriction enzymes, and band sizes expected for each genotype.

Marker Name	Primers used	R.E. Used	Band size Pvr1+	Band size pvr1	Band size pvr1[2]
Pvr1-S	Pvr1-S F+R	*Bsr-1*	133 + 578	711	**711**
pvr1-R1	Pvr1-S F+R	*Fnu4HI*	155+556	69+86+556	**155+556**
pvr1-R2	**Pvr1-R2 F+R**	***HindIII***	**412**	**412**	**32+380**

Pvr1-S F: 5'-GCTAATGAGGCAGATGATGAAGTTG-3'

Pvr1-S R: 5'-CAACCATAAATATACCCCGAGAAT-3'

Pvr1-R2 F 5'-GGGCTAAAATACGCTCATCTCCCTTC-3'

Pvr1-R2 R 5'-GGCTCAATTTTATGCTTGAAACAATGTAAGC-3'

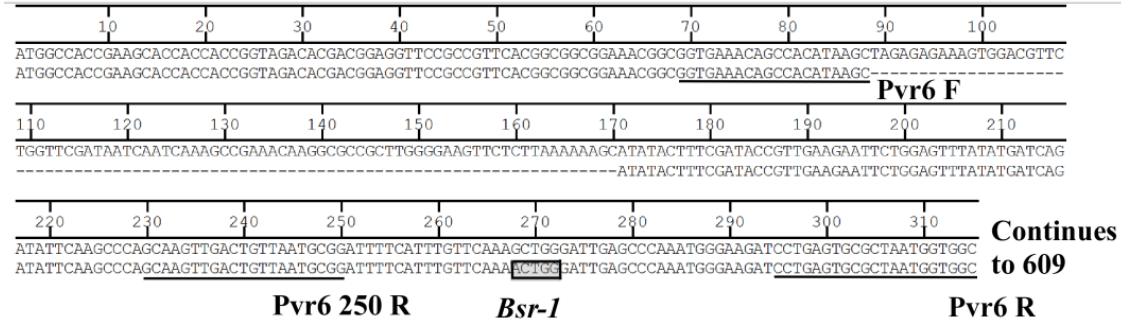

Figure AII.1: *Pvr6+* sequence aligned with *pvr6* mutant allele. Genotyping was performed by amplifying genomic DNA of pepper plants. Larger bands contained the wildtype allele, smaller bands contained the mutant.

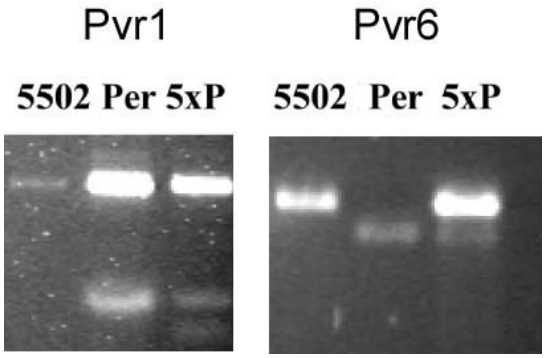

Figure AII.2: Gel images showing the molecular markers used to genotype for Pvr1 and Pvr6. Per = cultivar Perennial. 5xP = F_1 generated by crossing '5502' and 'Perennial'. 5xP has the genotype *Pvr1+pvr1*/*Pvr6+pvr6.*

Table AII.3: Observed and expected genotypic ratios for two loci. P value corresponds to a two-tailed Chi squared goodness of fit test.

	Pvr6+/Pvr6+	***Pvr6+/pvr6***	***pvr6/pvr6***	**P value**
Observed	16	43	30	
Expected	22.25	30	22.25	0.1051
	Pvr1+/Pvr1+	*Pvr1+/pvr1*	*pvr1/pvr1*	
Observed	6	14	6	
Expected	6.5	13	6.5	0.9260

