ANALYZING VIRUS GENOMIC VARIABILITY TO DESIGN AND TEST GENETIC CONSTRUCTS FOR RESISTANCE

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An understanding of the genomic diversity of plant pathogenic viruses is essential for devising control strategies. Over the last two decades, improved sequencing technologies and the discovery of RNA silencing have profoundly impacted our ability to understand the diversity of virus populations and develop resistant plants.

In Chapter 1 of this dissertation, I critically evaluate the literature regarding materials providing resistance against viruses and vectors in *Vitis* species and discuss their availability for disease management. This review indicates little or no useful resistance toward most virus diseases, and the critical need to develop resistant materials.

In Chapter 2, in order to gain insights into the evolutionary mechanisms of *Grapevine fanleaf virus* (GFLV), sequence information was obtained from fourteen isolates collected in naturally infected vineyards in California. My results indicated that some isolates result from interspecies recombination between GFLV and *Arabis mosaic virus*, and suggest that recombination and purifying selection are important evolutionary mechanisms in the genetic diversification of GFLV.

In Chapter 3, I designed various resistance constructs derived from GFLV based upon an analysis of sequence variability. These constructs were tested for resistance to GFLV using a transient expression system. Results indicated that some of these constructs are capable of reducing virus titers in GFLV-infected plants.

In Chapter 4, I reviewed the literature regarding environmental and human safety issues related to virus-resistant transgenic horticultural crops. My analysis suggests that the use of virus-resistant transgenic plants is a safe and effective way to control viral diseases.

In Chapter 5, I present the results of a survey for *Prunus necrotic ringspot virus* in an orchard of sour and sweet cherry trees. Sequence analysis of the viral coat protein gene from various isolates indicated one predominant and several minor molecular variants. Results revealed a higher rate of infection among sour cherry vs. sweet cherry trees, and also suggested that this virus may have been transferred from a single infected sour cherry tree into the orchard by pollen transfer.

In Chapter 6, I present conclusions regarding the implications of my research and suggest future directions of the work presented in the preceding five chapters.

BIOGRAPHICAL SKETCH

Jonathan Emery Oliver was born in St. Augustine, Florida the son of Lawrence L. Oliver and Celia Joyce Emery Oliver. Jonathan grew up in Palatka, Florida, and became interested in scientific research while in middle school as a member of the Future Farmers of America. As the Chapter's prepared public speaker, he was asked to give a speech on genetic engineering in agriculture.

In 2001, Jonathan graduated as salutatorian of Palatka High School, and he went on to study Microbiology and Cell Science at the University of Florida as a National Merit Scholar. During his sophomore year at UF, Jonathan became interested in adding a major in Plant Pathology. During his junior year, after expressing interest in plant research, Jonathan joined the Floriculture Biotechnology Lab working under Dr. David Clark. As part of the Undergraduate Scholars Research Program, Jonathan conducted research to look at the resistance of *SAG:Ipt* transgenic petunia to fungal and bacterial pathogens. In 2005, Jonathan graduated *summa cum laude* from the University of Florida with his Bachelors of Science Degree with majors in both Microbiology & Cell Science and Plant Pathology. He also minored in Chemistry and Plant Molecular and Cellular Biology.

To pursue his PhD, Jonathan chose to attend graduate school at Cornell University in the Department of Plant Pathology and Plant-Microbe Biology in the Fall of 2005. After performing laboratory rotations in the laboratories of Dr. Christine Smart, Dr. Gregory Martin and Dr. Rosemary Loria, Jonathan joined the laboratory of Dr. Marc Fuchs in Fall 2006. For his dissertation work, Jonathan studied the genetic variability of

plant viruses, and used the information to develop and test constructs that may confer transgenic resistance to *Grapevine fanleaf virus* in grapevines.

In December 2008, Jonathan married Miss Heidi-Ann Marie Hebebrand. Their first-born child, a son, Phillip Ryan Oliver, was born to them in April 2011.

To my mother who encouraged me to do all things “as unto the Lord.”

and

To my father who taught me that “without labor, neither knowledge nor wisdom can accomplish much.”

and

To God my heavenly father who has allowed me to think His thoughts after Him so that I may go forth and apply His thoughts in service.

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PREFACE

Viral pathogens can have significant impacts on the growth and reproduction of crop plants. Resistance to viruses is desirable for disease control; however, it is not available in all cases. One alternative means of protecting plants from viral infection is through the application of pathogen-derived resistance. The underlying mechanism behind pathogen-derived resistance against viruses is RNA silencing, an innate and potent defense mechanism. This mechanism is active in a nucleotide sequence dependent manner against a wide range of viral organisms. RNA silencing holds great promise for the development of resistant plants, in particular when traditional resistance has not been identified.

Due to the error-prone replication of their genomes, plant RNA viruses possess a high potential for genetic variation. Understanding the genetic variability amongst viral isolates can provide insights into the nature of plant virus populations and their spread as well as to predict their ability to overcome host resistances, both traditional and transgenic. Insights into genetic variability can also be exploited to design antiviral genetic constructs for engineering durable and broad-spectrum resistance in otherwise susceptible host plants.

In the following, I will present studies of two viruses, *Grapevine fanleaf virus* (GFLV) [in Chapter 2] and *Prunus necrotic ringspot virus* (PNRSV) [in Chapter 5] that were carried out to investigate virus populations within multiple vineyard sites (for GFLV) or a single orchard (for PNRSV) and speculate on the origins and variability amongst field isolates. For GFLV, I will examine the literature for host resistance - or

lack thereof - in *Vitis* species [Chapter 1], and discuss the use of GFLV sequence variability to design and test concatenated genetic constructs for their ability to provide resistance against divergent GFLV isolates. A high-throughput transient assay for testing the efficacy of constructs to suppress virus multiplication will be presented and its usefulness in identifying promising resistance constructs as compared to stably transformed plants will be discussed [Chapter 3]. Safety issues of virus-resistant transgenic plants in terms of their potential impact on the environment and human health will also be reviewed [Chapter 4]. Finally, conclusions regarding the future and potential use of my transient testing system to evaluate transgenic resistance constructs will be discussed, and directions for continued research in terms of advancing this innovative approach for practical control of virus diseases will be outlined [Chapter 6].

CHAPTER 1

Tolerance and Resistance to Viruses and Their Vectors in *Vitis* sp.: A Virologist's Perspective of the Literature[1]

ABSTRACT

Grapevines can be affected by many viruses and viral diseases. However, despite their long history of cultivation and breeding efforts, little useable resistance to viral diseases has been identified in *Vitis* species. As a result, management of viral diseases has largely relied upon prevention/exclusion or on the use of economically and/or environmentally consequential methods to manage vector populations. Resistance to dagger nematode vectors has been identified and successfully transferred to rootstock genotypes that are commercially available, but these resistant materials do not prevent virus translocation into the scion – although a significant delay in infection can allow for suitable production. In this review, we critically evaluate the literature regarding resistance against viruses and vectors in *Vitis* and *Muscadinia* species. We examine the challenges of breeding grapevines for virus resistance and the methodologies used to test for resistance. The availability of useful sources of resistance and tolerance toward both viruses and their vectors, or lack thereof, is evaluated and discussed in terms of disease management.

[1]Oliver, J.E. and Fuchs, M. 2011. Tolerance and resistance to viruses and their vectors in *Vitis* sp.: A virologist's perspective of the literature. *Am. J. Enol. Vitic*. *(in revision)*.

INTRODUCTION

A host of problems

Grapevines have been cultivated for thousands of years and on six continents (Burger et al. 2009). They are a highly valuable fruit crop with a $162 billion dollar annual impact on the U.S. economy alone (MFK Research 2007). Among the limiting factors affecting grapevine production are viruses. At least sixty virus species are known to affect grapevines (Martelli and Boudon-Padieu 2006), and when other known infectious agents including viroids (five), phytoplasmas (eight), and insect-transmitted xylematic bacteria (one) are included, these 74 intracellular pathogens represent the largest number ever found in a single crop (Martelli and Boudon-Padieu 2006). Viruses can be serious threats to grape production. Some viruses are transmitted from vine to vine by dagger nematodes or mealybugs and soft scale insects. Present management strategies rely on preventative approaches based upon the use of planting material derived from certified, virus-tested stocks. In vineyards where viruses and vectors are present, cultural practices, e.g. roguing, application of pesticides, and planting vines which possess resistance or tolerance to vectors, are used to mitigate the impact of viruses. However, these approaches are often costly, environmentally undesirable or of limited efficacy and the use of rootstocks with vector resistance or tolerance may have undesired effects on vine growth and quality. Nonetheless, the use of cultivars and rootstocks that are resistant or tolerant to viruses would be ideal to manage virus diseases (Maule et al. 2007). Despite the many viruses that can be a major constraint on grapevine production, and grapevines' high value and long history of

breeding/selection (Burger et al. 2009), little useable resistance to viruses has been identified (Laimer et al. 2009) even though it is obviously desirable. In this review, we will examine the potential reasons for this and the current state of knowledge regarding resistance or tolerance to viruses in *Vitis* and *Muscadinia* species.

Grapevine - A unique host

Grapevines (genera: *Vitis*) have a long history of cultivation, especially in Europe and the Middle East where the species of European grapevine (*Vitis vinifera*) is believed to have originated (Burger et al. 2009). *Vitis vinifera* is by far the most widely cultivated grape species; however, other *Vitis* species including *V. riparia, V. rupestris, V. candicans, V. x slavinii, V. longii, V. rufotomentosa, V. berlandieri, V. arizonica, V. x champinii, and V. labrusca,* as well as *Muscadinia rotundifolia*, are also important for other reasons. These include: their usefulness in breeding efforts, for grape production, and as rootstock materials to provide many important growth characteristics, including protection of grafted scion cultivars against pathogens, their vectors, and adverse soil conditions.

Grapevines are unique not only because of their perennial life cycle, long history of cultivation, and their extensive transfer between continents, but also because individual cultivars have been grafted and propagated vegetatively for substantial periods which has concurrently made it possible for viruses to easily be moved and disseminated over wide areas along with their grapevine hosts. Transfer of material began taking place long years before the discovery of viral pathogens in 1898 (Harrison and Wilson 1999), meaning the infection status of transferred vines was largely

unknown. These unique properties may partially explain why grapevine is afflicted with such a wide diversity of viral species. In addition, the long held preferences for a limited number of grapevine cultivars, due to their enological properties, have complicated efforts to integrate new genetic traits and resistances into cultivated elite varieties (Burger et al. 2009).

Resistance - What's in a name?

Resistance to grapevine viruses is the most desirable means of control, in particular for vector-transmitted viruses, once they have become established in a grape-growing area. However, the term "resistance" when it comes to viruses can refer to a range of plant reactions to virus infection, including, but not limited to: resistance to multiplication, reduced symptoms, delayed infection, or resistance to the virus vector. Often, the simple term "resistance" can be used interchangeably by plant breeders, virologists, physiologists, viticulturalists, or plant pathologists to refer to any one of these situations. This can cause a great deal of confusion (Buddenhagen 1981). For the purposes of this paper, we will differentiate between "resistance" and "tolerance" because both terms describe unique virus-host relationships which can differentially impact disease management and production.

Resistance refers to the plant's ability to limit virus multiplication (Fraile and Garcia-Arenal 2010) by interfering with the disease cycle within the host plant (Lecoq et al. 2004). Completely resistant or immune plants are unable to sustain virus replication (Lecoq et al. 2004), while completely susceptible plants do not impair pathogen infection. In between these extremes, plants more able to impair virus replication may

be described as showing *more resistance* than those plants less able to impair virus replication, which are, in turn, *more susceptible*. Though more resistant plants are often able to produce greater yields in the presence of the virus than more susceptible plants, this may not necessarily be true in all cases – as plants that are more able to impair replication, and thus show lower levels of virus infection, do not necessarily display fewer symptoms of virus infection (Hull 2002, Lapidot et al. 2006). However, a completely resistant plant would be unable to be infected, and of course would not be expected to show any viral symptoms. Because relative virus multiplication and yield are not always related, so-called "tolerance" to virus infection has been described.

Tolerance to virus infection is the ability of plants to reduce the damage caused by virus infection to produce a good crop in the presence of a virus (Fraile and García-Arenal 2010, Lecoq et al. 2004). Different degrees of tolerance can be identified (Peng and Moens 2003). *More tolerant* plants are able to produce a better crop when infected than *less tolerant* plants. *Completely tolerant* plants are capable of producing as good of a crop whether the pathogen is present or not, and *completely intolerant* or *susceptible* plants are unable to produce a crop when infected (Peng and Moens 2003). Most plants fall somewhere in between these extremes.

These definitions of "tolerance" and "resistance" can also be used to describe the plant's ability to interfere with vector multiplication or to avoid the harmful effects on yield in the presence of the vector, respectively. In the case of viruses transmitted by vectors, the system can be more complicated, in that a resistance or tolerance to a viral vector can appear to be a resistance to a viral disease if the vector's feeding is diminished or prevented – subsequently failing to allow efficient transmission of the

virus to the plant host. Once the virus gets in, if the plant cannot prevent virus replication, the virus would be able to replicate in the host and impact yield thereafter. Though not the result of true virus resistance, such a delay of infection may be significant to growers from an economic perspective (Raski et al. 1983) and so it cannot be discounted in a disease management scheme. The impact of such a delayed infection in terms of disease management should be given careful consideration on a case-by-case basis.

Evaluating resistance and tolerance to viruses

Resistance and tolerance evaluations are often made using a wide range of techniques and by looking at a diversity of parameters. Since virus symptoms can be quite variable between environmental conditions (Lider and Goheen 1986), reliance on symptoms alone to determine infection can be challenging and may not be an appropriate measure to assess resistance. A serological approach, such as enzyme linked immunosorbent assay (ELISA), is often used to assess the presence of viral proteins - often the coat protein or other structural proteins. Alternatively, nucleic acid based assays such as reverse transcription - polymerase chain reaction (RT-PCR) for RNA viruses or PCR for DNA viruses can be used to determine the accumulation of viral genomic material. Other methods, such as bioassays, can be employed to monitor infection with mechanically transmissible viruses. Bioassays rely on transfer of the virus from one host to another host (an indicator host) on which it is known to cause symptoms (or in which it can readily be detected by other means). For virus vector resistance evaluations, relative multiplication estimates are often made for nematodes

by comparing gall numbers or by counting numbers of individuals associated with roots or present in soil samples. For mealybugs and scale insects, similar approaches would likely be used for resistance evaluations, but we are not aware of ongoing research efforts in this area. By contrast, evaluations of tolerance often involve comparisons of yield between infected plants and uninfected plants of the same variety or cultivar. Ideally, these comparisons should be made under the same growing conditions. If not, natural variations in yield due to climatic effects, field location, or natural variations between cultivars or advanced breeding material can have a significant impact on yield in their own right. Vigor [as measured by pruning wood weight], weights and numbers of fruit clusters, and levels of soluble solids can be used to quantify virus impact on plant growth, yield, and fruit chemistry, as part of a tolerance evaluation.

In this paper, we critically examine the literature using the standards for tolerance or resistance evaluations to establish if tolerance or resistance exists in given grapevine genotypes. Utilizing the entire body of peer reviewed evidence for each individual genotype, we will discuss their relative resistance or tolerance, where appropriate, to either the virus vectors or the viruses themselves. Also, in each case we will discuss the usefulness – in a disease management context – of the respective sources of resistance or tolerance for each of the major virus groups.

Testing for Virus Resistance/Tolerance - A challenge with grapevines

Meaningful screens for resistance/tolerance can be challenging with grapevines. This is primarily due to the perennial nature of the crop and to the extreme difficulty or relative impossibility of mechanically inoculating grapevine tissue with viruses (Valat et

al. 2003). Unlike annual crops where resistance/tolerance screens can be carried out in a relatively short time (a few weeks to a few months) – often with more than one round of screening per year – the length of time necessary to evaluate a perennial crop like grapevine is substantially longer due to the fact that grapevines do not reach full production capacity for a few years after establishment and vines are expected to resist/tolerate pathogen or pest challenges over much longer time periods. Additionally, though many herbaceous hosts can be routinely infected with viruses by mechanical means or by *Agrobacterium tumefaciens*-mediated delivery of viral cDNA, both of which allow for large-scale routine inoculations in controlled experiments, these means of inoculation are ineffective and/or not possible with grapevine – with the exception of the delivery of *Grapevine virus A* (GVA) cDNA via *Agrobacterium tumefaciens* (Haviv et al. 2006, Muruganantham et al. 2009). Because of these limitations, the evaluation of grapevines for resistance or tolerance to viruses relies essentially on virus infection via graft inoculation or vector-mediated infection (Valat et al. 2003). Graft inoculations (Lahogue et al. 1995) and vector-mediated infection methods have their own difficulties. Vector-mediated infection studies, which more accurately mirror natural infection conditions in the vineyard, can be difficult due to low or inconsistent levels of inoculum present within a test vineyard (Gonsalves 1982, Harris 1988, Valat et al. 2003). Conversely, graft inoculation may be an inappropriate method of resistance screening in some cases, because the inoculation pressure may be too strong to allow for accurate identifications of virus resistance mechanisms (Esmenjaud and Bouquet 2009, Gonsalves 1982, Lahogue and Boulard 1996).

It is crucial that screens be realistic enough to mimic actual growing conditions, but precise enough to determine subtle differences between resistant varieties. Since individual grapevines may be expected to remain productive for longer periods than most fruit and vegetable crops, even delayed infections that take a substantial time to appear may be significant to growers. Therefore, precise measurements of the virus's impact on infected vines may be necessary to allow for growers to make appropriate disease management decisions. Likewise, due to the high value of grapes, and especially wine grapes, even a mild reduction in yield or alteration in fruit composition after virus infection may have a significant impact on growers – further highlighting the importance of precise evaluative parameters. Since a fully productive grapevine can take up a lot of space, controlled-environment experiments can be challenging, and realistic field studies are necessary for yield-related measurements. Conversely, since the varied growing conditions in field settings can influence disease symptom severity and yields, this can make it necessary for several years worth of field data to be collected before conclusions can be drawn. Also, since grapevines are grown in a wide variety of climates and soil conditions, proof of tolerance (i.e. no effects on yield) can be difficult and should involve testing in diverse climatic, soil, and cultivation conditions. Simply observing a given genotype in one field or growing it in a single soil type may mask potential virus-induced effects on yield.

GRAPEVINE VIRAL DISEASES

We are called legion - the many viral diseases of grapevine

Though grapevines can be infected with at least 60 different viral pathogens (Martelli and Boudon-Padieu 2006), the important viral diseases predominantly consist

of four larger groups of viruses (Table 1.1). These groupings include: the degeneration/decline disease complex - consisting of nepoviruses, many of which are transmitted by dagger nematodes; the leafroll disease complex - consisting of several ampeloviruses and other viruses, many of which are vectored by mealybugs or scale insects; the rugose wood disease complex - consisting of vitiviruses, which are transmitted by mealybugs or scale insects; and the fleck disease complex - consisting of marafiviruses and maculaviruses. Other viruses cause less extensive problems on grapevine (Table 1.1). In subsequent sections, we will look more closely at each of these viral disease groups with respect to the current status of knowledge regarding natural resistance/tolerance to the respective viruses or their vectors.

Degeneration/Decline complex: One of the most important viral diseases of grapevine is fanleaf degeneration/decline. Fanleaf degeneration/decline is caused by a number of viruses that belong to the genera *Nepovirus* and *Sadwavirus* in the family *Secoviridae* (Martelli and Boudon-Padieu 2006). Many of these viruses are specifically transmitted in a soilborne manner by ectoparasitic nematodes. The first virus confirmed to be transmitted by dagger nematodes was *Grapevine fanleaf virus* (GFLV) (Hewitt et al. 1958), which remains the most important viral cause of fanleaf degeneration. GFLV can result in severe crop losses of up to 80% and produce a range of symptoms, including vein banding, yellow mosaic, and epinastic distortion of leaves into a fanlike shaped leaf with narrowed interveinal sinuses. GFLV is naturally transmitted in a specific manner by the Longidorid nematode species *Xiphinema index* and is largely confined to grapevine (Andret-Link et al. 2004).

Table 1.1. Grapevine viruses, the disease they cause, and their vector if known.