Discussion

It appears that the genotype *pvr1pvr1*/*pvr6pvr6* is viable. Out of 85 F_2 plants successfully genotyped, 6 plants were obtained with this genotype. Although a Chi square goodness of fit test cannot be run since only the *pvr6pvr6* plants were genotyped for *pvr1*, this is not far away from the expected number of 5.3 (85 divided by 16). These F_2 plants were grown to maturity in the greenhouse and seed was harvested. No obvious phenotypic differences existed compared to other F_2 plants. Neither mutant pvr1 nor pvr6 protein is thought to bind the 5' cap of messenger RNA. Two possible reasons can explain the existence of these double recessive mutants. Either a third functional paralog of eIF4E exists that can compensate for mRNA cap binding, or else pvr1 retains cap-binding ability despite previous work suggesting otherwise (Kang et al. 2005a; Ruffel et al. 2002). The former explanation seems more likely since a third cap binding protein is known to exist in *Arabidopsis* (Ruud et al. 1998), but further work will be necessary to definitively conclude that this is the case.

REFERENCES

Caranta C, Palloix A, Gebre-Selassie K, Lefebvre V, Moury B, Daubeze A (1996) A complementation of two genes originating from susceptible *Capsicum annuum* lines confer a new and complete resistance to *Pepper veinal mottle virus*. Phytopathology 86:739-743

Charron C, Nicolai M, Gallois JL, Robaglia C, Moury B, Palloix A, Caranta C (2008) Natural variation and functional analyses provide evidence for co-evolution between plant eIF4E and potyviral VPg. Plant J 54:56-68

Hwang J, Li J, Liu WY, An SJ, Cho H, Her NH, Yeam I, Kim D, Kang BC (2009) Double mutations in eIF4E and eIFiso4E confer recessive resistance to Chilli veinal mottle virus in pepper. Mol Cells 27:329-36

Kang BC, Yeam I, Frantz JD, Murphy JF, Jahn MM (2005) The pvr1 locus in Capsicum encodes a translation initiation factor eIF4E that interacts with Tobacco etch virus VPg. Plant J 42:392-405

Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C (2002) A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). Plant J 32:1067-75

Ruffel S, Gallois J-L, Moury B, Robaglia C, Palloix A, Caranta C (2006) Simultaneous mutations in translation initiation factors eIF4E and eIF(iso)4E are required to prevent pepper veinal mottle virus infection of pepper. J Gen Virol 87:2089-2098

Ruud KA, Kuhlow C, Goss DJ, Browning KS (1998) Identification and characterization of a novel cap-binding protein from Arabidopsis thaliana. J Biol Chem 273:10325-30

Yeam I, Kang BC, Lindeman W, Frantz JD, Faber N, Jahn MM (2005) Allele-specific CAPS markers based on point mutations in resistance alleles at the pvr1 locus encoding eIF4E in Capsicum. Theor Appl Genet 112:178-86

APPENDIX III

The following is the basic protocol used for running Western Blots. A number of Western blots were run using several different antibodies with the intent of measuring the amount of transgenic protein in transgenic plants relative to the endogenous protein. These attempts failed, as described in Chapter III, and transgenic protein was measured by adding an HA tag to the N terminus of the transgenic protein. The purpose of this appendix is to document some of the failed attempts in order that other people may learn from these experiences.

Antibodies that were used to run Westerns included a rabbit polyclonal antibody that was developed against purified pepper eIF4E, as described in (Kang et al. 2005a). In addition, a mouse polyclonal antibody was developed against a peptide fragment of potato eIF4E. The mouse antibody was developed by Precision Antibody (project name 'Cor001'). Mice were injected with an amino acid peptide fragment of eIF4E that was highly conserved between potato, tomato, and pepper (CFKHKIEPKWEDPV). This peptide was synthesized by the company and bound to Keyhole Limpet Hemocyanin in order to increase the antigenicity. Both of these antibodies were used in an unpurified form by adding animal sera directly to the membrane. These antibodies were used in a number of Western blots under varying conditions (see below). Under certain blotting conditions several bands developed and in some cases there were bands that were of approximately the predicted size of eIF4E (26 kD). However, we were suspicious that they corresponded to eIF4E since they were only present under certain conditions and were often accompanied by other bands of varying sizes. Also, no differences were

observed between transgenic and nontransgenic potato plants and we strongly suspected that transgenic plants would be expressing an abundance of eIF4E protein. A typical Western blot is included using the two antibodies (Figure AIII.1).

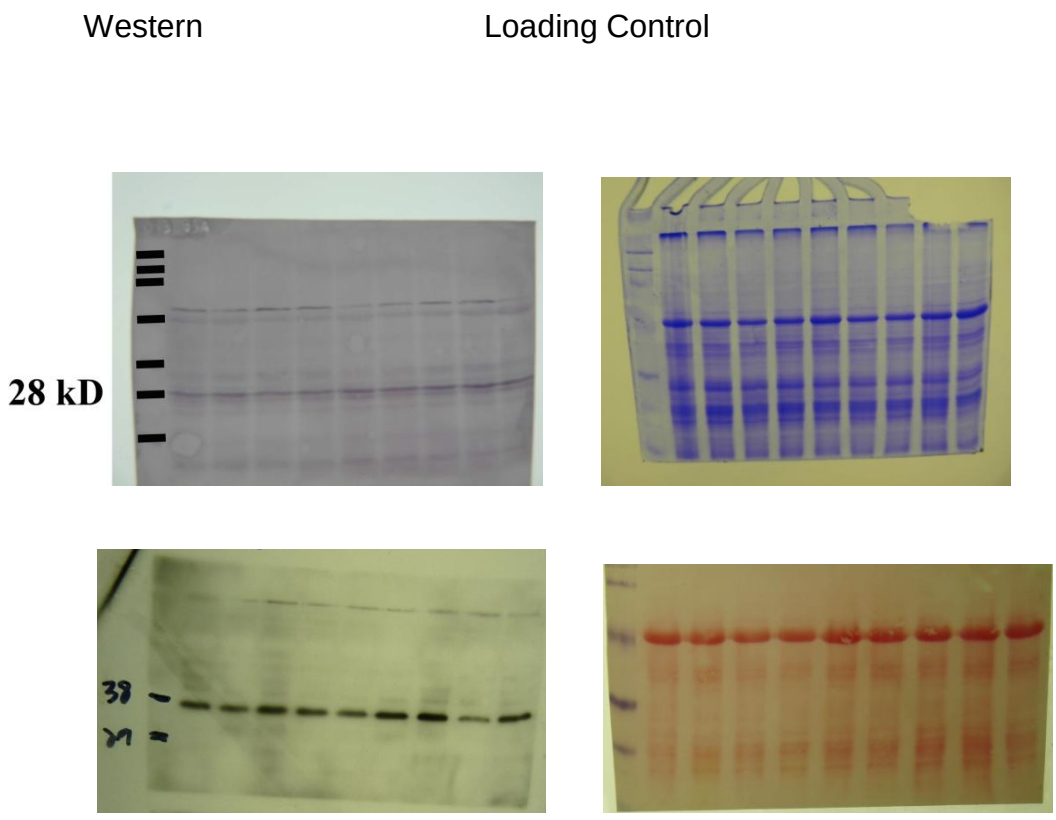

Figure AIII.1: Western and loading control containing transgenic (first 8 lanes after ladder) and a GUS control (last lane on right) ground leaf samples. A) Western and loading control using Cor001 antibody. B) Western and loading control using the rabbit polyclonal antibody developed in rabbit.

The size of the band using the pepper antibody was much too large to be eIF4E. However, the band with the Cor001 antibody was approximately the expected 26 kilodaltons. Further work was performed to determine whether

this band corresponded to eIF4E, or was a nonspecific band corresponding to some other protein of approximately the same size. In order to test this, the HA-tagged eIF4E protein was used from Chapter III. An immunoprecipitation experiment was performed where small anti-HA gel fragments (Sigma catalog number E6779) were used to purify eIF4E protein according to the manufacturer's instructions. Total protein and purified protein was then used to run a Western blot using the Cor001 antibody and an anti HA antibody (Figure AIII.2). The anti HA antibody detected the presence of the HA-tagged eIF4E in both the total protein sample (lane 6) and the purified sample (lane 8). The Cor001 anti eIF4E antibody, however, detected a band in the total protein sample (lane 1) but not one in the eIF4E-purified sample (lane 3). The band from lane 1 was also present in the total protein of the nontransgenic sample (lane 2), as we have seen previously (Figure AIII.1). This led us to conclude that the Cor001 antibody is detecting a nonspecific protein around the same size as eIF4E. Western results obtained using this antibody were disregarded.