Virus	Acronym	Family	Genus	Vector(s)
A. Degeneration/Decline Disease Complex				
Grapevine fanleaf virus	GFLV	*Secoviridae*	*Nepovirus*	*Xiphinema index*
Tomato ringspot virus	ToRSV		*Nepovirus*	*X. rivesi, X. americanum* and *X. californicum*
Tobacco ringspot virus	TRSV		*Nepovirus*	*X. americanum*
Peach rosette mosaic virus	PRMV		*Nepovirus*	*X. americanum and Longidorus diadecturus*
Blueberry leaf mottle virus	BLMoV		*Nepovirus*	Unknown
Grapevine Bulgarian latent virus	GBLV		*Nepovirus*	Unknown
Tomato blackring virus	TBRV		*Nepovirus*	*L. elongatus and L. attenuatus*
Artichoke Italian latent virus	AILV		*Nepovirus*	Unknown
Grapevine Tunisian ringspot virus	GTRV		*Nepovirus*	Unknown
Arabis mosaic virus	ArMV		*Nepovirus*	*X. diversicaudatum*
Raspberry ringspot virus	RpRSV		*Nepovirus*	*Paralongidorus maximus*
Grapevine deformation virus	GDefV		*Nepovirus*	Unknown
Grapevine chrome mosaic virus	GCMV		*Nepovirus*	Unknown
Grapevine Anatolian ringspot virus	GARSV		*Nepovirus*	Unknown
Cherry leafroll virus	CLRV		*Nepovirus*	Unknown
Strawberry latent ringspot virus	SLRSV		*Sadwavirus*	*X. diversicaudatum*
B. Leafroll Disease Complex				
Grapevine leafroll-associated virus-1	GLRaV-1	*Closteroviridae*	*Ampelovirus*	*Heliococcus bohemicus, Phenacoccus aceris, Pseudococcus maritimus, Pulvinaria vitis, Pu. innumerabilis* and *Parthenolecanium corni*
Grapevine leafroll-associated virus-2	GLRaV-2		*Closterovirus*	Unknown
Grapevine leafroll-associated virus-3	GLRaV-3		*Ampelovirus*	*Planococcus ficus, Pl. citri, Ps. longispinus, Ps. affinis, Ps. calceolariae, Ps. maritimus, Ps. viburni, Ps. comstocki, Ph. aceris, Pu. vitis, Pu. innumerabilis, H. bohemicus, Coccus longulus, Coccis Hesperidium, Prasaissetia nigra, Ceroplastes rusci* and *Saissetia sp.*
Grapevine leafroll-associated virus-4	GLRaV-4		*Ampelovirus*	*Pl. ficus*
Grapevine leafroll-associated virus-5	GLRaV-5		*Ampelovirus*	*Ps. longispinus, Pl. ficus* and *Ceroplastes rusci*
Grapevine leafroll-associated virus-6	GLRaV-6		*Ampelovirus*	Unknown
Grapevine leafroll-associated virus-7	GLRaV-7		*Unassigned*	Unknown
Grapevine leafroll-associated virus-9	GLRaV-9		*Ampelovirus*	*Ps. longispinus*
Grapevine leafroll-associated virus-De	GLRaV-De		*Ampelovirus*	Unknown
Grapevine leafroll-associated virus-Pr	GLRaV-Pr		*Ampelovirus*	Unknown
Grapevine leafroll-associated virus-Car	GLRaV-Car			*Ampelovirus* Unknown
C. Rugose Wood Disease Complex				
Grapevine rupestris stem pitting-associated virus	GRSPaV	*Betaflexiviridae*	*Foveavirus*	Unknown

Table 1.1 (Continued)

Grapevine virus A	GVA		*Vitivirus*	*Pl. citri, Pl. ficus, Ps. longispinus, Ps. affinis, H. bohemicus, Ph. aceris* and *N. innumerabilis*
Grapevine virus B	GVB		*Vitivirus*	*Pl. ficus, Ps. longispinus* and *Ps. affinis*
Grapevine virus D	GVD		*Vitivirus*	Unknown
Grapevine virus E	GVE		*Vitivirus*	*Ps. comstocki*
D. Fleck Disease Complex				
Grapevine redglobe virus	GRGV	*Tymoviridae*	*Maculavirus*	Unknown
Grapevine fleck virus	GFkV		*Maculavirus*	Unknown
Grapevine rupestris vein feathering virus	GRVFV		*Marafivirus*	Unknown
Grapevine asteroid mosaic-associated virus	GAMaV		*Marafivirus*	Unknown
Grapevine Syrah virus 1	GSyV-1		*Marafivirus*	Unknown
E. Other viruses				
Potato virus X	PVX	*Alfaflexiviridae*	*Potexvirus*	Unknown
Grapevine berry inner necrosis virus	GBINV	*Betaflexiviridae*	*Trichovirus*	Mites
Alfalfa mosaic virus	AMV	*Bromoviridae*	*Alfamovirus*	Aphids
Cucumber mosaic virus	CMV		*Cucumovirus*	Unknown
Grapevine angular mosaic virus	GAMoV		*Ilarvirus*	Unknown
Grapevine line pattern virus	GLPV		*Ilarvirus*	Unknown – seed borne
Tomato spotted wilt virus	TSWV	*Bunyaviridae*	*Bunyavirus*	Unknown
Grapevine vein clearing virus[a]	GVCV	*Caulimoviridae*	*Badnavirus*	Unknown
Bean common mosaic virus	BCMV	*Potyviridae*	*Potyvirus*	Unknown
Broadbean wilt virus	BBWV	*Secoviridae*	*Fabavirus*	Unknown
Carnation mottle virus	CarMV	*Tombusviridae*	*Carmovirus*	Unknown
Grapevine Algerian latent virus	GALV		*Tombusvirus*	Unknown
Petunia asteroid mosaic virus	PAMV		*Tombusvirus*	Unknown
Tobacco necrosis virus D	TNV-D		*Necrovirus*	Unknown
Tobacco mosaic virus	TMV	*Virgaviridae*	*Tobamovirus*	Unknown
Tomato mosaic virus	ToMV		*Tobamovirus*	Unknown
Sowbane mosaic virus	SoMV	Unassigned	*Sobemovirus*	Unknown
Raspberry bushy dwarf virus	RBDV	Unassigned	*Idaeovirus*	Unknown
Grapevine stunt virus	GSV	Unassigned	Unassigned	Unknown
Grapevine labile-rodshaped virus	GLRSV	Unassigned	Unassigned	Unknown
Putative new virus species[b]	na	*Reoviridae*	*Oryzavirus*	Unknown
Putative new virus species[b]	na	*Luteoviridae*	*Enamovirus*	Unknown
Putative new virus species[b]	na	*Endornaviridae*	*Endornavirus*	Unknown

na: not applicable; [a]Zhang et al. (2011); [b]Sabanadzovic (2009)

Xiphinema index, like many of the other nematode vectors of degeneration-causing viruses, can be an important pest of grapevine in its own right if populations are high. Since GFLV and its nematode vector have a relatively narrow host range, and since they were the first virus-vector combination identified in grapevine, much work has been done to develop methods to control GFLV (Andret-Link et al. 2004, Boubals and Pistre 1978, Bouquet et al. 2000, 2003a, 2003b, Bouquet and Danglot 1983, Bouquet 1981, 1983a, 1983b, Ipach et al. 2000, Jimenez Diaz and Goheen 1991, Lahogue and Boulard 1996, Lider and Goheen 1986, Raski et al. 1983, Smith et al. 2005, Staudt and Kassemeyer 1990, Walker and Jin 1998, 2000, Walker and Meredith 1990, Walker et al. 1985, 1989, 1991, Walker and Wolpert 1994). Resistance in grapevines toward other nepoviruses, by contrast, has been less extensively studied. Some of these other nepoviruses include: *Arabis mosaic virus* (ArMV), a virus closely related to GFLV, which can also cause fanleaf degeneration, has a much wider host range than GFLV, and is transmitted by another nematode species, *Xiphinema diversicaudatum*; *Tobacco ringspot virus* (TRSV), *Peach rosette mosaic virus* (PRMV), and *Tomato ringspot virus* (ToRSV), vectored by *Xiphinema americanum*; as well as several other nematode-vectored nepoviruses and a sadwavirus (Table 1.1a). Since most nepoviruses are transmitted within a vineyard only by nematode vectors, much effort toward controlling these pathogens has focused either on eliminating the nematode vector (Andret-Link et al. 2004, Raski et al. 1983) or on controlling virus transmission during vector feeding using resistant grapevine materials – especially rootstock genotypes that may be able to protect an otherwise susceptible scion. Investigations of resistance to GFLV or GFLV transmission are many (Becker 1989, Becker and Sopp 1990, Boubals and Pistre 1978,

Bouquet et al. 2000, 2003a, 2003b, Bouquet and Danglot 1983, Bouquet 1981, 1983a, 1983b, Ipach et al. 2000, Jimenez Diaz and Goheen 1991, Lahogue and Boulard 1996, Lider and Goheen 1986, Raski et al. 1983, Smith et al. 2005, Staudt and Kassemeyer 1990, Walker and Jin 1998, 2000, Walker and Meredith 1990, Walker et al. 1985, 1989, 1991, Walker and Wolpert 1994), with several reports of genotypes that are tolerant or less susceptible to the virus than others, or resistant to GFLV transmission. However, none of these studies have found resistance to GFLV that either prevents virus replication or movement into a susceptible grafted scion under field conditions. As of the start of 2011, approximately 24 publications have described studies of GFLV resistant cultivars, often with conflicting findings. By contrast, a substantially smaller number of studies have focused on other nepoviruses, i.e. ToRSV, PRMV and ArMV (Allen et al. 1982, Gonsalves 1982, Lahogue and Boulard 1996, Ramsdell et al. 1995), often with similarly conflicting results. A few studies have also focused on the nematode vector *X. americanum* (McKenry et al. 2001, 2004, McKenry and Anwar 2006, Ramsdell et al. 1996).

Theoretically, protection against nepoviruses could break down into at least four types: 1. Tolerance to the virus's nematode vector, 2. Resistance to the virus's nematode vector, 3. Tolerance to the virus itself, or 4. Resistance to the virus itself. Accordingly, resistance and/or tolerance to either nematodes or viruses has been described. Therefore, we will examine each of these types of resistance or tolerance and discuss the relevance or importance of cultivars or rootstocks displaying each type with respect to virus control - with a special emphasis on field studies.

Tolerance to nematode vectors: Tolerance to a nematode vector means a case where the cultivar or rootstock, in the presence of the nematode (and in the absence of any virus they transmit), either allows for the same level of production (grape yield/quality) as when the nematode vector is absent (completely tolerant), or more production than other cultivars or rootstocks allow for in the presence of the nematode (relatively more tolerance). Therefore, studies that look at some component of yield are necessary to prove whether a rootstock or cultivar is tolerant (Peng and Moens 2003, Trudgill 1991). Based on this criterion, several rootstocks or cultivars have been suggested to be tolerant to *X. index*, the nematode vector of GFLV, and *X. americanum*, the nematode vector of PRMV, ToRSV, and TRSV, or to exhibit relatively more tolerance in comparison with others in field studies (Table 1.2). In the case of vector tolerance, nematode feeding (probing) on rootstocks is still occurring; hence, these tolerant rootstocks (assuming viral replication is still possible) do not aid in the control of viruses, though these may be important to growers in areas where the nematode is present as the primary constraint on production in the absence of the virus.

Resistance to nematode vectors: When a nematode vector species is completely unable to reproduce on the host grapevine, then that grapevine may be called resistant to that nematode species. If the nematode vector is unable to reproduce as successfully on one cultivar or rootstock vs. another in similar growing conditions, then that rootstock or cultivar may be called relatively more resistant (Trudgill 1991). Necessarily, studies purporting this type of resistance must demonstrate lowered nematode reproduction rates or reduced nematode survival when associated with the supposedly resistant rootstocks (Peng and Moens 2003, Trudgill 1991).

Table 1.2. Grapevine materials with tolerance to nematode vectors of viruses.

Virus Vector	Species or Cross	Materials	Tolerance Indicated by:	Tolerance NOT Confirmed by:
X. americanum	*V. vinifera* Mourvedre x *V. rupestris* x *V. rupestris* Martin	1202C	[a]Ramsdell et al. 1996	
	V. solonis x V. riparia	1616C	[a]Ramsdell et al. 1996	
	V. riparia x V. rupestris	3309C	[a]Ramsdell et al. 1996	
	V. berlandieri x V. riparia	Teleki 5A	[a]Ramsdell et al. 1996	
		Kober 5BB	[a]Ramsdell et al. 1996	
	101-14 x Goldreisling	Foch	[a]Ramsdell et al. 1996	
	Seibel 5656 x Seibel 4986	Seyval	[a]Ramsdell et al. 1996	
	Seibel 6905 x Pinot de Corton	Vignoles	[a]Ramsdell et al. 1996	
	Seibel 4986 x Ungi blanc	Vidal	[a]Ramsdell et al. 1996	
X. index	*V. vinifera x M. rotundifolia*	O39-16	[a]Lider and Goheen 1986, [a]Walker et al. 1989, [a]1994	
		O43-43	[a]Lider and Goheen 1986, [a]Walker et al. 1989, [a]1994	
		O44-44	[a]Lider and Goheen 1986	
	V. rufotomentosa x V. vinifera	171-6	[a]Lider and Goheen 1986	
		171-13	[a]Harris 1988	
		171-52	[a]Harris 1988	
	V. x champinii			[b]Meredith et al. 1982
		Dog Ridge	[a]Harris 1988	
		Salt Creek	[a]Harris 1988	[b]Meredith et al. 1982
	1613C x *V. rupestris* Metallique	122-16	[a]Harris 1988	
	V. riparia x V. rupestris	101-14	[a]Harris 1988	
	V. berlandieri x V. riparia	SO4	[a]Harris 1988	
	V. slavinii x V. rupestris Metallique	88-113	[a]Harris 1988	
	V. rufotomentosa x V. candicans	142-40	[a]Harris 1988	
	V. berlandieri x V. rupestris	110R	[a]Harris 1988	
	V. arizonica x V. candicans	101-56	[a]Harris 1988	
	V. longii x (V. riparia Gloire x V. x champinii Ramsey)	106-38	[a]Harris 1988	
	V. candicans x 1613C	116-11	[a]Harris 1988	
		116-60	[a]Harris 1988	
	V. rufotomentosa x (V. riparia Gloire x Dog Ridge*)*	514-11	[a]Harris 1988	
	(V. riparia Gloire x Dog Ridge) x 1613C	112-2	[a]Harris 1988	
	V. arizonica		[b]Meredith et al. 1982	
	V. rufotomentosa		[b]Meredith et al. 1982	
	V. candicans		[b]Meredith et al. 1982	
	V. slavinii		[b]Meredith et al. 1982	
	V. solonis		[b]Meredith et al. 1982	
	V. riparia Gloire		[b]Meredith et al. 1982	

[a]Based on yield or vigor determination, [b]Based on other measure of host performance (root damage)

There has been some success in finding this type of resistance toward some nepovirus vectors in grapevine cultivars (Table 1.3). With respect to *X. index*, the nematode vector of GFLV, *Muscadinia rotundifolia* and hybrid crosses between *M. rotundifolia* and *Vitis* species reportedly show “high” resistance to *X. index* (Aballay et al. 1998, Bouquet et al. 2003a, 2003b, Esmenjaud et al. 2010, Staudt and Weischer 1992, Walker and Jin 1998, Walker et al. 1991) with few or no root-associated nematodes found and reactions to nematode feeding on roots that appear to be a hypersensitive response (Staudt and Weischer 1992). Similarly, the rootstock Börner (Hafner 1998), which results from a cross of *V. riparia* 183 Geisenheim and *V. cinerea* Arnold, has been reported to resist galling by *X. index* (Becker 1989, Becker and Sopp 1990, Sopp et al. 1998). Other rootstocks which are resistant to this nematode species include *Vitis arizonica* (Van Zyl et al. 2009, Weischer 1980, Wheeler and Walker 2005) and recent work with this species has led to the identification of the first major genetic locus responsible for resistance to an ectoparasitic nematode in grapevine (*XiR1*) [*Xiphinema index* resistance locus 1] (Hwang et al. 2010, Xu et al. 2008). This new genetic information will facilitate the use of marker-assisted selection in the breeding of new grapevine rootstocks with resistance to *X. index* (Xu et al. 2008). Many other genotypes and hybrids have resistance to *X. index* and *X. americanum* (Table 1.3), but only a fraction have been released for growers.

Table 1.3. Grapevine materials with resistance to nematode vectors of viruses**.**

Virus Vector	Species or Cross	Materials	Resistance Indicated by:	Resistance NOT confirmed by:
X. americanum	*V. vinifera x M. rotundifolia*	O39-16	McKenry et al. 2001, 2004	McKenry and Anwar 2006
X. index	*V. vinifera x M. rotundifolia*		Bouquet et al. 2000	
		Mtp 3146-1-87	Bouquet et al. 2000, 2003a, 2003b	
		O39-16	Aballay et al. 1998, McKenry and Anwar 2006, McKenry et al. 2001, 2004, Staudt and Kassemeyer 1990, Walker and Jin 1998, Walker et al. 1989, 1991	
		O43-43	[a]Staudt and Kassemeyer 1990, Walker et al. 1989	
		VRH 8771	Esmenjaud et al. 2010	
		VRH 97-99-79	Esmenjaud et al. 2010	
		NC 35-50	Esmenjaud et al. 2010	
		VMH 11-6-76	Bouquet et al. 2003a	
	V. riparia x V. cinerea	Börner	Becker and Sopp 1990, Becker 1989, Ipach et al. 2000, Sopp et al. 1998	
	V. riparia x V. rupestris	Schwarzmann	[a]Coiro et al. 1985, Harris 1983, [a]McKenry et al. 2001, McKenry and Anwar 2006	
		3309C	Boubals and Pistre 1978, Harris 1983	Bouquet et al. 2000, McKenry et al. 2001, 2004
	V. arizonica		Harris 1983, Kunde et al. 1968, Staudt and Kassemeyer 1990, Weischer 1980, Wheeler and Walker 2005	
		b40-14	Van Zyl et al. 2009	
		b42-26	Hwang et al. 2010, Xu et al. 2008	
	V. candicans		Harris 1983, Kunde et al. 1968, Weischer 1980	
		Solonis	Coiro et al. 1985	
	V. longii	Solonis		Kunde et al. 1968
	V. x champinii			Kunde et al. 1968
		Dog Ridge	Boubals and Pistre 1978, [b]Coiro et al. 1990	Aballay et al. 1998, Kunde et al. 1968, Malan and Meyer 1993, McKenry et al. 2001, McKenry and Anwar 2006
		Salt Creek	[b]Coiro et al. 1990	Boubals and Pistre 1978, Kunde et al. 1968, McKenry et al. 2001, McKenry et al. 2004, McKenry and Anwar 2006
	V. rupestris x M. rotundifolia		Walker and Jin 1998, 2000	
		8913-02	Walker and Jin 1998	
		8913-21	Walker and Jin 1998	
	V. berlandieri x V. rufotomentosa		Walker and Jin 1998, 2000	
	M. rotundifolia		Boubals and Pistre 1978, Sopp et al. 1998, Staudt and Weischer 1992	
		Noble	Bouquet et al. 2000	
		Carlos	Bouquet et al. 2000	
		Yuga	Bouquet et al. 2000	
	V. munsoniana		Staudt and Weischer 1992	
	V. riparia x V. berlandieri	161-49C	Boubals and Pistre 1978, [a]Coiro et al. 1985, [a]Malan and Meyer 1993	

Table 1.3 (Continued)

V. berlandieri x V. riparia	Teleki 5A	Coiro et al. 1985, [c]1990	
	Teleki 5C	Coiro et al. 1985	Boubals and Pistre 1978, Coiro et al. 1990, McKenry et al. 2004, McKenry and Anwar 2006, Sopp et al. 1988
	SO4	Boubals and Pistre 1978, Coiro et al. 1985	Coiro et al. 1990, Harris 1983, Malan and Meyer 1993, McKenry and Anwar 2006
V. riparia		Boubals and Pistre 1978, [a]Kunde et al. 1968	Walker and Jin 1998, 2000
	De Pailleìres	Coiro et al. 1985	
	Gloire de Montpellier	Boubals and Pistre 1978, [a]Coiro et al. 1985, [a]Kunde et al. 1968, [a]Staudt and Kassemeyer 1990	Esmenjaud et al. 2010, Malan and Meyer 1993
	Fabre	Coiro et al. 1985	
	Gran Glabre	[a]Coiro et al. 198	
V. berlandieri x V. rupestris	57 Richter	[a]Coiro et al. 1985	
	110 Richter	Boubals and Pistre 1978, [a]Coiro et al. 1985	Harris 1983
	770 Paulsen	[a]Coiro et al. 1985	
V. riparia x *V. vinifera* Carignon (Castel 15-612) x *V. rupestris* du Lot *V. berlandieri* x (Aramon -*V. rupestris* Ganzin 1)	Golia	Coiro et al. 1985	
	2413 Grimaldi	[a]Coiro et al. 1985	
	1045 Paulsen	Coiro et al. 1985	Malan and Meyer 1993
V. vinifera Trollinger x *V. riparia*	26G	Coiro et al. 1985	
V. solonis		Harris 1983, Kunde et al. 1968	
V. smalliana		Harris 1983, Kunde et al. 1968	
V. slavinii x *V. riparia* Gloire	86-10	Harris 1983	
V. riparia Gloire x *V. candicans*	91-39	Harris 1983	
V. arizonica x *V. candicans*	101-56	Harris 1983	
	101-9	Harris 1983	
V. longii x [*V. riparia* Gloire x *V. x champinii* Ramsey]	106-38	Harris 1983	
[*V. riparia* Gloire x Dog Ridge] x 1613C	112-2	Harris 1983	
	112-71	Harris 1983	
V. candicans x 1613C	116-11	Harris 1983	
	116-60	Harris 1983	
V. rufotomentosa		Harris 1983, Kunde et al. 1968	
V. rufotomentosa x *V. longii*	150-5	Harris 1983	
V. rufotomentosa x *V. vinifera*	171-13	Harris 1983	
	171-6	McKenry et al. 2001	
	171-52	Harris 1983	
V. rufotomentosa x *V. rupestris* Metallique	176-9	Harris 1983	
V. rufotomentosa x. *V. riparia* Gloire	513-4	Harris 1983	
V. rufotomentosa x [*V. riparia* Gloire x Dog Ridge]	514-11	Harris 1983	

Table 1.3 (Continued)

V. rufotomentosa x [*V. riparia* Gloire x *V. x champinii*]	515-1	Harris 1983	
V. rufotomentosa x *V. candicans*	142-40	Harris 1983	
	142-50	Harris 1983	
V. solonis x *V. candicans*	187-24	Harris 1983	
V. solonis x Othello	1613C	Coiro et al. 1990, [a]Harris 1983, [a]Kunde et al. 1968, Malan and Meyer 1993	Aballay et al. 1998, McKenry et al. 2001, McKenry and Anwar 2006, Boubals and Pistre 1978
V. slavinii		[a]Kunde et al. 1968	
V. cinerea			Kunde et al. 1968
	Arnold	Sopp et al. 1998	
1613C x Dog Ridge	Freedom	Harris 1983, Malan and Meyer 1993, McKenry and Anwar 2006, McKenry et al. 2001, 2004	
	Harmony	Aballay et al. 1998, [c]Coiro et al. 1990, Harris 1983, Malan and Meyer 1993, McKenry and Anwar 2006, McKenry et al. 2004	Lider and Goheen 1986, McKenry et al. 2001
V. rubra palmata		[a]Kunde et al. 1968	
VRH8771 x *140 Ruggeri*	RPG1	Esmenjaud et al. 2010	
V. vinifera Mourvedre x *V. rupestris* x *V. rupestris* Martin	1202C	Boubals and Pistre 1978	

[a]Moderate resistance, [b]Resistance to California *X. index* populations only, not other *X. index* populations tested, [c] Resistance to Italian, Israeli, and French *X. index* populations only, not to a Californian *X. index* population.

None of the rootstocks that are highly resistant to *X. index* have been found to prevent replication of GFLV (Boubals and Pistre 1978, Bouquet 1983a, 1983b, Bouquet et al. 2003a, 2003b, Bouquet and Danglot 1983, Ipach et al. 2000, Laimer et al. 2009, Malan and Meyer 1993, Walker et al. 1985), nor do they prevent the translocation of GFLV to susceptible scions, though some of them substantially delay the movement of GFLV and reduce the infection rate of test vines in field settings, possibly through a reduction in nematode populations and therefore subsequent feeding events. This has led a series of rootstocks such as Dog Ridge (Harris 1988), Ramsey (Harris 1988), Börner (Becker 1989), Schwarzman (McKenry et al. 2001) and O39-16 (Walker et al. 1989, 1991) to be recommended over time for fanleaf affected sites. In a 12-year field trial in *X. index* and GFLV-infected fields in California, Cabernet Sauvignon grafted onto O39-16 remained uninfected with GFLV for nine years before virus infection of the scion could be detected in the 10th year of the study (Walker et al. 1994, Walker and Wolpert 1994). Börner has also been reported to resist *X. index*-mediated transmission of GFLV (Becker 1989, Becker and Sopp 1990, Sopp et al. 1998), but longer-term studies have shown that Börner vines eventually do become infected (Esmenjaud and Bouquet 2009, Ipach et al. 2000). While in some cases a delay in infection may provide economic benefits to growers dealing with *X. index* and GFLV, ultimately, if the vines are infected, then this is not adequate for control of GFLV in a vineyard setting. In addition, some of these rootstocks have undesired viticultural characteristics. For example, O39-16 is highly vigorous (Walker and Jin 1998) and has poor rooting ability (Bouquet et al. 2003a, 2003b), while Börner and other promising species and muscadine hybrids have

been shown to be highly susceptible to lime-induced chlorosis (Bouquet 1991, Esmenjaud and Bouquet 2009).

It has been suggested that some other recently released rootstocks, RS-3 (Ramsey x Schwarzman) and RS-9 (Ramsey x Schwarzman) posses broad resistance to nematode species including the dagger nematodes *X. index* and *X. americanum* (Anwar and McKenry 2000, Anwar and McKenry 2002); however, at the time of compiling this review, no peer reviewed papers have been published describing their level of resistance to dagger nematodes or their competency at preventing nepovirus spread. The same is also true for GRN-1 (*V. rupestris x M. rotundifolia)*, GRN-2 (*V. rofotomentosa* x [Dog Ridge x Riparia Gloire]) x Riparia Gloire), GRN-3 and GRN-4 (*V. rofotomentosa* x [Dog Ridge x Riparia Gloire]) x *V. champinii* c9028) and GRN-5 ([Ramsey x Riparia Gloire] x *V. champinii* c9021) (Walker 2009). The purported resistance of those genotypes may have been delivered from the parentage of the rootstocks which has already been described as a source of nematode resistance or tolerance, i.e. Dog Ridge, Gloire de Montpellier, Ramsey, Schwarzmann, or *M. rotundifolia*.