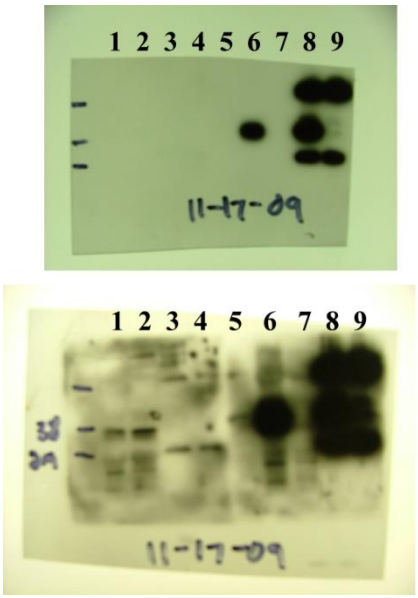

Figure AIII.2: Immunoprecipitation experiment. The two images are the same blot exposed for different lengths of time. Lanes 1 and 5 correspond to total protein of a transgenic potato plant leaf sample expressing HA-tagged eIF4E. Lanes 2 and 6 correspond to total leaf protein of a nontransgenic potato plant leaf sample. Lanes 3 and 7 correspond to the eIF4E purified sample using the anti HA gel fragments. Lanes 4 and 8 correspond to the nontransgenic protein sample that was exposed to the HA gel fragments (but eIF4E was not purified since only the transgenic eIF4E contains the HA tag). Lanes 1 through 4 were exposed to the Cor001 antibody. Lanes 5 through 8 were exposed to an anti-HA High Affinity antibody (Roche).

Western Protocol

Jason Cavatorta

April 2010

SDS-PAGE Gel casting

Materials

- Casting gel unit for electrophoresis
- 30% Acrylamide monomer (29.3g Acrylamide + 0.8g bis)
- Separating Buffer
    - 1.5M Tris-HCl buffer, pH 8.8 (18.7g Tris/100mL)
- Stacking Buffer
    - 0.5M Tris-HCl buffer, pH 6.8 (6.06g Tris/100mL)
- 20% (w/v) SDS
- 10% (w/v) Ammonium persulfate (APS)
- TEMED

NOTE: Acrylamide is a powerful/neurotoxin. Do not come in contact with the monomer. Always wear gloves.

- **Separating Gel (mix just prior to use) – ENOUGH FOR 2 GELS**
    - 6.8mL Acrylamide monomer
    - 4.5mL separating buffer
    - 5.8mL H_20
    - 90µl of 10% (w/v) SDS
    - 180µl 10% APS (in freezer under MM's bench)

- 9µl TEMED (oxidizing chemicals cabinet)

- **Stacking Gel (mix just prior to use) – ENOUGH FOR 2 GELS**
  - 1.0mL Acrylamide monomer
  - 2.0mL stacking buffer
  - 4.0mL H_20
  - 35µl 10% (w/v) SDS
  - 65µl 10% APS (in freezer under MM's bench)
  - 7µl TEMED (oxidizing chemicals cabinet)

Procedure

1. Assemble Mini PROTEAN 3 system glass slides using 1 short plate and 1 spacer plate. Put onto the loading gel unit.
2. Prepare a separating gel
3. Gently fill the center of the glass chamber with the solution by allowing the solution to run down the side of one of the spacers. Avoid introducing air bubbles. USE 7.5 mL PER GEL.
4. Immediately place a water layer on the gel to prevent formation of a curved meniscus. Using a 1mL pipette tip add 1 mL (500 µl down each side) of water. Add it slowly to the edge of the gel as before.
5. After 30 minutes pour off water layer. Excess water may be removed with blotting paper.
6. Prepare a stacking gel from the listed ingredients.
7. Insert appropriate comb and add stacking gel until it reaches the top of the short glass plate. You should have to add about 2 mL. Avoid trapping air bubbles beneath the comb.