Tolerance to nepoviruses: A cultivar or rootstock that can be infected by a nepovirus but does not suffer negative effects on yield or plant growth would be considered tolerant to the infecting virus. If the cultivar or rootstock does not suffer as much yield loss as other cultivars or rootstocks upon infection, then that cultivar or rootstock may be called relatively more tolerant to the nepovirus. Regarding tolerance to nepoviruses in rootstock or scion varieties, little published information is available. However, one small trial of American and interspecific hybrids did suggest tolerance to

PRSV in some cultivars when yield and growth data from infected and uninfected vines were compared (Ramsdell et al. 1995).

In the case of scions grafted to rootstocks, tolerance to the virus infection potentially could be provided by either the rootstock or the scion. In addition to being resistant to *X. index*, some studies have reported that susceptible scions grown on O39-16 and related hybrid rootstocks do not show the usual yield losses associated with GFLV infection, even when GFLV infection is detected in these scion materials (Walker and Wolpert 1994, Walker et al. 1994). The yields of scions grafted onto these rootstocks are less affected by GFLV infection than scions grafted onto more conventional rootstocks, which could indicate that these former rootstocks are capable of providing some level of tolerance to GFLV-infection to their grafted scions - perhaps through the production of phytohoromones such as cytokinin (Smith et al. 2005). Since these vines still get infected with GFLV, this type of a tolerance would not help in controlling the virus, but it would possibly provide economic and production benefits to growers. However, while tolerant materials are potentially desirable, virus symptoms and their effects on yield are notoriously variable (Lider and Goheen 1986) and seem to be affected by even relatively slight changes in climatic conditions or growing conditions. For this reason, and given that vines infected with GFLV tend to degenerate over time, it is especially important that realistic field trials of potentially tolerant materials be undertaken in a diversity of growing and climatic conditions, and over an extended period. Ideally, yields of both infected and uninfected vines grafted onto tolerant rootstocks should be evaluated before the virus tolerance of grafted scions can be concluded, though such a study may be challenging in a field setting due to soilborne

nematode transmission. To our knowledge, this type of comparative yield study has not been conducted with the reportedly GFLV tolerant rootstocks. Therefore, the potential of nepovirus tolerant rootstocks at providing virus tolerance to grafted scion varieties has not been conclusively shown.

Resistance to nepoviruses: When a nepovirus is completely unable to replicate effectively within a host grapevine, then that grapevine may be called resistant to that nepovirus. Several studies have looked at resistance to nepoviruses (Allen et al. 1982, Becker 1989, Boubals and Pistre 1978, Bouquet 1981, 1983a, 1983b, Bouquet et al. 2000, 2003a, 2003b, Bouquet and Danglot 1983, Dias 1980, Gonsalves 1982, Ipach et al. 2000, Jia and Walker 1995, Jimenez Diaz and Goheen 1991, Lahogue and Boulard 1996, Lider and Goheen 1986, Ramsdell et al. 1995, Smith et al. 2005, Staudt and Kassemeyer 1990, Stellmach and Berres 1986, Uyemoto et al. 1977, Walker and Jin 1998, 2000, Walker and Meredith 1990, Walker and Wolpert 1994, Walker et al. 1985, 1989, 1991). Some resistance has been reported to TRSV and ToRSV in a few interspecific hybrids derived from *V. labrusca* or *V. rupestris* (Allen et al. 1982, Gonsalves 1982, Uyemoto et al. 1977). Additionally, resistance to PRMV in the interspecific hybrid Seyval blanc has been reported (Ramsdell et al. 1995), and resistance to TBRV has also been reported in some cultivars of *V. vinifera*, *V. labrusca*, and interspecific hybrids (Dias et al. 1980); however, it should be noted that for both PRMV and TBRV, data to verify these claims are limited. Toward GFLV, resistance has been reported in some Middle Eastern cultivars of *Vitis vinifera*, other *Vitis* species, and muscadines (Becker 1989, Jimenez Diaz and Goheen 1991, Walker and Meredith 1990, Walker et al. 1985). However, little of these conclusions appear to have been followed

up in subsequent work, or, in some cases, have not been confirmed by subsequent studies (Esmenjaud and Bouquet 2009, Staudt and Kassemeyer 1990, Walker and Wolpert 1994), stressing the value of multiple-site vineyard trials. Studies of GFLV resistance involving graft inoculation, micrografting, or nematode inoculations in pots, have appeared to reveal differences between cultivars with regard to infectivity in the short term (Becker 1989, Becker and Sopp 1990, Boubals and Pistre 1978, Staudt and Kassemeyer 1990, Staudt 1997, Walker et al. 1985) only to have these varieties become infected in longer term studies (Bouquet et al. 2000, Esmenjaud and Bouquet 2009, Walker and Wolpert 1994), indicating that they are not resistant to virus replication. Accordingly, there are currently no rootstocks or cultivars available with resistance to GFLV in *Vitis* species nor any recognized sources of useful resistance (Laimer et al. 2009). Previously, muscadine grapes *(Muscadinia rotundifolia*) and *Muscadinia*-*Vitis* hybrids, including O39-16 and O43-43, were believed to possess resistance to GFLV (Walker et al. 1985, 1989); however, this resistance appears to be a resistance to *X. index* transmission of GFLV rather than a replication resistance (Bouquet et al. 1981, Staudt and Weischer 1992), since muscadine grapes and hybrids can be infected with GFLV – though at a low rate (Bouquet 1981, Bouquet et al. 2000, Staudt and Weischer 1992, Walker and Wolpert 1994). This lowered rate of infection is presumably due to the apparent hypersensitive reaction of these rootstocks to feeding by *X. index* (Sopp et al. 1998, Staudt and Weischer 1992). Likewise, only a few rootstocks and cultivars appear to have resistance to ToRSV and TRSV although some of the findings on rootstocks are conflicting (Allen et al. 1982, Gonsalves 1982, Stobbs et al. 1988, Uyemoto et al. 1977). Despite the fact that resistance acting on virus

replication or preventing virus movement from the rootstock to a scion variety would be economically beneficial to growers and allow for virus control in a field setting, no resistance in any source material has produced a grapevine that is resistant to GFLV or ArMV (Laimer et al. 2009).

Leafroll disease complex: Leafroll disease is an important viral disease of grapevine that occurs in all of the world's grape-growing areas. Leafroll can cause significant yield losses, delayed fruit ripening, and an overall decline in vine vigor (Martelli and Boudon-Padieu 2006). Leafroll is caused by at least 11 different viral species, the majority of which are transmitted by mealybugs and soft scale insects (Table 1.1b).

Of the relatively few published studies that have looked at leafroll resistance or tolerance (Ioannou et al. 1997, Kovacs et al. 2001, Lahogue and Boulard 1996), no leafroll resistant materials have been identified. Asymptomatic infections of cultivated *V. labrusca* and interspecific hybrids are common (Kovacs et al. 2001), and while latent infections with leafroll viruses may indicate some level of tolerance, neither hybrid vines nor *V. vinifera* cultivars are free from leafroll disease's negative effects on fruit quality (Kovacs et al. 2001, Wolpert and Vilas 1992). Observed differences in relative virus titers in host plants (Boscia et al. 1991, Credi and Santucci 1991, Ioannou et al. 1997, Kovacs et al. 2001) may suggest some differences in the ability of leafroll viruses to infect some cultivars. Nonetheless, there are currently no commercially available leafroll resistant cultivars, and no recognized sources of useful resistance (Martelli and Boudon-Padieu 2006). We are also not aware of any studies of resistance or tolerance

to mealybugs and soft scales - though, given the relative diversity of these vector species as compared to those transmitting nepoviruses, resistance to one or a few of these vectors may not be effective as a means of control for leafroll viruses.

Rugose wood disease complex: Several viruses are known to cause rugose wood disease on grapevine (Table 1.1c). Some of these are known to be vectored by mealybugs and scale insects. While these viruses cause less vigorous growth, delayed bud opening, and a general decline in vigor, this disease is named based upon the formation of “corky rugose wood” which occurs in certain cultivars. In these cultivars, a spongy-textured, thick, and corky layer of bark is formed above the graft union, and the stem is often marked by pits and grooves on scions and/or rootstocks (Credi et al. 1991, Martelli and Boudon-Padieu 2006). Though several rootstocks and cultivars of grapevine have been noted to be especially sensitive to rugose wood (Abracheva 1981, Credi et al. 1991, Téliz et al. 1980a, 1980b, 1981, Téliz and Valle 1980), there is not any commercially available resistance or recognized sources of useful resistance to these viruses or to their vectors (Martelli and Boudon-Padieu 2006).

Fleck disease complex: The fleck disease complex consists of at least four viruses (Table 1.1d) that have no known vectors. Vines affected by this disease may exhibit leaf wrinkling or twisting, and stunting, with reduced rooting ability or poor graft-take. Some fruit effects, including higher titratable acidity levels and lower soluble solids, may also be observed in co-infections of fleck virus with other viruses (Kovacs et al. 2001); however, asymptomatic infections with these viruses are quite common

(Martelli and Boudon-Padieu 2006), indicating there may be some tolerance to these viruses in *Vitis* species. There are currently no grapevines recognized to be resistant to these viruses (Martelli and Boudon-Padieu 2006), but further work is necessary before conclusions can be drawn.

Other viral diseases: There are several other viral agents known to infect grapevine (Table 1.1e). These cause a variety of diseases. However, presumably because these viruses seem to be less widespread and are of relatively low economic importance, little work has been done looking for resistance or tolerance to these viruses (Stobbs and Broadbent 1993).

CONCLUSIONS

Despite a long history of cultivation, a large number of viruses and viral diseases, and extensive efforts to identify useful sources of natural resistance to viruses or their vectors in wild and cultivated *Vitis and Muscadinia* germplasm, there remains no proven useful resistance to grapevine viruses. Though traditional breeding efforts have been successful at identifying sources of resistance to *X. index* and, to a lesser extent, to *X. americanum*, these materials do not prevent virus infection or movement from rootstocks to grafted scion cultivars. They may be very useful against the vector itself in those cases where it may be a significant economic problem in grape production, or to mitigate the impact of GFLV on yield by delaying the debilitating effects of virus infection so that these do not take place during early stages of grapevine development. However, these sources of nematode resistance are not sufficient to control GFLV in a

vineyard setting since (1) these materials can still be infected with nepoviruses and (2) *X. index* transmission of GFLV from these materials to other susceptible material can still occur. Recent advances in marker-assisted selection (Burger et al. 2009, Hwang et al. 2010, Xu et al. 2008) and grapevine genomic knowledge (Jaillon et al. 2007, Velasco et al. 2007) may prove to be invaluable in discovering solutions to virus problems; however, to date, no genes or genetic loci have been identified which confer resistance to any virus in grapevine (Fraile and Garcia-Arenal 2010, Martelli and Boudon-Padieu 2006, Maule et al. 2007). Continued efforts to find useful resistance to grapevine viruses must be undertaken, and the importance of realistic field trials should not be understated.

Unfortunately, virus resistance evaluations in grapevine are complicated by several factors, not the least of which are the difficulty of testing a perennial crop, and the difficulties involved in mechanically inoculating grapes with viruses. Though the first difficulty is unavoidable, resistance screens could be facilitated if a better delivery system were available for viruses (Valat et al. 2003). Though research into delivery systems has yielded some promising results using agro-infection to deliver GVA (Muruganantham et al. 2009), more progress is needed to develop a routine system for grapevine virus infection. Adapting agro-infiltration technology to other grapevine viruses would be desirable. An efficient system for virus delivery, such as agro-infiltration, may allow for more effective resistance screens to be set-up with quantified inoculum levels, consistent inoculum delivery, and potentially shorter screening times. This would be in stark contrast to the lack of control of virus inoculum load provided by the graft inoculation methods currently used or to the inconsistent distribution of viruses

and their vectors in field testing which at least in part accounts for the prolonged exposure necessary to ensure virus delivery in many resistance screens. The overwhelming majority of research efforts have focused on identifying resistance to GFLV and its nematode vector, but resistance to GFLV has not been found. Also, despite their increasingly important impacts on grape production, relatively little is known about other grapevine viruses and vectors including the mealybug-transmitted leafroll-associated viruses. Thus, there remains an urgent need to develop grapevine material with resistance to GFLV and other viruses.

For grapevine viruses where no source of resistance has been identified in spite of extensive study, genetic engineering is likely the only approach to achieve resistance. Efforts on engineering resistance to viruses in grapevine through pathogen-derived resistance or RNA-silencing based approaches have provided alternative means for developing resistance to many grapevine viruses, including GFLV, GCMV, GVA, GVB, ArMV, ToRSV, GBINV, and GLRaV-2 in herbaceous hosts (Andret-Link et al. 2004, Burger et al. 2009, Fuchs 2003, Laimer et al. 2009). However, in spite of promising results (Andret-Link et al. 2004, Burger et al. 2009, Laimer et al. 2009), the translation of laboratory knowledge to real world solutions has been quite slow (Laimer et al. 2009). Though little work has been done with transgenic resistance technologies under field conditions, a 3-year field trial of transgenic rootstocks indicated a few GFLV-resistant clones (Vigne et al. 2004). However, as has been observed in many of the studies of GFLV resistant materials examined in this review, three years of field testing are not necessarily adequate to confirm the efficacy of the engineered resistance, and longer-term field trials of transgenic materials are needed. It would be desirable to more

aggressively explore this innovative technology to engineer virus resistance because its safety and efficacy as a disease management option have been proven in other crops including summer squash, papaya and plum (Oliver et al. 2011). Efforts are ongoing in several countries to obtain virus-resistant transgenic grapevines (Laimer et al. 2009). Additionally, efforts to stack resistance to *X. index* and transgenic resistance to GFLV in a single rootstock to manage fanleaf degeneration are underway (Bouquet et al. 2003a, 2003b). Stacking resistance to both the virus and the vector in a single rootstock would allow for more efficient management of this virus/vector pathosystem and would therefore be ideal for growers. Of course, in order to assess the usefulness of these technologies, they should be assessed in realistic field settings where the virus and vector are present to more accurately mirror situations encountered by growers.

Preventative measures such as good sanitary practices and the use of virus-tested propagation material derived from certified stocks remain the most effective tools against viral diseases. Once viruses and their vectors become established in vineyard settings, their elimination can be cumbersome or nearly impossible; and, as we have demonstrated in this review, establishing a new vineyard with virus-resistant materials is not an option with currently available rootstocks and cultivars. Research is ongoing in many locations to develop new grapevine materials which will provide useful resistance against viruses.

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CHAPTER 2

Genetic Structure and Molecular Variability of *Grapevine fanleaf virus* Populations[2]

ABSTRACT

To gain insights into the evolutionary mechanisms of *Grapevine fanleaf virus* (GFLV) from the genus *Nepovirus*, family *Secoviridae*, the sequences of the complete coding region of RNA2, including genes $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$, and partial sequence from the RNA1-encoded gene $1E^{Pol}$ of 14 GFLV isolates from three naturally infected California vineyards were characterized. Phylogenetic analyses suggested two to three evolutionarily divergent lineages that did not reflect the vineyard origin of the isolates or an association with rootstock genotype or scion cultivar. Examination of the genetic variability of the California isolates alongside isolates worldwide, for which three RNA1 and 44 RNA2 coding sequences are available, revealed similar patterns of molecular evolution for the different regions within the GFLV genome but distinct selection constraints with the strongest pressure exerted on genes $2C^{CP}$ and $2B^{MP}$, an intermediate level of pressure exerted on gene $1E^{Pol}$, and the weakest pressure exerted on gene $2A^{HP}$. Some of the California isolates resulted from interspecies recombination events between GFLV and *Arabis mosaic virus* with crossover sites suspected in gene $1E^{Pol}$ and identified in genes $2A^{HP}$ and $2B^{MP}$; and intraspecies recombination events

[2]Oliver, J.E., Vigne E. and Fuchs, M. 2010. Genetic structure and molecular variability of *Grapevine fanleaf virus* populations. *Virus Res*. 152:30-40.

inferred in the four target genes but most frequently observed within gene $2C^{CP}$. This study suggested that purifying selection and recombination are important evolutionary mechanisms in the genetic diversification of GFLV.

INTRODUCTION

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration, one of the most devastating viral diseases of grapevines worldwide (Andret-Link et al. 2004a). GFLV is specifically transmitted by the ectoparasitic nematode species *Xiphinema index* and can be very difficult to control due to the extended persistence of viruliferous vectors in vineyard soils even in the absence of host plants (Demangeat et al. 2005). The virus causes problems on nearly every continent and in every region where grapes are grown and the nematode vector is present. In the United States, though GFLV-infected vines have been identified in California (Martelli and Hewitt 1963, McKenry et al. 2001, Taylor and Hewitt 1964), Washington (Mekuria et al. 2009), and Missouri (Lunden et al. 2010), the nematode vector *X. index* has been documented only in California (Hewitt et al. 1958, McKenry et al. 2001). GFLV is a member of the genus *Nepovirus* in the family *Secoviridae* (Sanfaçon et al. 2009). It has a bipartite plus-sense RNA genome (Andret-Link et al. 2004a). RNA1 consists of 7342 nts and codes for a M_r 253K polyprotein, which is cleaved by the virally encoded proteinase (also found on RNA1) into five individual proteins, including a protein of unknown function (1A), a putative helicase ($1B^{Hel}$), a viral protein genome-linked or VPg ($1C^{VPg}$), a proteinase ($1D^{Pro}$) and a putative RNA-dependent RNA polymerase (RdRp) ($1E^{Pol}$) (Andret-Link et al. 2004a). RNA2

consists of 3774 nts and codes for a polyprotein of M_r 122K, which is cleaved by the RNA1-encoded viral proteinase into three individual proteins, including a homing protein ($2A^{HP}$) necessary for RNA2 replication, a movement protein ($2B^{MP}$) and a coat protein ($2C^{CP}$) (Andret-Link et al. 2004a).

As with other RNA viruses, the GFLV RdRp has no proofreading mechanism. Therefore, its replication is error prone and GFLV exists as a quasispecies (Naraghi-Arani et al. 2001). Based upon genetic variation studies conducted on isolates from France, Germany, Iran, Slovenia, South Africa, Tunisia and the United States, divergence of up to 17% and 9% at the nucleotide and amino acid levels, respectively, has been observed within GFLV gene $2C^{CP}$ (Bashir et al. 2007, Boulila 2007, Fattouch et al. 2005, Liebenberg et al. 2009, Mekuria et al. 2009, Naraghi-Arani et al. 2001, Pompe-Novak et al. 2007, Pourrahim et al. 2007, Vigne et al. 2004, Wetzel et al. 2001). While several studies have examined the genetic diversity of gene $2C^{CP}$ and to a lesser extent gene $2B^{MP}$ – with over 300 complete or partial sequences of the two genes available in GenBank – sequence information from other parts of the genome is relatively limited.

Recombination can be an important factor in viral evolution (Garcia-Arenal et al. 2001, Moury et al. 2006); and in the case of GFLV, recombination has been reported to occur within RNA2 both between distinct genetic variants (Boulila 2007, Mekuria et al. 2009, Pompe-Novak et al. 2007, Vigne et al. 2004, 2005, 2009), and between GFLV and other closely related viruses from the genus *Nepovirus*, including *Arabis mosaic virus* (ArMV) (Jawhar et al. 2009, Mekuria et al. 2009, Vigne et al. 2008) and *Grapevine deformation virus* (GDefV) (Mekuria et al. 2009).

Characterizing the genetic structure of viral populations and the factors that contribute to their evolution may help improve understanding of new epidemics, phylogenetic relationships amongst isolates from various geographical origins, and pathogenicity changes that may result from variations between host genotypes. To characterize GFLV isolates in naturally infected vineyards in California and gain further insights into the mechanisms of GFLV evolution, we analyzed the genetic variation and phylogenetic relationships amongst isolates in four genomic regions, i.e. the RNA1-encoded $1E^{Pol}$ gene and the RNA2-encoded $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes, and investigated recombination between divergent sequence variants. Our analyses suggested that the RNA2 and $1E^{Pol}$ sequences from the GFLV variants from California that were examined in this study segregate into two evolutionarily divergent lineages for RNA2 and three lineages for $1E^{Pol}$ irrespective of host scion cultivar, rootstock genotype, or vineyard origin. In addition, they showed evidence of variable selection pressures exerted on different genes and frequent recombination events.

MATERIALS AND METHODS

Vineyard and leaf sample collection

Three vineyards in Lodi, California with a long history of GFLV infection and presence of the nematode vector *X. index* were selected for this study. These vineyards vary in age (10–40 years) and were established from source materials originating in California. Typical GFLV symptoms, such as foliar mosaic, yellowing and distortion as well as shot berries and reduced yield were observed on the majority of vines in these vineyards. No readily observable differences were recognized among

vines or vineyards regarding the severity and types of symptoms present, or between vineyards in terms of disease prevalence. Vineyard A (~2 ha) was established ~20 years ago and consisted of *Vitis vinifera* cv. Zinfandel grafted onto the rootstock Freedom (1613 Couderc [*V. solonis*×Othello]×*V. champinii*). Vineyard B (~1.5 ha) was established ~10 years ago and consisted of *V. vinifera* cv. Cabernet Sauvignon grafted onto the rootstock Freedom. Vineyard C (~3 ha) was established ~40 years ago and consisted of *V. vinifera* cv. Cabernet Sauvignon grafted onto the rootstock Dog Ridge (*V. champinii*). Vineyard A and vineyard B were located adjacent to one another with vineyard C positioned approximately one mile away. GFLV transmission via *X. index* has been determined to be occurring in these vineyards (Fuchs, unpublished). Leaf samples (8–10 per vine) were collected from the tip of symptomatic shoots on October 16th, 2007 for GFLV detection by double antibody sandwich (DAS)-enzyme linked immunosorbent assay (ELISA) and immunocapture (IC)-reverse transcriptase (RT)-polymerase chain reaction (PCR). We considered a GFLV isolate to be a viral culture from a single vine.

Virus detection by DAS-ELISA

GFLV was detected by DAS-ELISA in crude leaf extracts with specific antibodies (Bioreba, Reinach, Switzerland). A portion of 8–10 stacked leaves was torn and ground in 200 mM Tris–HCl pH 8.2, 140 mM NaCl, 2% polyvinylpyrrolidone 40, and 0.05% Tween 20 at a 1:5 ratio (w/v) using a semi-automated ball-bearing HOMEX tissue homogenizer (Bioreba, Reinach, Switzerland). Test conditions were according to the manufacturer's instructions (Bioreba, Reinach, Switzerland). Substrate hydrolysis was

recorded at 405 nm with an absorbance BioTek® ELx808TM microplate reader (BioTek, Winooski, VT). Samples were considered positive if their optical density (OD 405 nm) readings were at least twice those of healthy controls.

GFLV RNA1 and RNA2 characterization by IC-RT-PCR

GFLV was detected by IC-RT-PCR in plant sap from leaf samples that reacted positively with GFLV antibodies in DAS-ELISA. The immunocapture step was carried out using a 96-well microplate coated with specific GFLV antibodies (Bioreba, Reinach, Switzerland). Leaf material (250 mg) was crushed in the extraction buffer used for DAS-ELISA (2.5 ml) and crude sap (100 µl) was incubated in coated microtiter plates overnight at 4°C. After four washes with PBS (1×) and Tween 20 (0.05%), sterile water (10 µl) was added to each well before incubation at 70°C for 10 min followed by 5 min at 4°C. The RT step was carried out using the AMV RT enzyme (Promega Corporation, Madison, WI), 50 pmol of reverse RNA1 primer 5'-GTTATCCCAGTACCAAGAAT-3' and reverse RNA2 primer 5'-GAGGATCCCAGTAAAAAGAAAGGAAAA-3' for 1 h at 42°C, followed by 5 min at 99°C and 5 min on ice. Reverse primers were designed based on the full-length genomic sequences of GFLV strain F13 (Ritzenthaler et al. 1991, Serghini et al. 1990) to hybridize to nts 7106–7125 and nts 3742–3760 of RNA1 and RNA2, respectively.

PCR was carried out using the GoTaq DNA polymerase and 20 pmol of specific primers (Table 2.1) in a 50 µl final volume according to the manufacturer's protocol. PCR used a 2 min heating step at 94°C followed by 30 cycles of 1 min melting at 94°C, 1 min annealing at 50°C, and 2 min elongation at 72°C with a final extension of 7 min at

72°C. The reaction products were resolved by electrophoresis in 1.5% agarose gels in 90 mM Tris–borate, 2 mM EDTA, pH 8.0, stained with ethidium bromide and subsequently visualized under UV light.

Table 2.1. Primers used for IC-RT-PCR amplification of GFLV RNA1 and RNA2.

Genomic RNA	Sequence (5' to 3')	Hybridizing location (nts)[a]
RNA1	TTATTAGGGGAGAAGTGCC	4615-4633 (+)
	GTTATCCCAGTACCAAGAAT	7125-7106 (-)
	CTGGGGAAAAGGGAAAGGCG	5065-5084 (+)
	CGTGACCCTGTCGGCAATAG	5517-5536 (+)
	TGATAAATGCGCCTTTGGAA	5944-5963 (+)
	GAGGATCCGGTACCAGATGAATTGTGC	4736-4754 (+)
	GAGGATCCTCACCATGTAGATAAAGCT	6076-6058 (-)
RNA2	GCCTGTTGGGCTGCTGGGAAGAA[b]	266-288 (+)
	TGGGAAAGTGTGGAGGAAC[b]	1922-1940 (+)
	GCCTGGCAATCCTTGGGAATG[b]	2094-2074 (-)
	AAAGAGAGATCTGGGCGCAC[b]	3066-3047 (-)
	ATGAAAAATGTTTACGTTTTCTTAC	1-25 (+)
	ATGGGCAAATTTTATTATTCCAAC	233-256 (+)
	GAGGATCCCCCCAGCTCCCTACTTTAG[c]	573-591 (+)
	GAGGATCCTGGCAATTCGGCAAAGAGTGC[c]	703-683 (-)
	GAGGATCCTGATAGAAACGTTGATCTT[c]	1987-2005 (+)
	GAGGATCCCTTGAAGTCTGAGATCATA[c]	2146-2128 (-)
	GAGGATCCTTAGTGAGTGGAACGGGAC[c]	2802-2820 (+)
	GAGGATCCCTGACTTTGACCAGCAAGCA[c]	2947-2928 (-)
	GAGGATCCCAGTAAAAAGAAAGGAAAA[c]	3760-3742 (-)

[a]Hybridization location shown relative to GFLV-F13 reference sequences (Ritzenthaler et al. 1991; Serghini et al. 1990) with (+ or -) indicating forward or reverse.
[b]Adapted from Vigne et al. (2008)
[c]Primer developed previously with additional 7 bases and BamHI restriction site at 5' end

GFLV sequence determination and analyses

Overlapping viral cDNA amplicons obtained for each isolate by IC-RT-PCR were extracted from agarose gels with the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA) and cloned in pCR4®-TOPO® (Invitrogen, Carlsbad, CA). TOPO plasmids containing GFLV inserts were extracted from *E. coli* competent cells using the Promega Wizard® Plus SV Minipreps DNA Purification System and sequenced bidirectionally using M13F and M13R primers with the Big Dye Terminator Kit, AmpliTaq-FS DNA polymerase and an Applied Biosystem Automated 3730xl DNA Analyzer at the Cornell University DNA Sequencing Facility in Ithaca, NY.

Sequences were analyzed and compared using the DNASTAR Lasergene® v7.2 software package. The algorithm CLUSTAL W was used for alignment of nucleotide

sequences and the program SeaView (Galtier et al. 1996) was used for hand editing and construction of contigs. Fragments were assembled only if they had at least 99% nucleotide identity in the overlapping regions. Additional clones (one to three) of each fragment were characterized to confirm sequence integrity. Phylogenetic relationships were determined with neighbor-joining using the GFLV and nepovirus isolate sequences from GenBank listed in Table 2.2 (Ghanem-Sabanadzovic et al. 2005,

Table 2.2. GFLV and nepovirus sequences used in phylogenetic analyses.

Genome	Isolate	Accession #	Grape cultivar	Origin	Reference
RNA1	F13	NC_003615	Muscat	France	Ritzenthaler et al. 1991
	WAPN173	GQ332372	Pinot noir	USA (WA)	Mekuria et al. 2009
	WAPN6132	GQ332373	Pinot noir	USA (WA)	Mekuria et al. 2009
	CAZINA1	GU972558	Zinfandel	USA (CA)	This study
	CAZINA2	GU972559	Zinfandel	USA (CA)	This study
	CAZINA3	GU972560	Zinfandel	USA (CA)	This study
	CAZINA4	GU972561	Zinfandel	USA (CA)	This study
	CAZINA5	GU972562	Zinfandel	USA (CA)	This study
	CACSB1	GU972563	Cabernet Sauvignon	USA (CA)	This study
	CACSB2	GU972564	Cabernet Sauvignon	USA (CA)	This study
	CACSB3	GU972565	Cabernet Sauvignon	USA (CA)	This study
	CACSB5	GU972566	Cabernet Sauvignon	USA (CA)	This study
	CACSC1	GU972567	Cabernet Sauvignon	USA (CA)	This study
	CACSC2	GU972568	Cabernet Sauvignon	USA (CA)	This study
	CACSC3	GU972569	Cabernet Sauvignon	USA (CA)	This study
	CACSC4	GU972570	Cabernet Sauvignon	USA (CA)	This study
	ArMV-NW	AY303786	Pinot gris	Germany	Wetzel et al. 2004
RNA2	F13	NC_003623	Muscat	France	Serghini et al. 1990
	NW	AY017338	Huxel	Germany	Wetzel et al. 2001
	GHu	EF426852	Gloriae Hungariae	Hungary	Vigne et al. 2008
	B19a	AY780903	Chardonnay	France	Vigne et al. 2004
	A10a	AY780902	Chardonnay	France	Vigne et al. 2004
	A17a	AY780899	Chardonnay	France	Vigne et al. 2004
	A17b	AY780900	Chardonnay	France	Vigne et al. 2004
	A17d	AY780901	Chardonnay	France	Vigne et al. 2004
	WACH911	GQ332364	Chardonnay	USA (WA)	Mekuria et al. 2009
	WAPN57	GQ332367	Pinot noir	USA (WA)	Mekuria et al. 2009
	WAPN173	GQ332368	Pinot noir	USA (WA)	Mekuria et al. 2009
	WAPN8133	GQ332369	Pinot noir	USA (WA)	Mekuria et al. 2009
	WAPN165	GQ332365	Pinot noir	USA (WA)	Mekuria et al. 2009
	WAPN6132	GQ332366	Pinot noir	USA (WA)	Mekuria et al. 2009
	WAME1492	GQ332370	Merlot	USA (WA)	Mekuria et al. 2009
	WACF2142	GQ332371	Cabernet franc	USA (WA)	Mekuria et al. 2009
	Vol[a]	DQ922652-79	Volovnik	Slovenia	Pompe-Novak et al. 2007
	CAZINA1	GU972571	Zinfandel	USA (CA)	This study
	CAZINA2	GU972572	Zinfandel	USA (CA)	This study
	CAZINA3	GU972573	Zinfandel	USA (CA)	This study
	CAZINA4	GU972574	Zinfandel	USA (CA)	This study
	CAZINA5	GU972575	Zinfandel	USA (CA)	This study
	CACSB1	GU972576	Cabernet Sauvignon	USA (CA)	This study
	CACSB2	GU972577	Cabernet Sauvignon	USA (CA)	This study
	CACSB3	GU972578	Cabernet Sauvignon	USA (CA)	This study
	CACSB4	GU972579	Cabernet Sauvignon	USA (CA)	This study
	CACSB5	GU972580	Cabernet Sauvignon	USA (CA)	This study
	CACSC1	GU972581	Cabernet Sauvignon	USA (CA)	This study
	CACSC2	GU972582	Cabernet Sauvignon	USA (CA)	This study
	CACSC3	GU972583	Cabernet Sauvignon	USA (CA)	This study
	CACSC4	GU972584	Cabernet Sauvignon	USA (CA)	This study
	ArMV-NW	AY017339	Pinot gris	Germany	Wetzel et al. 2001
	ArMV-U	X81814	Syrah	France	Loudes et al. 1995
	GDefV	AY291208	Dimrit	Turkey	Ghanem-Sabanadzovic et al. 2005

[a]Sequences included Vol45c1, Vol47c1, Vol47c2, Vol47c3, Vol47c4, Vol47c5, Vol49c1, Vol49c2, Vol50c1, Vol50c2, Vol51c1, Vol51c2, Vol51c3, Vol51c4, Vol51c5, Vol52c1, Vol54c1, Vol54c2, Vol54c3, Vol55c1, Vol55c2, Vol55c3, Vol57c1, Vol57c2, Vol57c3, Vol57c4, Vol57c5, Vol57c6

Loudes et al. 1995) along with ArMV strain NW (Wetzel et al. 2001, 2004) that was used as an outgroup [RNA1: AY303786; RNA2: AY017339]. Six Slovenian isolates (Vol45c1, Vol55c3, Vol47c1, Vol50c2, Vol52c1, and Vol51c3) were chosen to represent the three clades into which 28 Slovenian isolates were previously reported to cluster (Pompe-Novak et al. 2007). The robustness of the inferred evolutionary relationships was assessed by 1000 bootstrap replicates. Branches with boot-strap support values less than 750 out of 1000 were not considered to be strongly supported. Phylogenetic trees were visualized using TreeView© v1.66 (Page 1996).

Recombination analysis using SISCAN and RDP3

Suspected recombination events among GFLV isolates based on phylogenetic relationships and sequence analyses were confirmed using the algorithm SISCAN (Gibbs et al. 2000). The program RDP v3.41 (Martin et al. 2005) was also used to screen sequences for potential recombination events according to the default parameters.

Synonymous/non-synonymous mutation rate calculations

The ratios of the rate of synonymous (d_S) to the rate of non-synonymous (d_N) mutations for each GFLV gene characterized in this study, both excluding and including sequences of recombinant variants, were calculated using SNAP (Korber 2000, http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html). The d_S/d_N was used as an indicator of the evolutionary direction and strength of the respective GFLV genes.

The reading frame of queried sequences was established by comparison with sequences of GFLV strain F13 (Ritzenthaler et al. 1991, Serghini et al. 1990).

RESULTS

Serological detection of GFLV isolates in California vineyards

The presence of GFLV was confirmed in symptomatic leaves from randomly selected vines in three California vineyards by DAS-ELISA. Fourteen of the vines whose leaves reacted to GFLV antibodies in DAS-ELISA were selected for subsequent molecular characterization. Samples CAZINA1, CAZINA2, CAZINA3, CAZINA4, and CAZINA5 were from five individual vines in vineyard A, samples CACSB1, CACSB2, CACSB3, CACSB4, and CACSB5 were from five individual vines in vineyard B, and samples CACSC1, CACSC2, CACSC3, and CACSC4 were from four individual vines in vineyard C. Isolates were identified with “CA” indicating their California origin, “ZIN” or “CS” standing for the scion cultivars Zinfandel and Cabernet Sauvignon, respectively, and “A”, “B”, or “C” indicating their vineyard origin.

GFLV RNA1 and RNA2 sequences from California isolates

The RNA1-encoded gene $1E^{Pol}$ was characterized for 13 of the 14 GFLV isolates selected for this study. Full-length nucleotide sequences (2475 nts) were obtained for isolates CAZINA5 and CACSC3 and partial nucleotide sequences (ranging in length from 1154 to 2362 nts) were obtained for 11 GFLV isolates (CAZINA1, CAZINA2, CAZINA3, CAZINA4, CACSB1, CACSB2, CACSB3, CACSB5, CACSC1, CACSC2 and CACSC4). Sequences of gene $1E^{Pol}$ obtained in this study are available in GenBank as

accession numbers GU972558–GU972570. No PCR amplicons were obtained for GFLV isolate CACSB4 from vineyard B despite repeated attempts. An 1140 nt region that was obtained for each of the 13 isolates within gene $1E^{Pol}$ was used in subsequent analyses to allow for equivalent comparisons between all isolates.

From the sequencing efforts on RNA2, the coding region of all 14 isolates and all but the final 12 nts of the 3' non-coding region [versus GFLV strain F13] were obtained. In addition, for isolates CAZINA4, CACSB5, and CACSC1, contig sequences including the entire 5' non-coding region were obtained resulting in a total contig length of 3762 nts. The contigs of the other 11 isolates (CAZINA1, CAZINA2, CAZINA3, CAZINA5, CACSB1, CACSB2, CACSB3, CACSB4, CACSC2, CACSC3, and CACSC4) had a total length ranging from 3455 to 3534 nts. The predicted RNA2 coding region [determined versus GFLV strain F13] of each of the 14 isolates was used in subsequent analyses to allow for equivalent comparisons between all isolates. This region ranged in length from 3327–3333 nts with inter-isolate variability due to differences in the length of gene $2A^{HP}$. RNA2 sequences obtained in this study are available in GenBank as accession numbers GU972571–GU972584.

Genetic variability among California GFLV isolates

Analysis of the partial nucleotide sequences from RNA1-encoded gene $1E^{Pol}$ and deduced protein sequences indicated a maximum divergence between isolates of 22.9 and 18.8% with an average of 11.5±8.3% and 9.9±6.5%, respectively (Table 2.3). Analyses of the coding RNA2 nucleotide sequence and deduced protein sequence showed a maximum divergence between isolates of 17.1 and 10.1%, respectively. The

average divergence among isolates was 10.8±5.1% and 6.0±2.9% at the nucleotide and amino acid level, respectively (Table 2.3).

Table 2.3. Nucleotide and amino acid sequence divergence values for the GFLV RNA2 coding region, RNA2-encoded genes $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ and RNA1-encoded gene $1E^{Pol}$.

Vineyard/Scion/ Rootstock[a]	Nucleotide and Amino Acid Divergence[b]									
	RNA2 ORF		$2A^{HP}$		$2B^{MP}$		$2C^{CP}$		[c]$1E^{Pol}$	
Vineyard A	11.3±3.4	(15.8)	11.9±3.8	(16.2)	13.2±7.9	(23.2)	9.9±3.5	(14.1)	10.8±9.8	(22.9)
	5.9±2.0	*(9.0)*	*11.5±4.0*	*(17.9)*	*5.2±4.6*	*(11.2)*	*3.7±1.1*	*(5.3)*	*9.3±7.9*	*(18.8)*
Vineyard B	10.4±6.0	(16.5)	11.3±7.9	(19.0)	13.8±11.0	(23.1)	7.9±3.7	(12.7)	12.2±9.9	(18.0)
	5.9±3.5	*(8.7)*	*11.7±7.3*	*(18.6)*	*6.6±5.1*	*(10.8)*	*2.8±1.3*	*(4.3)*	*10.3±7.8*	*(15.2)*
Vineyard C	12.7±6.0	(16.9)	11.7±5.7	(15.7)	15.5±10.6	(22.7)	11.4±3.4	(14.6)	11.9±10.3	(17.9)
	7.1±3.4	*(9.6)*	*12.3±5.5*	*(16.0)*	*7.6±5.1*	*(11.2)*	*4.4±1.6*	*(5.6)*	*9.9±8.3*	*(14.9)*
C. Sauvignon	10.7±5.9	(17.1)	10.6±6.7	(19.2)	13.0±10.6	(23.1)	9.4±4.4	(14.7)	11.0±8.9	(18.3)
	6.1±3.4	*(10.1)*	*11.2±6.3*	*(18.6)*	*6.3±5.0*	*(11.5)*	*3.7±1.9*	*(6.6)*	*9.4±7.2*	*(15.8)*
Zinfandel	11.3±3.4	(15.8)	11.9±3.8	(16.2)	13.2±7.9	(23.2)	9.9±3.5	(14.1)	10.8±9.9	(22.9)
	5.9±2.0	*(9.0)*	*11.5±4.0*	*(17.9)*	*5.2±4.6*	*(11.2)*	*3.7±1.1*	*(5.3)*	*9.3±7.9*	*(18.8)*
Dog Ridge	12.7±6.0	(16.9)	11.7±5.7	(15.7)	15.5±10.6	(22.7)	11.4±3.4	(14.7)	11.9±10.3	(17.9)
	7.1 ±3.4	*(9.6)*	*12.3± 5.5*	*(16.0)*	*7.6±5.1*	*(11.2)*	*4.4±1.6*	*(5.6)*	*9.9±8.3*	*(14.9)*
Freedom	10.8±4.8	(16.5)	11.4±5.8	(19.0)	13.8±9.5	(23.5)	8.8±3.5	(14.8)	10.7±8.7	(22.9)
	6.0±2.8	*(9.2)*	*11.5±5.9*	*(18.9)*	*6.1±4.8*	*(11.5)*	*3.3±1.3*	*(5.6)*	*9.3±6.8*	*(18.8)*
Overall (Nt)[d]	**10.8±5.1**	**(17.1)**	**11.0±5.9**	**(19.2)**	**13.4±9.7**	**(23.7)**	**9.3± 3.9**	**(15.3)**	**11.5±8.3**	**(22.9)**
Overall (AA)[d]	***6.0±2.9***	***(10.1)***	***11.3±5.9***	***(19.9)***	***6.1±4.8***	***(11.5)***	***3.6±1.7***	***(6.6)***	***9.9±6.5***	***(18.8)***

[a]Grouping of GFLV isolates by vineyard (A, B, and C), scion (Cabernet Sauvignon and Zinfandel), or rootstock (Dog Ridge and Freedom)
[b]Average divergence within each group listed as mean±SD. Maximum divergence is indicated in parentheses. Nucleotide divergence is listed in regular font and amino acid divergence is listed in italics.
[c]Values calculated using the 1,140 nt region common to all isolates, and excluding isolates CACSB1 & CACSC4. When these isolates are included, the overall nucleotide and amino acid divergence for gene $1E^{Pol}$ changes to 17.1±11.6 (32.8) and 14.2±9.2 (27.1), respectively.
[d]Average of all pairwise comparisons

Analyses of the individual GFLV genes examined in this study revealed no significant differences in terms of nucleotide or amino acid divergence when examining isolates by scion cultivar, rootstock, or vineyard origin no matter which genomic region was examined (Table 2.3).

Lower levels of diversity at the nucleotide and amino acid levels were observed in gene $2C^{CP}$ than in genes $1E^{Pol}$, $2B^{MP}$ or $2A^{HP}$ — with gene $2B^{MP}$ having less divergence at the protein level than genes $2A^{HP}$ or $1E^{Pol}$ (Table 2.3).

Phylogenetic analyses

Neighbor-joining phylogenetic trees with 1000 bootstrap replicates generated from the GFLV RNA1-encoded $1E^{Pol}$ gene sequences (1140 nts) and the entire RNA2 coding nucleotide sequence of California isolates revealed segregation into three divergent evolutionary lineages for the $1E^{Pol}$ gene and two evolutionary divergent lineages for the RNA2 coding sequence. For the $1E^{Pol}$ sequences, a predominant clade comprised of the sequence variants of seven isolates (CAZINA1, CAZINA2, CAZINA3, CAZINA4, CACSC1, CACSB3 and CACSB5) and two minor clades comprised of four (CACSC2, CACSC3, CACSB2 and CAZINA5) and two (CACSB1 and CACSC4) isolates were observed (data not shown). For the RNA2 coding nucleotide sequence, a clear segregation into two predominant clades composed of eight isolates (CAZINA1, CAZINA2, CAZINA4, CAZINA5, CACSB2, CACSB5, CACSC1, and CACSC2) and six isolates (CAZINA3, CACSB1, CACSB3, CACSB4, CACSC3, and CACSC4) was observed (Figure 2.1). Neither tree provided support for segregation of California isolates by vineyard origin, scion variety, or rootstock genotype. Phylogenetic trees generated using $1E^{Pol}$ protein sequences also supported these conclusions (data not shown).

Examination of the phylogenetic relationships among GFLV isolates from this study alongside isolates from France (Ritzenthaler et al. 1991, Serghini et al. 1990, Vigne et al. 2004), Hungary (Vigne et al. 2008), Germany (Wetzel et al. 2001), Slovenia (Pompe-Novak et al. 2007) and Washington State (Mekuria et al. 2009) for which full-length sequences are published, including three full-length RNA1 sequences,

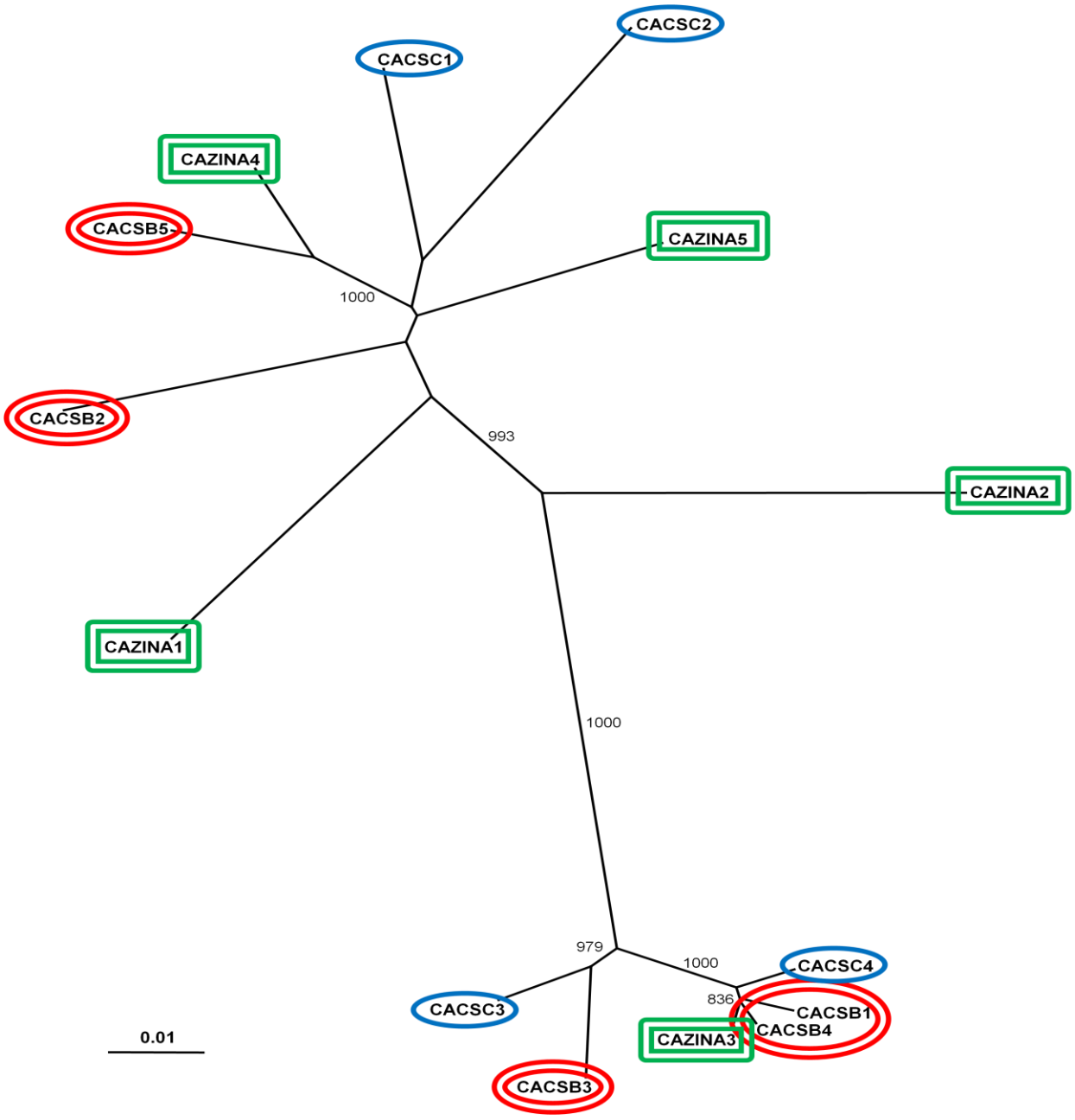

Figure 2.1. Phylogenetic tree showing relationships among GFLV isolates from three naturally infected vineyards in California within the RNA2 coding sequences. The vineyard origins (A, B and C) for these isolates are indicated by green, red, and blue shapes, respectively. The host scion variety (Zinfandel and Cabernet Sauvignon) is indicated by a rectangle or a circle, respectively, whereas the host rootstock (Freedom or Dog Ridge) is indicated by double-lines or single lines, respectively. Phylogenetic tree is based on nucleotide sequence. Bootstrap values greater than 750 out of 1000 are shown. Scale bars represent a genetic distance of 0.01.