8. Allow gels to polymerize for 30 minutes prior to use.

Running the Gel

Materials

- Polyacrylamide gel
- Protein standards
- BSA (10mg/mL)
- 4x-SDS Sample Buffer (10mL)
    - 2.5mL stacking buffer
    - 4.0mL glycerol
    - 0.8g SDS
    - 0.08mL 0.5% Bromophenolblue (5mg/mL) (just add till very blue)
    - Bring to 8mL with water
    - ***Add beta-mercaptoethanol to final concentration of 20% before use***
    - ***Make 2x by diluting 1:1 with H_20***

- 5X-SDS Electrophoresis Running Buffer
    - 15g Tris base
    - 72g glycine
    - 1L H_20
    - ***Dilute to 1x and add 5mL 20% SDS per liter before use***

Procedure

- Bacterial protein
    - Pellet 1mL of an *E. coli* culture resuspended in 100µl H_20
    - Add 100µl 2x sample buffer
    - Boil for 5 min and centrifuge at 12,000rpm for 10 min
    - Recover the supernatant and load 12µl (or 20µl for induced cells) for SDS-PAGE
    -
- Yeast Protein (Modified from Kushnirov, V. 2000. Yeast 16:857-860)
    - Pellet 3 mL of liquid culture (make sure it is galactose CM so that protein expression is turned on) OR scrape some yeast cells off an agar plate – I have had best luck with the former method
    - Resuspend cells in 100 microliters of distilled water
    - Add 100 microliters of 0.2 Molar NaOH
    - Incubate for 5 min at room temp
    - Pellet
    - Resuspend in 50 microliters of SDS sample buffer (I use 100 microliters for pelleted cells from liquid culture)
        - SDS Sample Buffer: 0.06M Tris-HCl pH 6.8, 5% glycerol, 2% SDS, 4% beta-mercaptopethanol, 0.0025% bromophenol blue (I just keep adding drops from a saturated solution into the buffer until the buffer looks dark enough)
    - Boil for 3 min
    - Pellet

- Use 6 microliters per lane

- Plant Protein
  - Grind samples in Liquid N_2 and take 1 PCR strip tube cap amount of sample (or 2 leaf-discs)
  - Add 200µl 2x sample buffer and vortex
  - Boil for 5 min (use cap collars so they don't open up) and centrifuge at 12,000rpm for 10 min
  - Recover the supernatant and load 12µl for SDS-PAGE

Procedure

1) Remove combs from gel and rinse the wells with distilled water. Drain off this water and load gel onto the appropriate slab unit for running the electrophoresis.
2) Fill the wells and the chamber in between the gels with running buffer. Put at least 300 mL running buffer outside the wells. The bottom of the gel should be immersed but the top uncovered. The electrodes should reach into the buffer in the upper chamber.
3) Add samples to the bottom of the wells. Include 5 µl of a protein standard.
4) Assemble the top of the electrophoresis apparatus and connect the system to an appropriate power source. Be sure that the cathode (+) is attached to the appropriate electrode (they are marked).
5) Turn on the power supply and run the gel at 20mA constant current per 1.5mm gel

6) When the tracking dye reaches the separating gel layer increase the current to 30 mA per 1.5mm gel.
7) Continue applying the current until the tracking dye reaches the bottom of the gel. For most samples running for another half of an hour is appropriate.
8) Turn off and disconnect the power supply. Disasemble the gel apparatus and remove the gel from the glass plates.
9) One gel will be used for Coomassie Blue staining to show that equal amounts of protein were loaded. The other gel will be used to transfer the protein to a membrane.

Coomassie Blue Staining Procedure

Materials

- Staining solution
    - 0.2% (w/v) Coomasie Brilliant Blue R 250 in destaining solution
- Destaining solution (1L)
    - 400 mL methanol
    - 500 mL distilled $H_{2=}$
    - 100 mL glacial acetic acid
    -

Procedure

1) Place a gel in at least 10 volumes of Coomasie Blue staining solution for 2 hours. Agitate gently to distribute the dye evenly over the gel. Stain may be re-used but will stain slower during subsequent uses.

2) Place he gels into a destaining solution for at least 1 hour. Put a kimwipe in the destaining solution on one edge to help absorb the dye.
3) If the background is still deeply stained at the end of the hour, move the gel to fresh destaining solution as often as is necessary. Place the gels into containers filled with water for storage.