11 full-length RNA2 sequences, and 33 additional full-length RNA2 coding sequences (Table 2.2), confirmed our previous observations with California isolates and inferred several evolutionary distinct lineages for each gene with ArMV-NW as an outgroup. The sequences of GFLV isolates grouped into three phylogenetically distinct clades (with bootstrap values over 750) for gene $1E^{Pol}$ (Figure 2.2), six distinct clades with an additional seven isolates that did not appear to group with other isolates (with high bootstrap support) for gene $2A^{HP}$ (Figure 2.3), seven distinct clades with an additional seven ungrouped isolates for gene $2B^{MP}$ (Figure 2.4), and nine distinct clades with an additional six ungrouped isolates for gene $2C^{CP}$ (Figure 2.5).

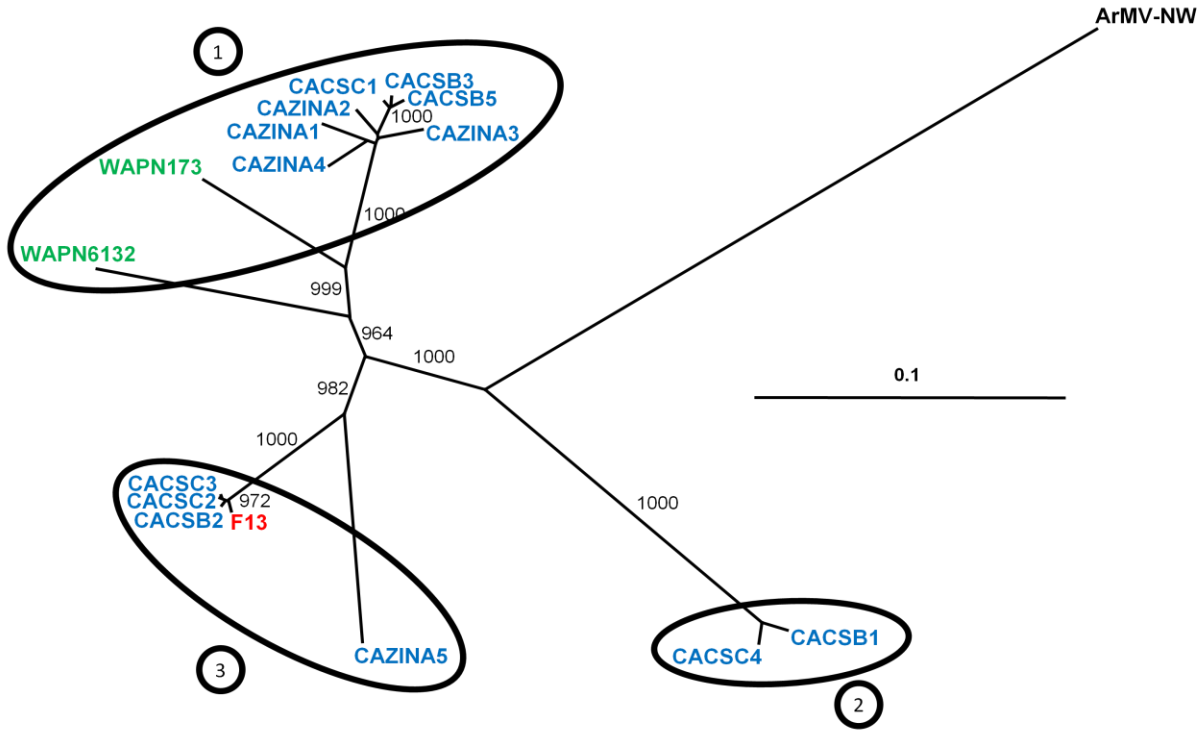

Figure 2.2. Phylogenetic tree showing genetic relationships among GFLV isolates from California and various origins worldwide within the RNA1-encoded partial sequence from gene $1E^{Pol}$. The geographic origins of these sequences are indicated by the following colors: blue for California isolates, red for French isolates, and green for isolates from Washington State, USA. Distinct clades (numbered) are shown within black circles. Phylogenetic tree is based on nucleotide sequence. Bootstrap values greater than 750 out of 1000 are shown. Scale bars represent a genetic distance of 0.1.

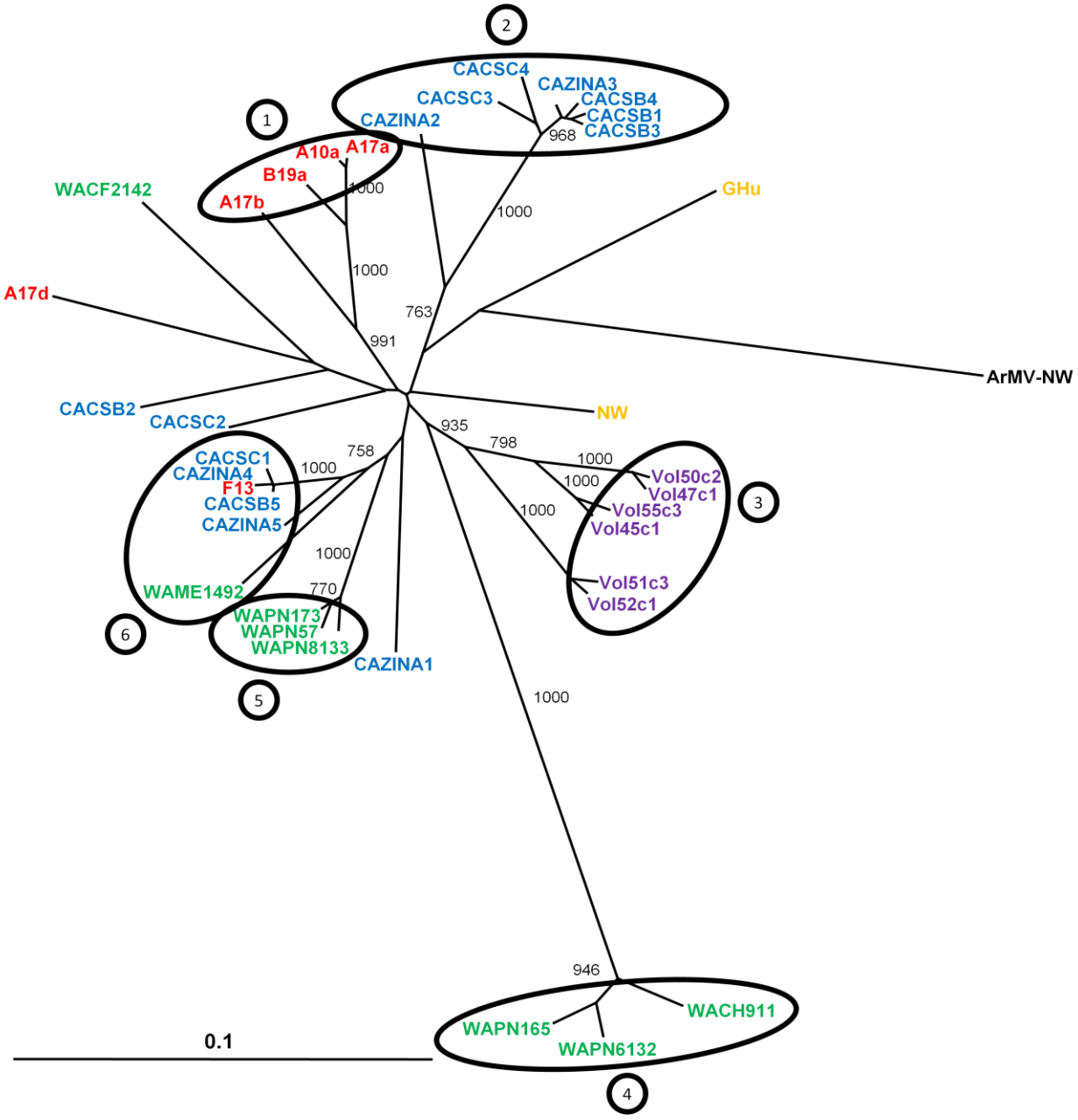

Figure 2.3. Phylogenetic tree showing genetic relationships between GFLV isolates from California and various origins worldwide within the RNA2-encoded gene $2A^{HP}$. The geographic origins of these sequences are indicated by the following colors: blue for the California isolates, red for French isolates, green for isolates from Washington State, USA, purple for isolates from Slovenia, and orange for other European isolates. Distinct clades (numbered) are shown within black circles. Phylogenetic tree is based on nucleotide sequence. Bootstrap values greater than 750 out of 1000 are shown. Scale bars represent a genetic distance of 0.1.

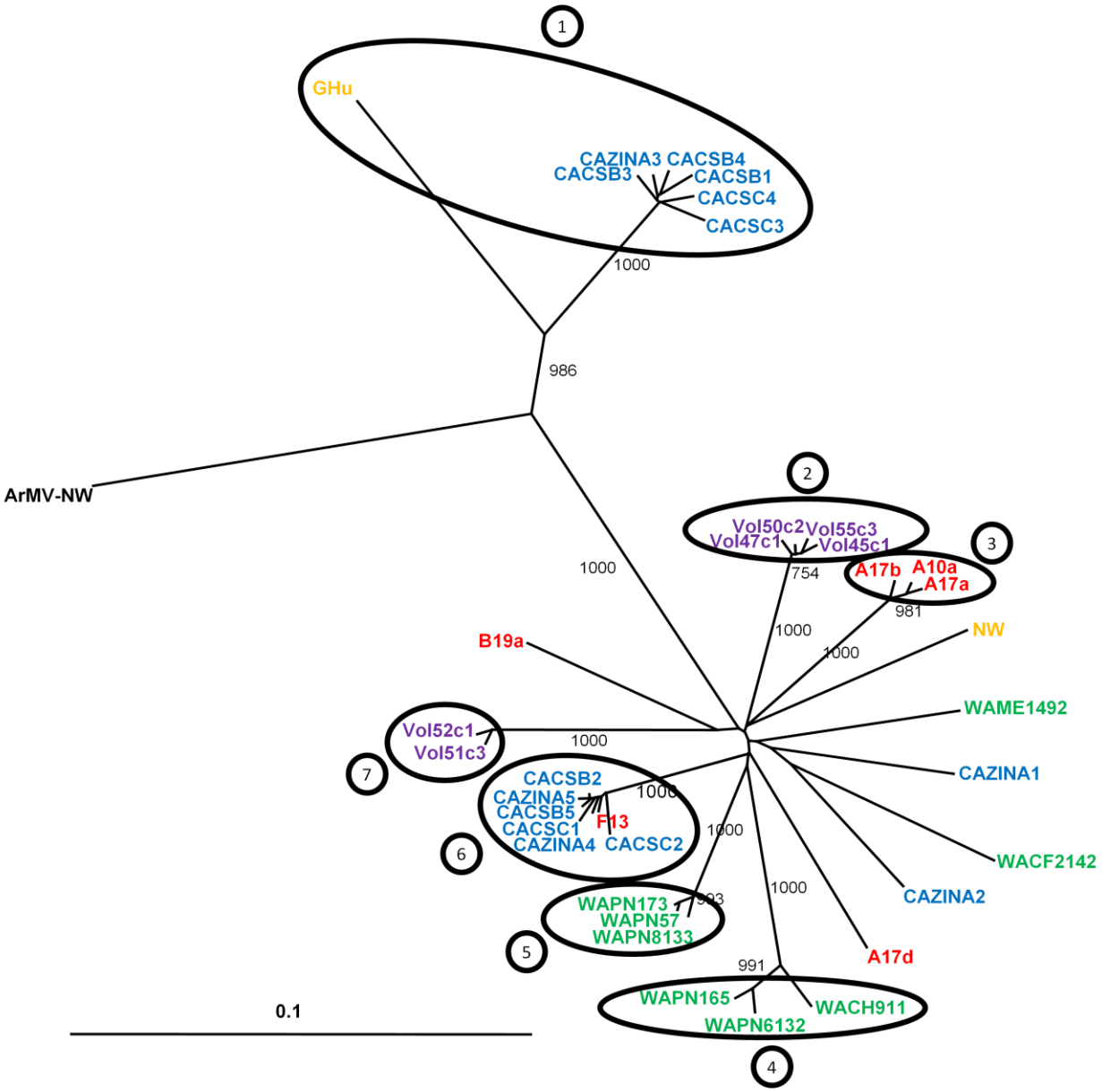

Figure 2.4. Phylogenetic tree showing genetic relationships among GFLV isolates from California and various origins worldwide within RNA2-encoded gene $2B^{MP}$. The geographic origins of these sequences are indicated by the following colors: blue for the California isolates, red for French isolates, green for isolates from Washington State, USA, purple for isolates from Slovenia, and orange for other European isolates. Distinct clades (numbered) are shown within black circles. Phylogenetic tree is based on nucleotide sequence. Bootstrap values greater than 750 out of 1000 are shown. Scale bars represent a genetic distance of 0.1.

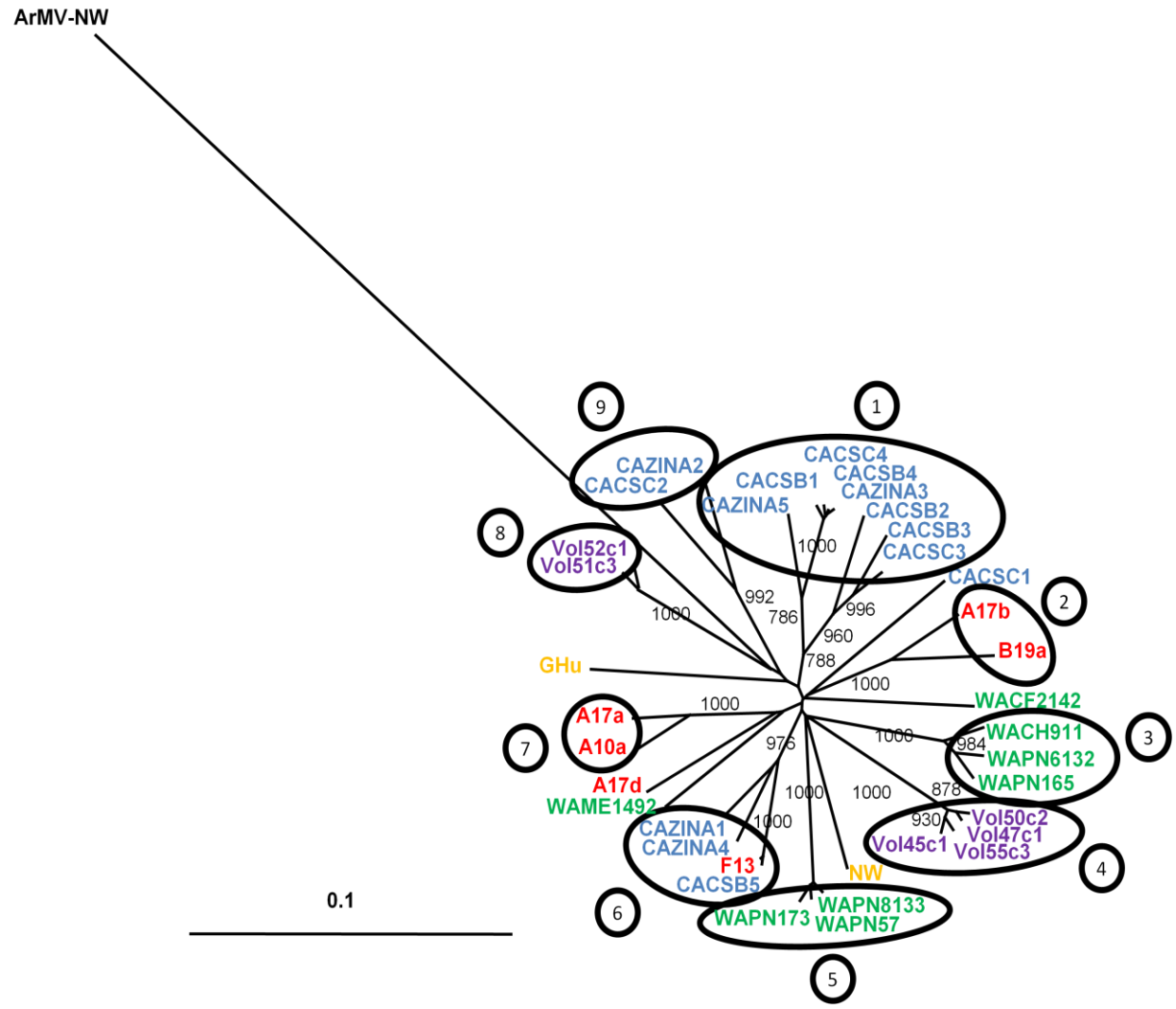

Figure 2.5. Phylogenetic tree showing genetic relationships of GFLV isolates from California and various origins worldwide within the RNA2-encoded gene $2C^{CP}$. The geographic origins of these sequences are indicated by the following colors: blue for the California isolates, red for French isolates, green for isolates from Washington State, USA, purple for isolates from Slovenia, and orange for other European isolates. Distinct clades (numbered) are shown within black circles. Phylogenetic tree is based on nucleotide sequence. Bootstrap values greater than 750 out of 1000 are shown. Scale bars represent a genetic distance of 0.1.

Within some of these clades, subclade organization was also observed and, though some clades consisted exclusively of isolates from a given geographic origin, other isolates from the same geographic origin clustered by themselves on a different branch or with isolates from other geographic origins (Figures 2.2–2.5, Table 2.2).

Therefore, these phylogenetic analyses did not support clear segregation by geographic origin for genes $1E^{Pol}$ (Figure 2.2), $2A^{HP}$ (Figure 2.3), $2B^{MP}$ (Figure 2.4), and $2C^{CP}$ (Figure 2.5), nor for the RNA2 coding sequence (Figure 2.6). Phylogenetic trees based on amino acid sequences provided further support for these conclusions (data not shown). Interestingly, gene $1E^{Pol}$ of isolates CACSB1 and CACSC4 segregated with the ArMV $1E^{Pol}$ gene with significant bootstrap support, suggesting that these two isolates might have originated from recombination events between GFLV and ArMV. Likewise, phylogenetic trees for the RNA2-encoded genes $2A^{HP}$ (Figure 2.3) and $2B^{MP}$ (Figure 2.4) as well as the RNA2 full coding sequences (Figure 2.6) revealed that these two isolates (CACSB1 and CACSC4) and four other isolates (CAZINA3, CACSB3, CACSB4, and CACSC3) formed a group of six California isolates that branched together with ArMV-NW and GFLV strain GHu with significant bootstrap support. These results suggested that these six GFLV isolates might be recombinants between GFLV and ArMV. By contrast, for gene $2C^{CP}$, sequences of GFLV isolates did not segregate with ArMV-NW (Figure 2.5).

Sequence analyses of the RNA2 sequences obtained in this study along with GDefV [GenBank accession # AY291208] did not indicate significant relationships (data not shown).

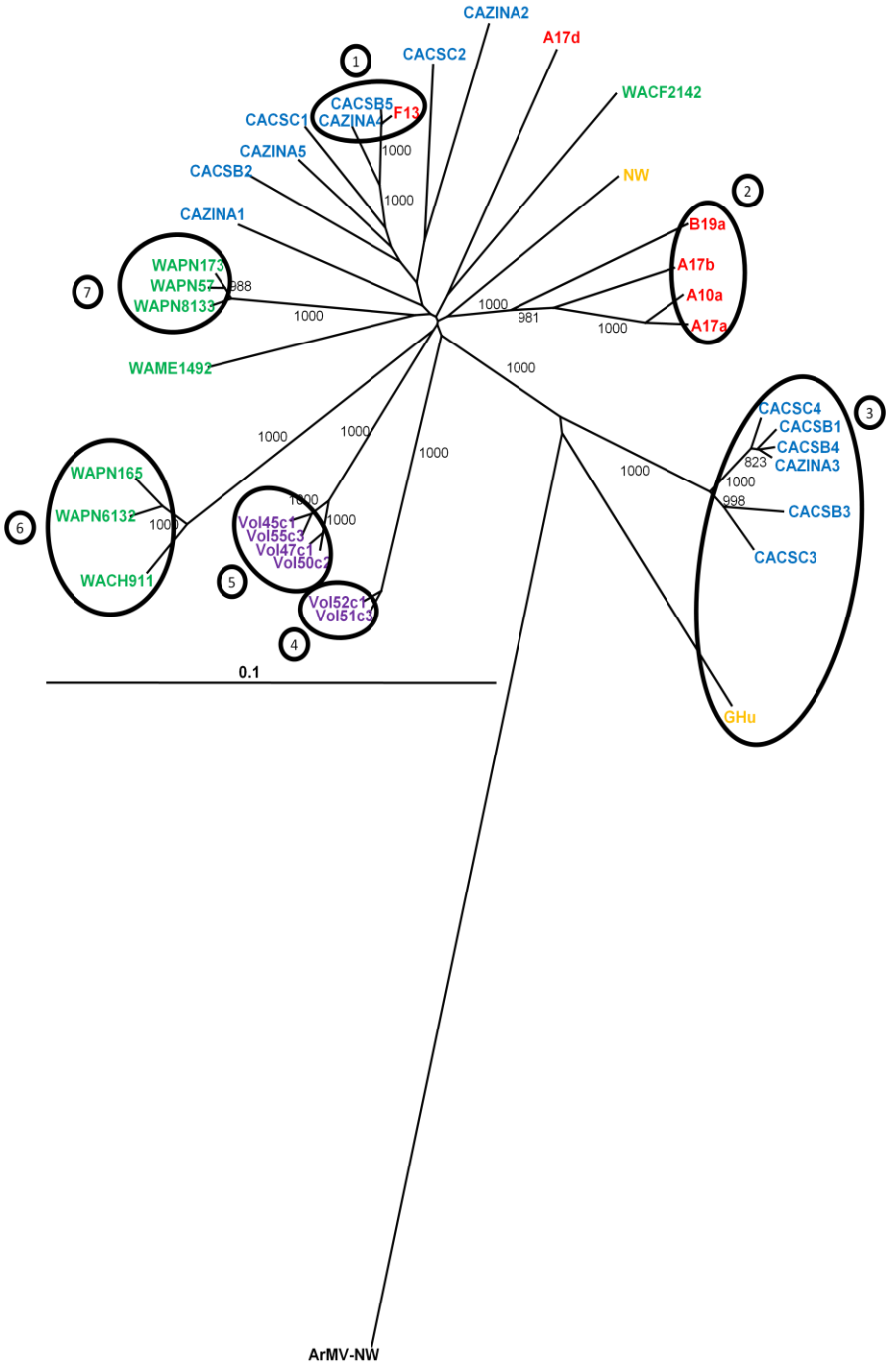

Figure 2.6. Phylogenetic tree showing relationships among GFLV isolates from California and various origins worldwide within RNA2 coding sequences. The geographic origins of these sequences are indicated by the following colors: blue for the California isolates, red for French isolates, green for isolates from Washington State, USA, purple for isolates from Slovenia, and orange for other European isolates. Distinct clades (numbered) are shown within black circles. Phylogenetic tree is based on nucleotide sequence. Bootstrap values greater than 750 out of 1000 are shown. Scale bars represent a genetic distance of 0.1.

Recombination events in GFLV genomic RNAs

SISCAN analysis was performed to confirm recombination events suspected by phylogenetic analyses. Interspecies recombination between GFLV and ArMV was indicated within RNA2 of isolates CACSB1, CACSC4, CAZINA3, CACSB3, CACSB4, and CACSC3 (Figure 2.7). The crossover region was determined to extend from nts 855 to 1930 within the 3' end of gene $2A^{HP}$ and into the 5' end of gene $2B^{MP}$.

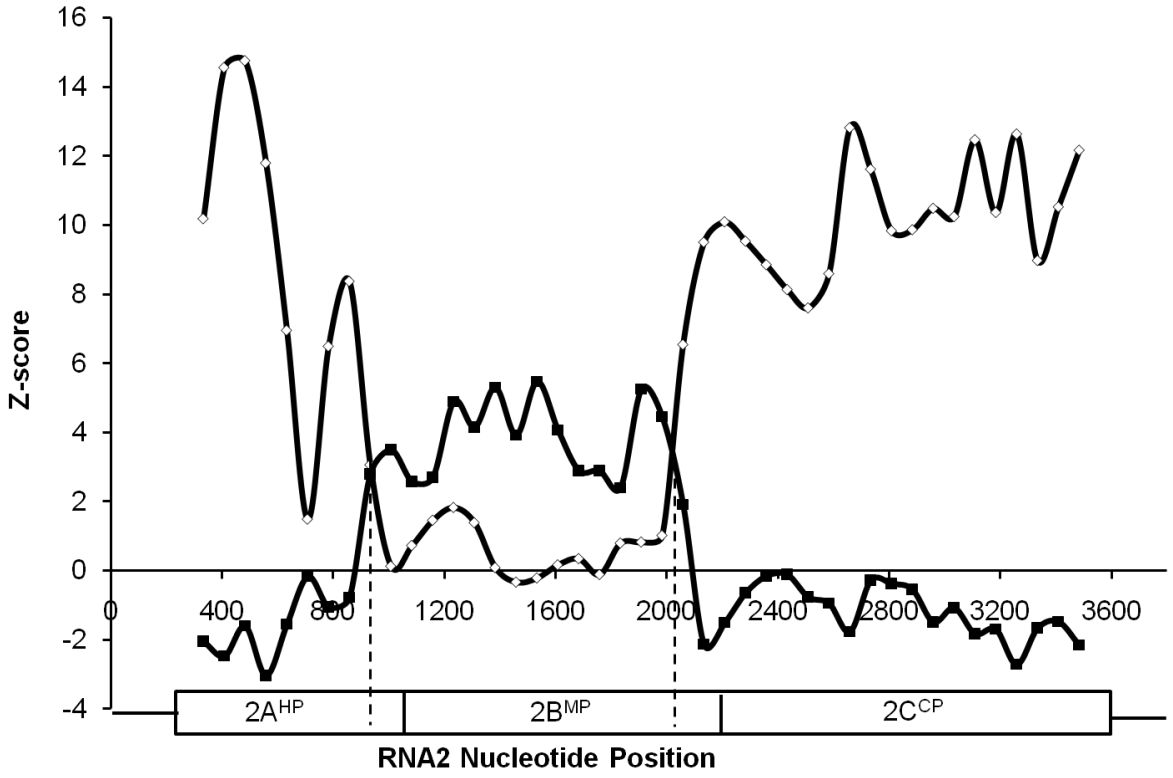

Figure 2.7. SISCAN analysis of the aligned nucleotide sequence from the RNA2 coding region of recombinant CACSB1 with ArMV-U (Syrah) (closed squares) and CACSB2 (open diamonds). The window covered 200 nt positions and moved through the alignment with a step size of 75 nts. Graphs are based on Z-values using the total nucleotide identity scores. Recombination crossover sites are indicated by a dotted line within the GFLV RNA2 coding region.

This crossover region is in a similar location as a crossover previously indicated for interspecies recombinant GFLV strain GHu (Table 2.4, Vigne et al. 2008). No support

for recombination events between other pairs of isolates was observed. Interspecies recombinant isolates were observed in all three vineyards examined in this study: isolate CAZINA3 was from vineyard A, isolates CACSB1, CACSB3, and CACSB4 from vineyard B, and isolates CACSC3 and CACSC4 from vineyard C. SISCAN analysis yielded poor Z-score values for all suspected regions of crossover in gene $1E^{Pol}$ from isolates CACSB1 and CACSC4 (data not shown). Similarly, p-values obtained with RDP3 were not significant (at $p < 0.05$) regardless of the GFLV and ArMV isolates used as putative major and minor parents. Therefore, recombination in gene $1E^{Pol}$ between GFLV and ArMV for isolates CACSB1 and CACSC4 could not be confirmed.

Table 2.4. Interspecies recombination crossover sites identified within GFLV RNA2.

Recombinant Isolate	Non-GFLV Parent	Recombination Sites[a]	Genomic Region	Reference
WACH911	GDefV	220-489	5' UTR & $2A^{HP}$	Mekuria et al. 2009
WAPN165	GDefV	230-488	5' UTR & $2A^{HP}$	Mekuria et al. 2009
WAPN6132	GDefV	230-489	5' UTR & $2A^{HP}$	Mekuria et al. 2009
WACF2142	GDefV	1299-1578	$2B^{MP}$	Mekuria et al. 2009
WACH911	ArMV	192-463	5' UTR & $2A^{HP}$	Mekuria et al. 2009
WAPN165	ArMV	191-540	5' UTR & $2A^{HP}$	Mekuria et al. 2009
WAPN6132	ArMV	192-468	5' UTR & $2A^{HP}$	Mekuria et al. 2009
WACF2142	ArMV	510-656	$2A^{HP}$	Mekuria et al. 2009
YM-7	ArMV	499-661[b]	$2A^{HP}$	Jawhar et al. 2009
YM-5	ArMV	499-661[b]	$2A^{HP}$	Jawhar et al. 2009
YM-C1	ArMV	499-661[b]	$2A^{HP}$	Jawhar et al. 2009
GHu	ArMV	1-105[b]	5' UTR	Vigne et al. 2008
GHu	ArMV	874-1935[b]	$2A^{HP}$ & $2B^{MP}$	Vigne et al. 2008
CAZINA3	ArMV	855-1930[b]	$2A^{HP}$ & $2B^{MP}$	This study
CACSB1	ArMV	855-1930[b]	$2A^{HP}$ & $2B^{MP}$	This study
CACSB3	ArMV	855-1930[b]	$2A^{HP}$ & $2B^{MP}$	This study
CACSB4	ArMV	855-1930[b]	$2A^{HP}$ & $2B^{MP}$	This study
CACSC3	ArMV	855-1930[b]	$2A^{HP}$ & $2B^{MP}$	This study
CACSC4	ArMV	855-1930[b]	$2A^{HP}$ & $2B^{MP}$	This study

[a]Nucleotide locations of recombination crossover regions corresponding to the non-GFLV parent as identified using the software package RDP3
[b]For consistency amongst comparisons, nucleotide locations were adjusted to correspond to their location relative to the GFLV-F13 reference sequence (Serghini et al. 1990)

Intraspecies recombination amongst GFLV isolates characterized in this study was also indicated by RDP3 analysis (at $p < 0.05$). Three unique recombination events (12 total events) were inferred within gene $1E^{Pol}$ of nine isolates from vineyard A (CAZINA1, CAZINA3, CAZINA4, and CAZINA5), B (CACSB5) and C (CACSC1, CACSC2, CACSC3, and CACSC4) (Table 2.5). Eleven unique recombination events

(22 total events) were inferred within RNA2, with five occurring entirely within gene $2C^{CP}$ of six isolates (CAZINA1, CAZINA4, CAZINA5, CACSB3, CACSB5, and CACSC1), three within genes $2A^{HP}$ and $2C^{CP}$ of eight isolates (CAZINA3, CAZINA5, CACSB1, CACSB2, CACSB3, CACSB4, CACSC3, and CACSC4), one within genes $2A^{HP}$ and $2B^{MP}$ of five isolates (CAZINA4, CAZINA5, CACSB2, CACSB5, and CACSC1), one within genes $2B^{MP}$ and $2C^{CP}$ of one isolate (CACSC2), and one within gene $2A^{HP}$ of another isolate (CACSB2) (Table 2.5). Thirteen total RNA2 recombinant isolates were

Table 2.5. Intraspecies recombination crossover sites identified within California GFLV isolates by RDP3.

Genomic RNA	Recombinant isolate[a]	Major Parent	Minor Parent	Crossover (nts)[b]	Gene(s)
RNA1	CAZINA1	CAZINA2	CAZINA4	1104-1497	$1E^{Pol}$
	CACSB5	CAZINA2	CAZINA4	1095-1322	$1E^{Pol}$
	CACSC1	CAZINA2	CAZINA4	1092-1338	$1E^{Pol}$
	CAZINA5, CACSC2, CACSC3	CACSC1	CAZINA2	439-2475	$1E^{Pol}$
	CAZINA1, CAZINA2, CAZINA3, CAZINA4, CACSB5, CACSC1	CACSC2	CAZINA5	2142-2475	$1E^{Pol}$
RNA2	CACSB2	CACSC1	CACSC4	503-594	$2A^{HP}$
	CACSB5, CAZINA4	CAZINA1	CACSC2	692-1973	$2A^{HP}$ & $2B^{MP}$
	CACSC1	CAZINA1	CACSC2	692-1983	$2A^{HP}$ & $2B^{MP}$
	CAZINA5	CAZINA1	CACSC2	692-1889	$2A^{HP}$ & $2B^{MP}$
	CACSB2	CAZINA1	CACSC2	686-1977	$2A^{HP}$ & $2B^{MP}$
	CACSB3	CACSB2	CACSB5	687-2058	$2A^{HP}$ & $2C^{CP}$
	CACSC3	CACSB2	CACSB5	683-2058	$2A^{HP}$ & $2C^{CP}$
	CACSB4, CAZINA3, CACSB1	CACSB2	CACSB5	687-2027	$2A^{HP}$ & $2B^{MP}$
	CACSC4	CACSB2	CACSB5	694-2027	$2A^{HP}$ & $2B^{MP}$
	CAZINA5	CAZINA1	CACSB5	242-2071	$2A^{HP}$ & $2C^{CP}$
	CACSB2	CAZINA2	CACSB5	248-2069	$2A^{HP}$ & $2C^{CP}$
	CACSC2	CACSC1	CAZINA2	1984-2918	$2B^{MP}$ & $2C^{CP}$
	CACSB3	CACSC3	CACSC1	2497-2603	$2C^{CP}$
	CACSB5, CAZINA4, CAZINA1	CAZINA3	CACSC3	2090-2858	$2C^{CP}$
	CACSC1	CAZINA2	CACSC3	2164-2746	$2C^{CP}$
	CACSC1	CACSB5	CACSC2	2919-3566	$2C^{CP}$
	CAZINA5	CACSB3	CACSB1	2072-2819	$2C^{CP}$

[a]Crossovers indicated by RDP3 to be a single event are grouped by shading.
[b]Crossover locations are reported based on alignment versus the full GFLV-F13 RNA2 sequence, and based on alignment versus the full GFLV-F13 $1E^{Pol}$ gene sequence only.

identified in vineyards A (CAZINA1, CAZINA3, CAZINA4, and CAZINA5), B (CACSB1, CACSB2, CACSB3, CACSB4, and CACSB5) and C (CACSC1, CACSC2, CACSC3, and CACSC4) (Table 2.5). SISCAN analyses confirmed intraspecies recombination events for recombinants in RNA1 and RNA2 whose major and minor parents were identified by RDP3 (data not shown).

SNAP analysis

To gain insights into the evolutionary forces and constraints acting on GFLV genes, d_S/d_N ratios amongst California isolates for the RNA2 coding region, gene $2A^{HP}$, gene $2B^{MP}$, gene $2C^{CP}$, and the partial sequence obtained from gene $1E^{Pol}$ were calculated using SNAP analysis. Different d_S/d_N ratios were obtained for each gene but all values were greater than 1 (Table 2.6). These results suggested that all genomic

Table 2.6. Synonymous (d_S)/non-synonymous (d_N) mutation rate ratios within GFLV RNA1-encoded gene $1E^{Pol}$ and RNA2-encoded genes $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$.

	With Interspecies Recombinant Isolates						Without Interspecies Recombinant Isolates[a]					
	California			Worldwide[b]			California			Worldwide[b]		
Gene	d_S	d_N	d_S/d_N	d_S	d_N	d_S/d_N	d_S	d_N	d_S/d_N	d_S	d_N	d_S/d_N
[c]$1E^{Pol}$	0.767	0.081	8.51	0.771	0.085	8.41	0.444	0.054	7.33	0.538	0.066	7.62
$2A^{HP}$	0.293	0.064	4.69	0.352	0.089	4.95	0.245	0.046	5.14	0.304	0.049	5.77
$2B^{MP}$	0.620	0.043	18.39	0.550	0.026	35.91	0.206	0.006	26.25	0.369	0.009	43.48
$2C^{CP}$	0.461	0.018	26.10	0.566	0.020	26.09	0.533	0.021	24.57	0.519	0.019	24.13

[a]Excludes recombinant isolates listed in Table 2.4 as well as suspected recombinants CACSB1 and CACSC4 for $1E^{Pol}$ calculations
[b]Includes all GFLV isolates listed in Table 2.2
[c]Only 1,140 nt region common to all isolates used in analysis

regions are under purifying selection but subjected to distinct constraints with the strongest pressures exerted on gene $2B^{MP}$ (d_S/d_N = 26.25) and gene $2C^{CP}$ (d_S/d_N = 24.57). They further indicated that gene $2A^{HP}$ is subjected to the weakest selection strength (d_S/d_N = 5.14) followed by gene $1E^{Pol}$ (d_S/d_N = 7.33). Analysis of d_S/d_N ratios for each gene of the 14 isolates characterized in this study alongside 44 other isolates from France, Hungary, Germany, Slovenia and Washington State (Table 2.6) were found to be similar, though a somewhat weaker negative selection was noted for gene $2B^{MP}$ from California isolates (d_S/d_N = 26.25) versus worldwide isolates (d_S/d_N = 43.48) (Table 2.6). A comparison of d_S and d_N values calculated both with and without interspecies recombinant isolates indicated lower overall levels of both synonymous and non-synonymous mutations for the non-recombinants. This partially accounts for the

differences observed in the d_S/d_N ratios for gene $2B^{MP}$ between worldwide isolates and the California isolates sequenced in this study.

DISCUSSION

Despite the fact that GFLV research has a rich history – it was the first plant virus ever to be shown to be transmitted by a nematode vector (Hewitt et al. 1958) – our knowledge of the virus's genetic structure and evolutionary mechanisms is limited. While there is an abundance of information on the genetic diversity within gene $2C^{CP}$ and to a lesser extent within gene $2B^{MP}$, there is relatively little sequence information from other parts of the genome.

Like wine grapes, GFLV is believed to be native to Asia—around the Caspian Sea- from which it spread worldwide likely through the distribution and use of infected grape propagation material (Bashir et al. 2007). Little is known on the genetic relatedness of Asian, European and American GFLV populations. Likewise, despite the significant economic impact of GFLV in California vineyards, currently no sequence information from California isolates – outside of genes $2C^{CP}$ and $2B^{MP}$ (Naraghi-Arani et al. 2001, Sanchez et al. 1991) – is available. Also, there have been few studies carried out to examine the genetic variability of GFLV at the vineyard level (Pompe-Novak et al. 2007, Vigne et al. 2004, 2009), especially in vineyard settings where natural spread by *X. index* occurs. This study, by providing information on the genetic diversity and variability of GFLV within both RNA1 ($1E^{Pol}$) and RNA2 in three vineyard settings in California where nematode-mediated transfer of the virus is known to occur, addresses an area where knowledge on GFLV is currently lacking.

Our phylogenetic analyses showed segregation of the RNA2 and $1E^{Pol}$ sequences from California GFLV isolates into two and three distinct groups, respectively. A segregation of isolates into at least two evolutionary lineages of molecular variants has been seen previously for gene $2C^{CP}$ of GFLV isolates from France (Vigne et al. 2004, 2009), South Africa (Liebenberg et al. 2009) and Tunisia (Fattouch et al. 2005). This is consistent with the evolution of viral RNA populations (Garcia-Arenal et al. 2001, Huynen et al. 1996, Moury et al. 2006).

Phylogenetic relationships indicated no apparent segregation amongst California GFLV isolates by scion, rootstock, or vineyard origin. The lack of segregation by host genotype is likely due to the fact that there is no recognized resistance to GFLV in *Vitis* species (Andret-Link et al. 2004a). Lacking resistance, grapevine hosts are unlikely to pose as a bottleneck on genetic diversity and select for certain genetic variants. The rootstocks Dog Ridge and Freedom, onto which the Zinfandel and Cabernet Sauvignon vines that were characterized in this study were grafted, are resistant to the nematode vector of GFLV, *X. index* (Harris 1983, Kunde et al. 1968, McKenry et al. 2001). Freedom has been reported to be resistant to *X. index* in multiple environments, while some studies have found Dog Ridge to be susceptible (McKenry et al. 2001) or give it a low resistance rating (Kunde et al. 1968). Resistance to *X. index*, however, does not prevent GFLV infection because transmission can still occur in these rootstock genotypes, as confirmed in this study. Therefore, a lack of phylogenetic segregation based on host genotype is not unexpected.

The lack of segregation of GFLV isolates by vineyard may be consistent with multiple introductions of the virus to the vineyards, movement between vineyards of

either infected vines or the nematode vector, or the innate tendency of a highly variable RNA virus to tend toward heterogeneity. The three vineyards surveyed in this study were established at different times from different source materials originating in California, and vary in age from 10 to 40 years. Also, they are owned and operated by the same vineyard manager, so one possible explanation for the lack of segregation by vineyard is that the virus came into each vineyard from multiple sources and was subsequently mixed amongst the three vineyards due to transfer of soil containing viruliferous nematode vectors, possibly on farm machinery (Villate et al. 2008). Mixing of virus isolates would tend to cause the population to appear ubiquitous between vineyards.

Phylogenetic relationships amongst GFLV isolates worldwide did not indicate segregation by geographic origin, except for a few lineages (Figure 2.6). This is likely due to a variety of factors including the aforementioned lack of host resistance in *Vitis* species, the uncontrolled exchange of grapevine planting material and budwood worldwide, and also the limited origins from which substantial genomic sequence information is available outside of genes $2B^{MP}$ and $2C^{CP}$ (i.e. five countries – France, Hungary, Germany, Slovenia and United States).

Based on the genetic divergence of each gene examined in this study at both the nucleotide and amino acid levels, the quasispecies nature of GFLV (Naraghi-Arani et al. 2001) is evident. The nucleotide divergence for each gene is relatively similar at an average of approximately 11%. This level of variation at the nucleotide level is a common sight in RNA viruses (Garcia-Arenal et al. 2001, Moury et al. 2006) and is likely the result of the lack of a proofreading mechanism provided by the RdRp when

replicating the viral genome. This is a likely explanation for the overall wide level of divergence at the nucleotide level within vineyards (maximum divergence of 16.9% for RNA2 and 22.9% within gene $1E^{Pol}$ – without suspected recombinant isolates CACSB1 or CACSC4) that was similar to levels seen in worldwide comparisons. Within gene $1E^{Pol}$, isolates CACSB1 and CACSC4 were much more divergent from the other isolates than the other isolates were to one another. In fact, as much or more nucleotide divergence was observed between isolates within a single vineyard surveyed in this study as has been seen previously in comparing other sequences from multiple regions or countries (Bashir et al. 2007, Fattouch et al. 2005, Liebenberg et al. 2009, Mekuria et al. 2009, Vigne et al. 2004, 2009, Wetzel et al. 2001, 2002).

SNAP results indicated strong purifying selection exerted on genes $2C^{CP}$ and $2B^{MP}$ and limited divergence at the amino acid level (3.6% and 6.1%, respectively) – versus genes $2A^{HP}$ and $1E^{Pol}$. The fact that protein $2C^{CP}$ determines vector transmission (Andret-Link et al. 2004b) and proteins $2B^{MP}$ and $2C^{CP}$ are involved in cell-to-cell and systemic movement (Belin et al. 1999, Van Lent and Schmitt-Keichinger 2006) likely serves as a strong evolutionary constraint on these two genes. The fact that protein $2C^{CP}$ is highly conserved at the amino acid level has implications for immunological detection of GFLV for diagnostic purposes. The relatively low level of amino acid changes supports the continued use of antibodies targeting GFLV virions in immunological assays. By targeting a protein that is less likely to vary from isolate to isolate, the reliability of the tests will be assured.