Transfer to Membrane

Materials

- SDS-PAGE gel that has had protein run out on it (see above)
- Protein transfer membrane (we use "Immun-Blot PVDF Membrane for Protein Blotting" from Bio-rad)
- Sponges
- Whatman paper
- Transfer "sandwich" apparatus (the black and white one with holes in it)
- Transfer electric box
- Electricity source
- Transfer buffer (2L): 25mM Tris base (6.06g), 192mM Glycine (28.8g), 10% methanol – Stable for 1 year in a tightly capped bottle
    - ***Reusable***
- 1xPBS buffer (For **10x** (1L) = 1.3M NaCl (76g), 70mM Na_2HPO_4 (10g), 30mM NaH_2PO_4 (4.1g)
- 1xPBS buffer + 3% tween
- Blocking buffer = 1xPBS + 3% BSA + 1% blocking agent or milk

- For 40mL PBS add 1.2g BSA and 0.4g blocking agent
- Alternative = 1xPBS + 5% (2g/40mL) blocking agent

- Primary antibody (found on second shelf up in -80 in autoclave room)
- Secondary antibody (found in Western Kit)

Procedure

1) Prepare the PVDF membrane by:
   - Cutting it to size
   - Placing it in methanol for 15 sec
   - Placing it in water for 2 min
   - Placing it in transfer buffer for 5 to 10 minutes
2) Transfer protein to membrane
   - Place the “sandwich” apparatus open black side down
   - Layer in the following order
     i. Sponge (bottom)
     ii. Whatman paper
     iii. Gel
     iv. Membrane
     v. Whatman paper
     vi. Sponge (top)
   - Close apparatus
   - Place in transfer box, side that opens facing down and black side facing the black side
   - Fill with transfer buffer (this may be re-used several times)
   - Attack to electricity source with 100 volts and watts and amps maxed out on the 200 scale

- Run for 1 hour to 1 hr 15 min

3) Treat membrane after transfer
    - Take out from sandwich and place in methanol for 10 sec
    - Let dry on Whatman paper(15 min)
    - Place in methanol for 10 sec
    - Rinse in 1X PBS 3 times for 5 minutes each
    - Put in Blocking Buffer with 1:1000 (40 µl for 40 mL) Primary Antibody (anti-rabbit for eIF4E-*capsicum*) overnight at 4 degrees
        i. **OR** use Alternative Blocking Buffer overnight at 4 degrees with no primary antibody then add primary antibody the next day for 1-2 hours at room temp
4) Wash membrane
    - Wash in 1xPBS twice for 5 min each
    - Wash in 1xPBS-Tween twice for 5 min each
    - Wash in 1xPBS twice for 5 min each
    - Place in blocking buffer + secondary antibody (1:5000 or 8µl for 40mL) for 30 minutes to 2 hours (start with less then do with longer time if needed)
    - Wash in 1xPBS twice for 5 min each
    - Wash in 1xPBS-Tween twice for 5 min each
    - Wash in 1xPBS twice for 5 min each
5) Develop membrane
    - Put the blot in 1:1 mixed ECL development solution from Western Kit (1mL solution 1 and 1mL solution 2)
    - Shake 1 min

- Place on a light-proof folder under plastic wrap with minimum liquid
- Expose 1 and 3 min
- Develop in the dark by placing film in:
    i. Development sol'n for 30 sec to 2 min
    ii. Water for 2 min
    iii. Fix solution for at least 2 min
    iv. Wash in water and hang to dry

ALTERNATIVE (used in summer 2009):

Block in TBS-T 0.05% Tween 5% blocking agent

Rinse off milk

Incubate with 1:5K primary AB in TBS-T 1 hour

Rinse TBS-T 5 min 3x

Incubate with 1:5K secondary AB in TBS-T 1 hour

Rinse TBS-T 5 min 3x

<u>1L of 10X TBS-T</u>

80g NaCl

200ml 1M Tris pH7.4 and 5 ml Tween 20

REFERENCES

Kang BC, Yeam I, Frantz JD, Murphy JF, Jahn MM. (2005). The *pvr1* locus in *Capsicum* encodes a translation initiation factor eIF4E that interacts with *Tobacco etch virus* VPg. Plant J 42: 392-405.