The levels of divergence within genes $2A^{HP}$ and $1E^{Pol}$ were similar at the nucleotide level, but higher at the amino acid level, versus those of genes $2B^{MP}$ and

$2C^{CP}$. This differs from previous reports which indicated a higher level of nucleotide divergence within gene $2A^{HP}$ versus the level observed in genes $2B^{MP}$ and $2C^{CP}$ (Mekuria et al. 2009, Pompe-Novak et al. 2007, Vigne et al. 2008, Wetzel et al. 2001). The SNAP results indicated a negative selection exerted on gene $2A^{HP}$ but a weaker negative selection than those on genes $2B^{MP}$ or $2C^{CP}$. This may indicate that the exact amino acid sequence is less important to the overall function of protein $2A^{HP}$ in RNA2 replication, suggesting it might be more genetically flexible. Previous research has indicated that gene $2A^{HP}$ can vary in length between GFLV isolates (Mekuria et al. 2009, Pompe-Novak et al. 2007, Vigne et al. 2008), further supporting the idea that it may be able to function with more variation at the amino acid level than other RNA2 proteins. Based on our analyses, protein $1E^{Pol}$ appears to have intermediate levels of divergence relative to the RNA2-encoded proteins. However, it is important to note that the 1140 nucleotide region from gene $1E^{Pol}$ that was used for sequence analyses does not include domains I through VII of the eight conserved protein domains identified previously in the RdRp of positive-strand RNA viruses (Koonin 1991), so it is possible that the amino acid conservation would be higher if the entire gene $1E^{Pol}$ could have been analyzed for all isolates. More sequences of gene $1E^{Pol}$ for isolates of diverse origins are needed to verify this hypothesis and to more precisely assess the genetic variability within this genomic region.

Some of the California isolates characterized in this study result from recombination between GFLV and ArMV, a related nepovirus that has previously been shown to recombine with GFLV in genes $2A^{HP}$ and $2B^{MP}$ (Jawhar et al. 2009, Mekuria et al. 2009, Vigne et al. 2008). Phylogenetic analyses and sequence comparisons of

genes $2A^{HP}$ and $2B^{MP}$ clearly showed isolates CAZINA3, CACSB1, CACSB3, CACSB4, CACSC3, and CACSC4 segregating with ArMV (Figures 2.3 and 2.4). Isolates CACSB1 and CACSC4 also segregated with ArMV-NW within gene $1E^{Pol}$ (Figure 2.2). Putative recombination events were confirmed in genes $2A^{HP}$ and $2B^{MP}$, but could not be confirmed in gene $1E^{Pol}$. Unexpectedly, our analyses indicated that the recombination crossover locations near the 3' end of the $2A^{HP}$ and near the 3' end of the $2B^{MP}$ were similar to those reported for GFLV strain GHu (Table 2.4, Vigne et al. 2008). Since few GFLV/ArMV interspecies recombinants have been sequenced (Table 2.4), the fact that strain GHu and the six recombinant isolates from this study share crossover locations seems to be more than a coincidence. This seems to suggest that either strain GHu and the recombinant California isolates are descended from a single recombination crossover event, or that these recombination crossover sites are in some way favored recombination sites within GFLV RNA2. While the recombinant isolates examined in this study are the most similar to GFLV strain GHu at the nucleotide level as compared to all other nepovirus strains (data not shown) their nucleotide sequence differences (12.0% and 11.6% within and outside the crossover region, respectively) are substantial, which may point to a separate recombination event giving rise to the California recombinants rather than a common recombination event giving rise to both the California isolates and strain GHu. This observation further supports the notion that the recombination crossovers observed here are hotspots. The idea that these might be favored recombination sites has some support from the conservation of movement protein domains in nepoviruses (Mushegian 1994) and recombination reported to have occurred between the movement protein genes of GFLV and GDefV (Mekuria et al.

2009) and the nepoviruses *Grapevine chrome mosaic virus* and *Tomato black ring virus* (Le Gall et al. 1995). This raises the possibility that recombination in the movement protein gene of nepoviruses might play a role in host adaptation and specialization, perhaps in a similar manner to which recombination within the movement associated N-terminal domain of the coat protein of some potyviruses can affect host specialization (Wylie and Jones 2009).

In contrast to interspecies recombination, intraspecies recombination amongst California isolates appeared to be quite frequent with the majority of detected crossover events within gene $2C^{CP}$. One may therefore conjecture that while intraspecies recombination can be a frequent occurrence within the GFLV genome, interspecies recombination might face more rigorous constraints on where it may occur (and still yield a viable recombinant). The fact that protein $2C^{CP}$ is responsible for nematode transmission (Andret-Link et al. 2004b) may explain why interspecies recombination has not been detected within the gene encoding the structural protein (Table 2.4) and also why interspecies recombination, at least to our knowledge, has not been shown to occur within the coat protein gene of any nepoviruses.

The identification of interspecies GFLV/ArMV recombination within GFLV isolates from California (this study) and Washington State (Mekuria et al. 2009) is quite puzzling. ArMV is present in European vineyards where it is transmitted by the nematode species *Xiphinema diversicaudatum*. However, in California and Washington State, neither ArMV nor *X. diversicaudatum* have ever been documented, and ELISA testing of more than 500 vines from vineyards A, B, and C surveyed in this study failed to show the presence of ArMV (data not shown). So it is unlikely that the interspecies recombinant

isolates originated from recombination within the United States. Since GFLV strains GHu from Hungary (Vigne et al. 2008) and YM-7 from Italy (Jawhar et al. 2009) have been identified as interspecies GFLV/ArMV recombinants, one possible scenario is that American recombinants originated in Europe and were introduced to the United States via infected grapevine propagation material. Though, at least with the three vineyards surveyed in this study, no recent introduction of propagation material has taken place and the propagation material that was used originally was of U.S. origin, so such importations from Europe likely occurred some time ago. Introductions of GFLV/ArMV recombinant isolates from Europe into California GFLV populations have likely fixed the recombinant isolates within local virus populations. Previous observations have indicated that recombinants between GFLV and ArMV, including GFLV strain GHu, which are used in cross-protection trials to manage GFLV (Komar et al. 2008), produce less severe symptoms in grapevines than non-recombinant GFLV (Legin et al. 1993, Vigne et al. 2008). Perhaps the nucleotide and amino acid changes resulting from recombination between GFLV and ArMV have been maintained because they provide a selective advantage to the corresponding recombinant isolates due to the relative mildness of symptoms that they confer in grapevine and/or their potential to protect against more severe isolates. Such GFLV isolates may have a higher likelihood to be overlooked because vines infected with milder isolates may be more difficult to detect visually, possibly giving them an advantage when vineyard managers remove infected vines in efforts to eliminate GFLV from their vineyards. Also, the relatively limited sequence information available for ArMV and GFLV may have failed to reveal intimate relationships between these two nepoviruses, and more extensive sequence knowledge

may indicate that recombination between GFLV and ArMV is commonplace. The potential long-term impact of recombinant GFLV/ArMV isolates on vine growth and yield in the U.S. is unknown.

Notwithstanding, some GFLV isolates from California vineyards A, B and C are closely related to GFLV strain F13 from France in the four genomic regions characterized in this study (Figures 2.2–2.5). To our knowledge, this is the first report of a close genetic relatedness of strain F13 and other GFLV isolates, providing evidence for a close genetic relationship of some California and European GFLV isolates. In addition, the recombinant isolates sequenced in this study share the same GFLV/ArMV recombination crossover locations as those found within Hungarian recombinant GFLV strain GHu. These findings support the notion that the introduction of wine grape cultivars from France, Hungary, and other European countries to California beginning in the early 1830s not only started the tremendous expansion of the California wine industry in the nineteenth century (Pinney 1989), but potentially also disseminated European GFLV isolates to a new grape-growing region.

CONCLUSIONS

The following main conclusions are supported by our findings. First, analysis of 14 California GFLV isolates together with 44 isolates from France, Germany, Slovenia and Washington State indicated no clear association between genetic diversity and vineyard origin, rootstock, or scion variety for either RNA2 or RNA1-encoded gene $1E^{Pol}$. Second, there was as much or more genetic divergence among California isolates than had been documented previously amongst any GFLV isolates worldwide. Third, strong

negative selection constraints were observed for genes $2C^{CP}$ and $2B^{MP}$, while selection was substantially weaker on gene $2A^{HP}$ and intermediate on gene $1E^{Pol}$. Fourth, interspecies recombination between GFLV and ArMV was determined for 6 of the 14 California isolates characterized with crossover sites identified in genes $2A^{HP}$ and $2B^{MP}$ and suspected in gene $1E^{Pol}$. The recombination crossover locations within genes $2A^{HP}$ and $2B^{MP}$ appeared to correspond to the crossover locations in GFLV strain GHu, suggesting potential hotspots for recombination. Finally, intraspecies recombination was identified for 13 of the 14 California isolates, and in each of the four genomic regions examined. Together, this study indicated that purifying selection and recombination are important evolutionary mechanisms in the genetic diversification of GFLV.

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CHAPTER 3

Designing Resistance Constructs Derived from *Grapevine Fanleaf Virus* and Developing a Fast and High-Throughput Method to Test Their Effect on Virus Multiplication

ABSTRACT

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration disease, a devastating and widespread disease of grapevines. Since resistance to GFLV is not available in wild or cultivated *Vitis* sp., transgenic resistance would be desirable. Based on an analysis of GFLV genomic variability, conserved regions within the two genomic RNAs have been identified, and varied genetic constructs derived from GFLV have been generated with the aim of conferring resistance in transgenic grapevine rootstocks. In order to reduce the time and expense involved in the production and testing of transgenic grapevines for resistance to GFLV, a high-throughput approach has been developed for evaluating the antiviral potential of candidate constructs. This approach utilizes an *Agrobacterium tumefaciens*-mediated delivery system to achieve transient expression in *Nicotiana benthamiana*, a systemic host of GFLV, and allows for screening putative resistance constructs over a considerably shorter time frame than testing transgenic grapevines. Many of the genetic constructs reduced virus titers in agroinfiltrated plant tissues, as shown by enzyme-linked immunosorbent assays and semi-quantitative RT-PCR, with differential levels of antiviral activity observed amongst constructs. To test whether the

transient approach is an accurate predictor of the antiviral competency of constructs, transgenic *N. benthamiana* were also produced and utilized in resistance screening assays. Results from comparative resistance evaluations using the transient expression system and stable *N. benthamiana* transformants suggest that the transient expression system can be valuable in predicting the success of GFLV transgene constructs in stable transformants.

INTRODUCTION

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration, a devastating disease of grapevine that can result in up to 80% yield losses in vineyard settings. GFLV has a bipartite, positive-sense single-stranded RNA genome. Each genomic RNA is expressed as a polyprotein that is cleaved into individual proteins. RNA1 codes for five proteins that are involved in proteolytic processing and replication, and RNA2 codes for three proteins that are involved in movement, capsid formation and RNA2 replication. GFLV is specifically vectored by the ectoparasitic dagger nematode, *Xiphinema index*, in a soilborne manner (Andret-Link et al. 2004).

Resistance to GFLV would be desirable for fanleaf control; however, no source of resistance to GFLV has been identified in wild or cultivated grapevines (Oliver and Fuchs 2011). To date, management of GFLV has primarily relied on prevention through certification schemes and the use of planting material derived from clean, virus-tested stocks. Control of the nematode vector *X. index* is another component of GFLV management strategies, although this approach can be challenging due to the relative

lack of effective nematicides as well as their harsh environmental consequences. Prolonged fallow periods (up to 10 years) can reduce nematode populations in infested soils, but lengthy fallow periods are not practical in most high-value grape-growing areas (Andret-Link et al. 2004). Grapevines with resistance to *X. index* have been identified and rootstocks resistant to this dagger nematode have been developed. However, a single feeding event can theoretically result in virus transmission by the nematode. Therefore, nematode resistant rootstocks do not prevent GFLV infection of scions in a vineyard setting (Oliver and Fuchs 2011).

To manage GFLV, alternative means of control are needed. For this reason, transgenic grapes resulting from the application of the concept of pathogen-derived resistance have been developed to confer resistance to GFLV. These transgenic materials primarily express the virus's coat protein gene (Gambino et al. 2010, Krastanova et al. 1995, Maghuly et al. 2006, Mauro et al. 1995, Valat et al. 2006, Xue et al. 1999), and results from a three year field trial indicate that some of these materials are resistant to GFLV (Vigne et al. 2004). The mechanism behind the process of pathogen-derived resistance is believed to be RNA silencing through its antiviral pathways (Prins et al. 2008).

Transgenic resistance to viruses has proven to be an effective alternative means of obtaining resistant plants, in particular in cases where there is no natural resistance (Oliver et al. 2011). However, developing this type of resistance, especially in fruit crops such as grapes, can be time-consuming and, hence, costly. The tissue culture techniques used to generate the numerous transgenic lines necessary to identify a virus-resistant individual, as well as the greenhouse and field space necessary to test

these lines, can be quite expensive and labor intensive – especially in the case of perennial crops.

In recent years, knowledge regarding RNA silencing has led to the discovery of transgene designs capable of stimulating the RNA silencing machinery more effectively, thereby increasing the proportion of resistant individuals among a population of transgenic lines produced following *Agrobacterium tumefaciens*-mediated transformation (Prins et al. 2008, Wesley et al. 2001). Likewise, improved construct designs, including the use of conserved regions of the viral genome, may result in resistance that is more effective, durable, and broad-spectrum (Bucher et al. 2006). However, since the time and effort necessary to test numerous transgenic lines developed from each potential construct design can still be prohibitive in perennial crops, more expedient and high-throughput methods are needed to streamline the testing of transgene constructs for their relative effectiveness at conferring resistance to viral challenge.

In this study, we analyzed the genetic variability of GFLV and identified conserved fragments that were concatenated and cloned in a plant expression cassette. Agroinfiltration was explored as a high-throughput and fast system for testing the capacity of these constructs to interfere with GFLV multiplication following their transient expression in the model host *Nicotiana benthamiana*. The robustness and versatility of this transient expression system was determined by comparing the performance of transgenic constructs in patch assays and stable transgenic plants.

MATERIALS AND METHODS

Analysis of GFLV sequence information

GFLV RNA1 and RNA2 sequences available in GenBank (Table 3.1) were aligned using the algorithm Clustal W.

Table 3.1. GFLV RNA1 and RNA2 sequences used for alignments and identification of conserved regions.

Sequences and GenBank Accession Numbers[1]						
RNA1	**RNA2**					
D00915	AB222862	DQ922675	AY370995	AY371023	AY997699	*C19b*
EF528585	AF438354	DQ922679	AY370994	AY371001	AY997698	*C18c*
NC_003615	AF438351	DQ922677	AY370957	AY371000	AY997697	*CGHu*
[2]AY303786	AF438353	DQ922665	AY370962	AY370999	AY997694	*C1a*
[2]NC_006057	AF438352	DQ922678	AY370979	AY371024	AY997693	*C2c*
[2]AJ630200	AF438355	DQ922669	AY370971	AY371006	AY997696	*C18a*
	AF438349	DQ922664	AY370958	AY371002	AY997695	*C18b*
	AF438350	DQ922671	AY370953	AY370961	DQ513336	*C15b*
	AF438356	DQ922666	AY370967	AY370955	DQ513332	*C4a*
	AF438344	DQ922667	AY370965	AY370977	DQ513335	*C13b*
	AF438348	AY780900	AY370981	AY370973	DQ513333	*C16a*
	AF438357	DQ922652	AY370983	AY370970	DQ513334	*C3b*
	DQ386866	DQ922661	AY370982	AY370947	AY821657	*C6c*
	DQ286901	DQ922660	AY371017	AY370949	AF304015	*C5a*
	DQ286916	DQ922659	AY371022	AY370956	AF304013	*C14a*
	DQ286915	DQ922658	AY371016	AY370959	AF304014	*C12c*
	DQ286913	DQ922657	AY371004	DQ526452	X60775	*C9a*
	DQ286912	DQ922654	AY371014	AY370952	AY017338	*C9b*
	DQ286914	DQ922656	AY371012	AY370987	NC_003623	*C17b*
	DQ286911	DQ922655	AY370993	U11768	X16907	*AB3b_g7_*
	DQ286910	DQ922653	AY370986	AY370968	DQ922662	*AB6b_f2_*
	DQ286909	AY780903	AY371019	AY370951	DQ922670	*AB22a__I15_*
	DQ286908	DQ922676	AY371021	AY370966	DQ922668	*AB14a__I51_*
	DQ286907	DQ922674	AY371020	AY371011	DQ922673	*AB9a__g37_*
	DQ286906	DQ922663	AY371018	AY371027	DQ922672	*AB23a_G109_*
	DQ286905	DQ672566	AY370998	AY370997	AY780902	*AB21a_G122_*
	DQ286904	DQ672565	AY370975	AY371026	AY780899	*AB21b_G127_*
	DQ286903	DQ672567	AY370974	DQ362926	AY780901	*ABGHu*
	DQ286902	AY464090	AY370976	DQ362930		*AB17a__I78_*
	AF418579	DQ362932	AY370992	DQ362933		*AB19a_G62_*
	AY525605	AY371007	AY370960	AY371025		*AB22b__I16_*
	AY525606	AY371008	AY370991	AJ318415		*AB8b_G26_*
	AY594177	AY370948	AY370990	DQ362923		*AB10a_G134_*
	AY942809	AY371015	AY370963	DQ362925	[3]*Other Segs:*	*AB1a_f13_*
	AY942808	AY371013	AY370954	DQ362935	*C1b*	*AB1b_f14_*
	AY942807	AY371003	AY370989	DQ362928	*C1c*	*AB4a_G85_*
	AY942806	AY370964	AY370984	DQ362920	*C3d*	*AB2b__H28_*
	AY942805	AY370944	AY370988	DQ362929	*C6d*	*AB18a_f28_*
	AY942813	AY370943	AY370985	DQ362927	*C8b*	*AB16a_G49_*
	AY942812	AY370942	AY370980	DQ362931	*C8c*	*AB11a__H37_*
	AY942811	AY370941	AY370945	DQ362934	*C8d*	*AB13a__I37_*
	AY942810	AY371010	AY370978	DQ362921	*C8a*	*AB13b__I39_*
	AY942804	AY371009	AY370972	DQ362924	*C10b*	*AB8a__g25_*
	AY942803	AY370969	AY370950	DQ362922	*C3c*	*Chilean*
	AY942802	AY370946	AY370996	AY371005	*C19a*	*C_FL34*

[1]GenBank accession numbers accessed on May 30th, 2007.
[2]RNA1 sequences from *Arabis mosaic virus*, a virus closely related to GFLV.
[3]Sequences in *italics* were made available by some collaborators prior to their submission to GenBank.

Hand editing and alignment visualization was performed using SeaView. Following alignment, regions of at least 25 nts in length were identified where 85% of the nucleotide positions were conserved amongst 95% of the aligned sequences. Expanded regions of at least 100 nts in length that consisted of at least one or more of these smaller regions identified above were chosen for cloning.

Engineering and cloning of GFLV constructs

cDNA clones of GFLV strain F13 RNA1 (plasmid pMV13) and RNA2 (plasmid pVecP2) (Viry et al. 1993) were used as templates to amplify conserved regions using specific primer pairs. Primers carried various restriction sites at the 5' end to facilitate cloning (Table 3.2a). PCR was carried out using the GoTaq DNA polymerase and 10 pmol of primers in a 20 µl final volume according to the manufacturer's protocol (Cloning Phase I). PCR used a 2 min heating step at 94°C followed by 30 cycles of 1 min melting at 94°C, 1 min annealing at 50°C, and 2 min elongation at 72°C with a final extension of 7 min at 72°C. Resulting PCR fragments were cleaned up using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and digested using appropriate restriction enzymes including NheI, SpeI, XbaI, AgeI, BspEI, NgoMIV, SalI, XhoI, NotI, and PspOMI. Restriction digest products were cleaned up using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and ligated together using T4 DNA Ligase (New England Biolabs, Ipswich, MA) according to manufacturer's protocols. Following ligation and a new PCR run to amplify the desired product, amplicons were resolved by electrophoresis in 1.5% agarose gels in 90 mM Tris–borate, 2 mM EDTA, pH 8.0,

stained with ethidium bromide and subsequently visualized under UV light followed by gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Table 3.2. Primers for PCR and RT-PCR assays used for (A) Cloning Phase I, (B) Cloning Phase II, and (C) Resistance evaluation.

Purpose	Sequence (5' to 3')	Binding Location vs. GFLV-F13
A. Cloning Phase I Primers		
Frag. #1 – Forward	GAGCTAGCGTCGACGGTACCAGATGAATTGTGC	4736-4754 (RNA1)
Frag. #1 – Reverse	GAACCGGTCTCGAGTCATACCACGTCTGAACCA	4921-4939nts (RNA1)
Frag. #2 – Forward	GAGCTAGCGTCGACCTTGTTGAGAGTAAAATTT	5291-5309nts (RNA1)
Frag. #2 – Reverse	GAACCGGTCTCGAGACTCAAAATTTGCTCCGTA	5468-5486nts (RNA1)
Frag. #3 – Forward	GAGCTAGCGTCGACTACCTATGGTGATGATAATG	5744-5763nts (RNA1)
Frag. #3 – Reverse	GAACCGGTCTCGAGTCACCATGTAGATAAAGCT	6058-6076nts (RNA1)
Frag. #4 – Forward	GAGCGGCCGCTCCGGACCCCAGCTCCCTACTTTAG	573-591nts (RNA2)
Frag. #4 – Reverse	GAACTAGTGGGCCCTGGCAATTCGGCAAAGAGTGC	683-703nts (RNA2)
Frag. #5 – Forward	GAGCGGCCGCTCCGGATGATAGAAACGTTGATCTT	1987-2005nts (RNA2)
Frag. #5 – Reverse	GAACTAGTGGGCCCCTTGAAGTCTGAGATCATA	2128-2146nts (RNA2)
Frag. #6 – Forward	GAGCCGGCGGGCCCTTAGTGAGTGGAACGGGAC	2802-2820nts (RNA2)
Frag. #6 – Reverse	GATCTAGAGCGGCCGCCTGACTTTGACCAGCAAGCA	2928-2935nts (RNA2)
Frag. #7 – Forward	GAGCCGGCGGGCCCAGAAGAAATTGAGATTGGT	3127-3145nts (RNA2)
Frag. #7 – Reverse	GATCTAGAGCGGCCGCACCACTATGCAATCCATGG	3223-3241nts (RNA2)
Frag. #8 – Forward	GAGCCGGCGGGCCCTTTAGCTTTTATGGTAGAA	3527-3545nts (RNA2)
Frag. #8 – Reverse	GATCTAGAGCGGCCGCCAGTAAAAAGAAAGGAAAA	3742-3760nts (RNA2)
B. Cloning Phase I Primers		
Frag. #1 – Forward	GAGGATCCGGTACCAGATGAATTGTGC	4736-4754 (RNA1)
Frag. #1 – Reverse	GAGGATCCTCATACCACGTCTGAACCA	4921-4939nts (RNA1)
Frag. #2 – Forward	GAGGATCCCTTGTTGAGAGTAAAATTT	5291-5309nts (RNA1)
Frag. #2 – Reverse	GAGGATCCACTCAAAATTTGCTCCGTA	5468-5486nts (RNA1)
Frag. #3 – Forward	GAGGATCCTACCTATGGTGATGATAATG	5744-5763nts (RNA1)
Frag. #3 – Reverse	GAGGATCCTCACCATGTAGATAAAGCT	6058-6076nts (RNA1)
Frag. #4 – Forward	GAGGATCCCCCCAGCTCCCTACTTTAG	573-591nts (RNA2)
Frag. #4 – Reverse	GAGGATCCTGGCAATTCGGCAAAGAGTGC	683-703nts (RNA2)
Frag. #5 – Forward	GAGGATCCTGATAGAAACGTTGATCTT	1987-2005nts (RNA2)
Frag. #5 – Reverse	GAGGATCCCTTGAAGTCTGAGATCATA	2128-2146nts (RNA2)
Frag. #6 – Forward	GAGGATCCTTAGTGAGTGGAACGGGAC	2802-2820nts (RNA2)
Frag. #6 – Reverse	GAGGATCCCTGACTTTGACCAGCAAGCA	2928-2935nts (RNA2)
Frag. #7 – Forward	GAGGATCCAGAAGAAATTGAGATTGGT	3127-3145nts (RNA2)
Frag. #7 – Reverse	GAGGATCCACCACTATGCAATCCATGG	3223-3241nts (RNA2)
Frag. #8 – Forward	GAGGATCCTTTAGCTTTTATGGTAGAA	3527-3545nts (RNA2)
Frag. #8 – Reverse	GAGGATCCCAGTAAAAAGAAAGGAAAA	3742-3760nts (RNA2)
C. RT-PCR Primers		
GFLV-Forward	GAGGATCCTTAGTGAGTGGAACGGGAC	2802-2820nts (RNA2)
GFLV-Reverse	GAGGATCCACCACTATGCAATCCATGG	3223-3241nts (RNA2)
NptII-Forward	CTTGGGTGGAGAGGCTATTCG	n/a
NptII-Reverse	CGTCGCTTGGTCGGTCATTT	n/a
Rbc1-Forward	TACTTGAACGCTACTGCAG	n/a
Rbc1-Reverse	CTGCATGCATTGCACGGTG	n/a

If needed, a similar approach was used with other primers with differing restriction sites (Table 3.2b) until desired combinations of the initial fragments were obtained (Cloning

Phase II). Each of these resulting concatenate fragment combinations was then cloned into pCR4®-TOPO® (Invitrogen, Carlsbad, CA). Recombinant TOPO plasmids were extracted using the Promega Wizard® *Plus* SV Minipreps DNA Purification System and sequenced at the DNA sequencing and genotyping Life Sciences Core Laboratories Center at Cornell University. Sequences were analyzed using Clustal W and SeaView.

Concatenated fragments in TOPO plasmids were digested with BamHI and cloned into pEPT8, a plant expression vector with a double *Cauliflower mosaic virus* (CaMV) 35S promoter and a CaMV 35S terminator (Ling et al. 1997). Each construct was digested with HindIII for subsequent cloning into the binary vector pGA482G which carries the selectable marker gene neomycin phosphotransferase II (*nptII*) (Pang et al. 2000). The resulting purified plasmid was sequenced to verify its conformity using FinchTV, Clustal X, and SeaView and then transformed into *Agrobacterium tumefaciens* strain C58Z707 via electroporation at 25 µFD, 2.5 kV, and 200 Ω in a 0.1 cm cuvette using a Bio-Rad Gene Pulser^{TM}.

The gene encoding enhanced green fluorescence protein (*eGFP*) (Yang et al. 1996) was cut out of the pTRL2-eGFP plasmid using NcoI and BamHI restriction sites, cloned into pEPT8 and then into pGA482G using BglII.

In addition, two constructs, FL-CP and FL-CP(u), which had been previously cloned into pGA482G and transformed into *Agrobacterium tumefaciens* C58Z707 as described above, were used in subsequent testing. These two constructs consist of the full-length coat protein gene of GFLV isolate Cf57 in sense-translatable and sense-untranslatable forms, respectively (Xue et al. 1999).

Transient assay system

Plant material

N. benthamiana seedlings were grown under greenhouse conditions (16 hrs of light per day at ~75°C) until the four to six leaf stage for infiltration with *A. tumefaciens*. Leaves from greenhouse-grown *N. benthamiana* were also used for *A. tumefaciens*-mediated transformation.

Bacterial preparation and agroinfiltration

A. tumefaciens transformants with plasmids containing the constructs of interest or *eGFP* were grown on Luria broth supplemented with gentamycin (50 µg/mL) and prepared according to the specifications of Dinesh-Kumar et al. (2003) for infiltration of *N. benthamiana*. Infiltration was carried out using a needle-less syringe in two lower true leaves per plant, one of which received the control treatment with eGFP and the other of which received the construct of interest (Figure 3.1).

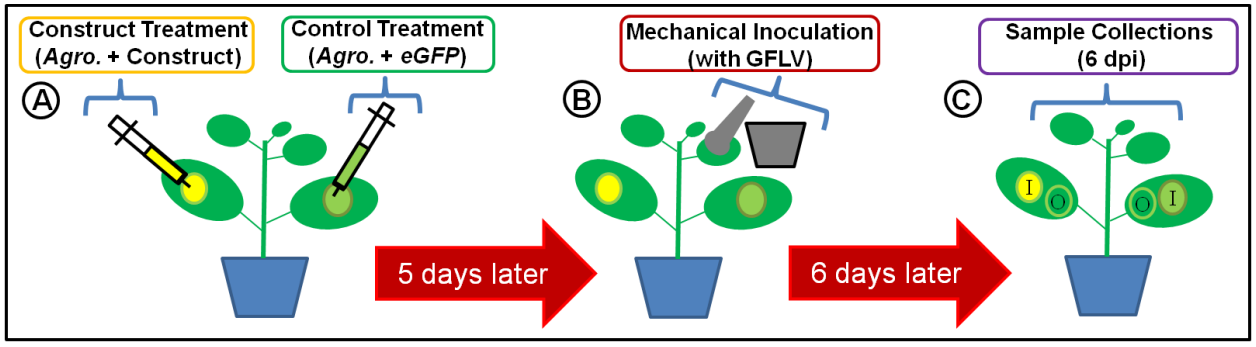

Figure 3.1. Pictorial outline of transient assay methods showing: (A) agroinfiltrations, (B) mechanical inoculations with GFLV, and (C) sample collections. Two leaves were agroinfiltrated per plant with one receiving the construct of interest [construct leaf] and the other receiving the *eGFP* control [control leaf]. Sample collections were carried out at 6 dpi with two leaf disks collected per leaf: one from inside (I) the agroinfiltrated zone and one from outside (O) the infiltrated zone as shown.

Other plants agroinfiltrated with eGFP on both lower leaves were also used for comparisons between plants. Agroinfiltration experiments were repeated at least three times.

Mechanical inoculations with GFLV-F13 or GHu

Five days after lower leaves were agroinfiltrated, upper leaves of *N. benthamiana* plants were mechanically inoculated with GFLV strain F13 (Vuittenez et al. 1964) or GHu (Huss et al. 1989) using 1:50 dilutions of crude extracts of infected *N. benthamiana* leaves prepared in 50 mM phosphate inoculation buffer pH 7.0. Mechanical inoculations took place on upper leaves to prevent damage to the agroinfiltrated leaves via the inoculation technique (Figure 3.1). GFLV-F13 causes a symptomless systemic infection in *N. benthamiana* while GFLV-GHu induces mosaic symptoms that fade away at 10-14 days post-inoculation (Huss et al. 1989).

Monitoring of GFLV infection and sample collections

Six days after *N. benthamiana* were mechanically inoculated with GFLV, leaf samples were collected with a number 11 cork borer for subsequent testing. Samples consisted of four leaf disks per plant (~50 mg each) with two discs taken per leaf: one within the agroinfiltrated zone and one outside the agroinfiltrated zone (Figure 3.1). In addition, the remainder of each of the agroinfiltrated leaves (minus the two leaf disks) was saved for subsequent GFLV detection by ELISA along with a single top leaf from each plant used to verify systemic GFLV infection by ELISA. Thirteen days post-GFLV

infection an additional leaf sample consisting of a single top leaf was collected for subsequent ELISA testing. Alternatively, additional plants were used in some experiments to allow for the entire lower leaf to be taken for subsequent testing without removal of leaf disks. Following weighing of each sample, all samples collected were immediately frozen at -80°C until testing could be performed.

ELISA testing

Crude leaf extracts of each sample were tested by double antibody sandwich (DAS) enzyme linked immunosorbent assay (ELISA) with specific antibodies (Bioreba, Reinach, Switzerland). Leaves were ground in 200 mM Tris-HCl pH 8.2, 140 mM NaCl, 2% polyvinylpyrrolidone 40, and 0.05% Tween 20 at a 1:10 ratio (w/v) using a semi-automated ball-bearing HOMEX tissue homogenizer (Bioreba, Reinach, Switzerland). Substrate hydrolysis was recorded at 405 nm with an absorbance BioTek® ELx808TM microplate reader (BioTek, Winooski, VT). Samples were considered positive if their optical density (OD 405 nm) readings were at least twice those of healthy controls. Expression of *nptII* was also monitored by DAS-ELISA using specific antibodies (Agdia, Inc., Elkhart, Indiana). Interpretation of absorbance value data was identical as for GFLV ELISA.

Total RNA extraction and RT-PCR detection of RNA abundance

Total RNA was extracted from *N. benthamiana* leaf disk samples using the E.Z.N.A® Plant RNA Kit (Omega Bio-Tek, Norcross, GA). RT-PCR was carried out using the QIAgen OneStep RT-PCR Kit (Qiagen, Valencia, CA) and 10 pmol of primers

(Table 3.2c) in a 20 µl final volume according to the manufacturer's protocol. Initial quantities of total RNA were standardized using the NanoDrop ND-1000 Spectrophotometer. Specific primers were used to check relative virus RNA abundance, construct transcript abundance, as well as *nptII* transcripts. *Rbc1* (Ribulose 1,5-biphosphate carboxylase) was used as an internal control. RT-PCR used a 30 min reverse transcription step at 42°C, with a 15 min heating step at 95°C followed by 26 to 34 cycles of 1 min melting at 94°C, 1 min annealing at 60°C, and 1 min elongation at 72°C with a final extension of 10 min at 72°C. The reaction products were resolved by electrophoresis in 1.5% agarose gels in 90 mM Tris–borate, 2 mM EDTA, pH 8.0, stained with ethidium bromide and subsequently visualized under UV light. A 100 bp DNA Ladder (New England Biolabs, Ipswich, MA) was used as a size standard.

N. benthamiana **stable transformation**

N. benthamiana were transformed with GFLV constructs of interest using *Agrobacterium tumefaciens*-mediated transformation according to methods adapted from Horsch et al. (1988). Regenerate T_0 *N. benthamiana* plants were selected with 100 µg/mL kanamycin.

Evaluation of GFLV resistance in transgenic *N. benthamiana*.

At the ~6 leaf stage, transgenic *N. benthamiana* plants were mechanically inoculated with GFLV-F13 or GFLV-GHu using a 1:200 dilution in the inoculation buffer described previously. Upper-fully expanded leaf samples were collected from *N. benthamiana* stable transformants before GFLV inoculations and at 7 and 14 days post-

infection (dpi) for ELISA testing using GFLV specific antibodies. Testing was performed for nptII and GFLV on the day of mechanical inoculation and 7 dpi, while only GFLV was tested at 14 dpi.

RESULTS

Conserved GFLV genomic regions and engineering of concatenate constructs

Eight conserved regions matching the selection criteria, i.e. stretches of at least 25 nts in length with 95% conservation of at least 85% of the nucleotide positions, were identified by alignments of GFLV RNA1 and RNA2 sequences (Figure 3.2).

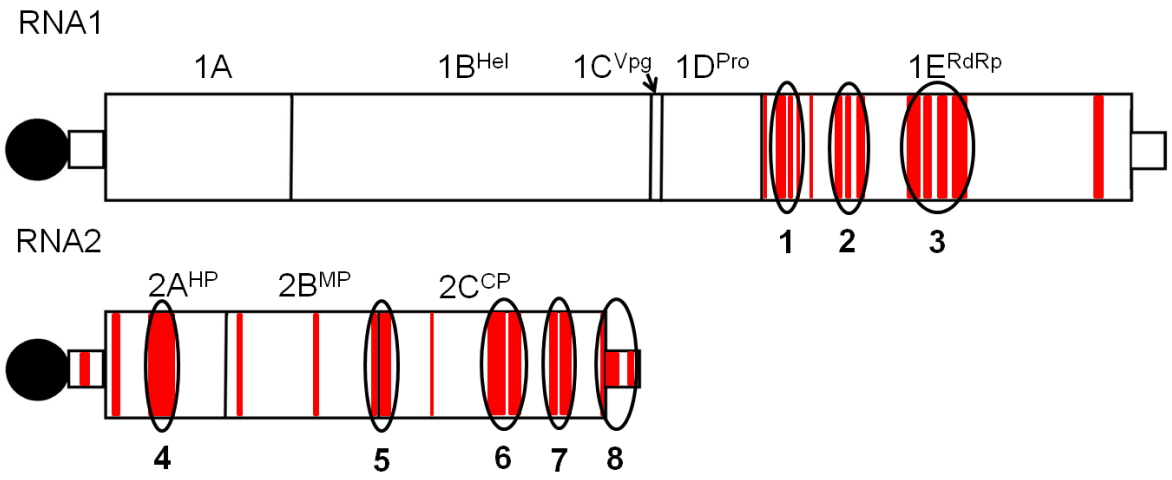

Figure 3.2. Location of conserved regions on the two genomic RNAs of GFLV strain F13. The large boxes represent open reading frames and small boxes at the 5' and 3' of each genomic RNA represent untranslated regions. The filled-in black circles at the 5' end of the genomic RNA represent the VPg. Proteins obtained after proteolytic cleavage are indicated on top. RNA1 codes for proteins 1A (unknown function), 1B (a putative helicase), $1C^{VPg}$ (the viral protein genome-linked), $1D^{Pro}$ (proteinase) and $1E^{Pol}$ (RNA-dependent RNA polymerase). RNA2 codes for proteins $2A^{HP}$ (a homing protein indispensable for RNA2 replication), $2B^{MP}$ (movement protein) and $2C^{CP}$ (coat protein). Conserved regions identified from the sequence analysis are highlighted in red. The black circled sequences indicate the regions selected for construct development.

These conserved regions were designated with numbers one through eight (denoted in Figure 3.2 as numbered circles). Three of these conserved regions (numbered "1", "2", and "3") were within the 5' half of the RNA1-encoded RNA-dependent RNA polymerase gene ($1E^{Pol}$) and five (numbered "4", "5", "6", "7", and "8" respectively) were within RNA2, including one within the homing protein gene ($2A^{HP}$), one consisting of a portion spanning the movement protein gene ($2B^{MP}$) and the coat protein gene ($2C^{CP}$), two entirely within gene $2C^{CP}$, and one including the 3' portion of gene $2C^{CP}$ with some of the 3' untranslated region (3' UTR) of RNA2 (Figure 3.2). After identification, the eight conserved regions were cloned and ligated into concatenate constructs which consisted of at least three of the cloned conserved regions. The following fragments were constructed: 1+2+3, 1+6+8, 2+4+5, 3+7+5, 4+6+3, 5+8+2, 6+7+8, 7+1+4, and 3+7+5+1+6+8, where "1+2+3" indicates that cloned region "1" (Table 3.3) was ligated to cloned regions "2" and "3" respectively, and "1+6+8" indicates that cloned region "1" was ligated to cloned regions "6" and "8", etc.

Table 3.3. Conserved GFLV regions #1-8 with their sizes and locations versus the GFLV-F13 genome.

Cloned Region	Location on RNA1 or RNA2	Total Length	Gene(s) Included	Conserved Regions Included
1	4743-4935 nts (RNA1)	193 nts	RdRp ($1E^{Pol}$)	4743-4807 nts (65 nts), 4851-4875 nts (25 nts), and 4903-4935 nts (33 nts)
2	5300-5483 nts (RNA1)	184 nts	RdRp ($1E^{Pol}$)	5300-5342 nts (43 nts), 5368-5393 nts (26 nts), and 5431-5483 nts (53 nts)
3	5746-6073 nts (RNA1)	328 nts	RdRp ($1E^{Pol}$)	5746-5813 nts (68 nts), 5824-5855 (32 nts), 5868-5920 nts (43 nts), and 6001-6073 nts (73 nts)
4	582-700 nts (RNA2)	119 nts	Homing Protein ($2A^{HP}$)	582-700 nts (119 nts)
5	1991-2137 nts (RNA2)	147 nts	Movement ($2B^{MP}$) and Coat Protein ($2C^{CP}$)	1991-2021 nts (31 nts) and 2088-2137 nts (50 nts)
6	2811-2935 nts (RNA2)	125 nts	CP ($2C^{CP}$)	2811-2874 nts (64 nts) and 2904-2935 nts (32 nts)
7	3132-3232 nts (RNA2)	101 nts	CP ($2C^{CP}$)	3132-3174 nts (33 nts) and 3207-3232 nts (26 nts)
8	3531-3757 nts (RNA2)	227 nts	CP ($2C^{CP}$) and 3' UTR	3531-3588 nts (58 nts) and 3692-3757 nts (67nts)

Most concatenates were generated with fragments from different GFLV-encoded genes rather than from within a single gene. For example, constructs consisted of at least three cloned regions from multiple genes like F-s and F-a "1+6+8" ($1E^{Pol}$ and $2C^{CP}$), E-s and E-a "2+4+5" ($1E^{Pol}$, $2A^{HP}$ and $2B^{MP}$), H-s and H-a "3+7+5" ($1E^{Pol}$, $2B^{MP}$ and $2C^{CP}$), D-s "3+7+5+1+6+8" ($1E^{Pol}$, $2B^{MP}$, $2C^{CP}$ and 3' UTR), C-s and C-s "4+6+3" ($1E^{Pol}$, $2A^{HP}$ and $2C^{CP}$), A-s and A-a "5+8+2" ($1E^{Pol}$, $2B^{MP}$, $2C^{CP}$ and 3'UTR), and B-s "7+1+4" ($1E^{Pol}$, $2A^{HP}$ and $2C^{CP}$). A few constructs originated all from within the same gene as in the case of I-s and I-a "1+2+3" ($1E^{Pol}$) and G-s and G-a "6+7+8" ($2C^{CP}$).

These fragments were cloned into pGA482G and transformed into *A. tumefaciens* for plant transformation. Sense and antisense versions (relative to original coding orientation) of each concatenated fragment combination were cloned, with the exception of 7+1+4 and 3+7+5+1+6+8 which were only cloned in sense orientation. For ease of reference, the combinations listed above were given an alphabetic designation (Table 3.4), where "-s" and "-a" refer to sense and antisense orientation, respectively.

Table 3.4. Alphabetic designation and description of concatenated constructs designed from GFLV RNA1 and RNA2 sequence as well as other constructs used in this study.

Concatenated GFLV and Other Constructs	Sense (-s) or Antisense (-a) Orientation	Alphabetic Designation
5+8+2	sense	A-s
5+8+2	antisense	A-a
7+1+4	sense	B-s
4+6+3	sense	C-s
4+6+3	antisense	C-a
3+7+5+1+6+8	sense	D-s
2+4+5	sense	E-s
2+4+5	antisense	E-a
1+6+8	sense	F-s
1+6+8	antisense	F-a
6+7+8	sense	G-s
6+7+8	antisense	G-a
3+7+5	sense	H-s
3+7+5	antisense	H-a
1+2+3	sense	I-s
1+2+3	antisense	I-a
Full length $2C^{CP}$	*sense-translatable*	FL-CP
Full length $2C^{CP}$	*sense-untranslatable*	FL-CP(u)
Enhanced Green Fluorescence Protein	*sense*-translatable	eGFP

Resistance Evaluation in Transient Assay System

Based upon preliminary results (data not shown), in order to maximize construct expression at the time of GFLV inoculation and allow for detectable systemic spread of GFLV before sample collection, respectively, agroinfiltration took place five days prior to GFLV mechanical inoculations and sample collection took place six days post-inoculation (Figure 3.1). Preliminary results also emphasized the importance of treating control plants/leaves with *A. tumefaciens* rather than infiltration buffer or no treatment alone. Presumably, systemic host defenses triggered by agroinfiltration can reduce relative virus titers (Pruss et al. 2008), making the use of *A. tumefaciens* control treatments (such as *eGFP* in our case) essential for data interpretations. Additionally, differences in virus titer between different portions of the plant, specifically higher virus titers in upper leaves relative to lower leaves, made it essential to choose adjacent lower leaves of comparable sizes and relative positions for comparisons.

Using these leaves for our analysis, *nptII* expression was shown in agroinfiltrated tissues by DAS-ELISA testing (OD 450 nm). Also, the presence of construct (Figure 3.3) and *nptII* transcripts (data not shown) was detected by RT-PCR with greatest relative abundance inside the agroinfiltrated zones. These results indicated transcript expression and accumulation from the binary vectors used for agroinfiltration experiments.

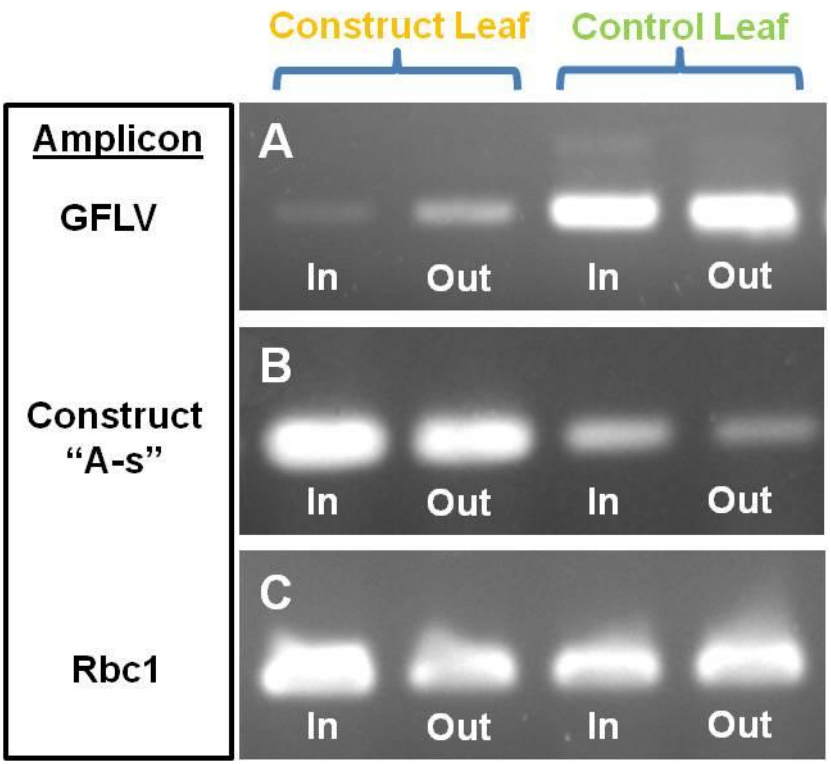

Figure 3.3. Representative results from semi-quantitative RT-PCR showing: (A) Lower relative GFLV RNA2 abundance in a leaf agroinfiltrated with construct A-s versus a control-infiltrated leaf at six days post-inoculation, (B) Relative construct "A-s" transcript in a construct-infiltrated leaf versus a control-infiltrated leaf, (C) *Rbc1* (Ribulose 1,5-biphosphate carboxylase gene) internal RT-PCR control. Two leaves were agroinfiltrated per plant with one leaf receiving the construct treatment and the other leaf receiving the control (*eGFP*) treatment. Results shown are from a single plant that received the construct "A-s" treatment. The sampling locations within the two leaves are shown with "In" for samples within the infiltrated zone and "Out" for samples taken from outside the infiltrated zone. Primers used to amplify construct "A-s" amplicon were: Frag. #8 – Forward & Frag. #2 – Reverse (See Table 3.2).

DAS-ELISA results for GFLV suggested relatively reduced levels of virus accumulation in agroinfiltrated leaves receiving the anti-GFLV construct versus those agroinfiltrated with *A. tumefaciens* containing an eGFP construct at six days post-inoculation. The average relative virus titers for ten of the constructs tested in four experiments is depicted in Figure 3.4.

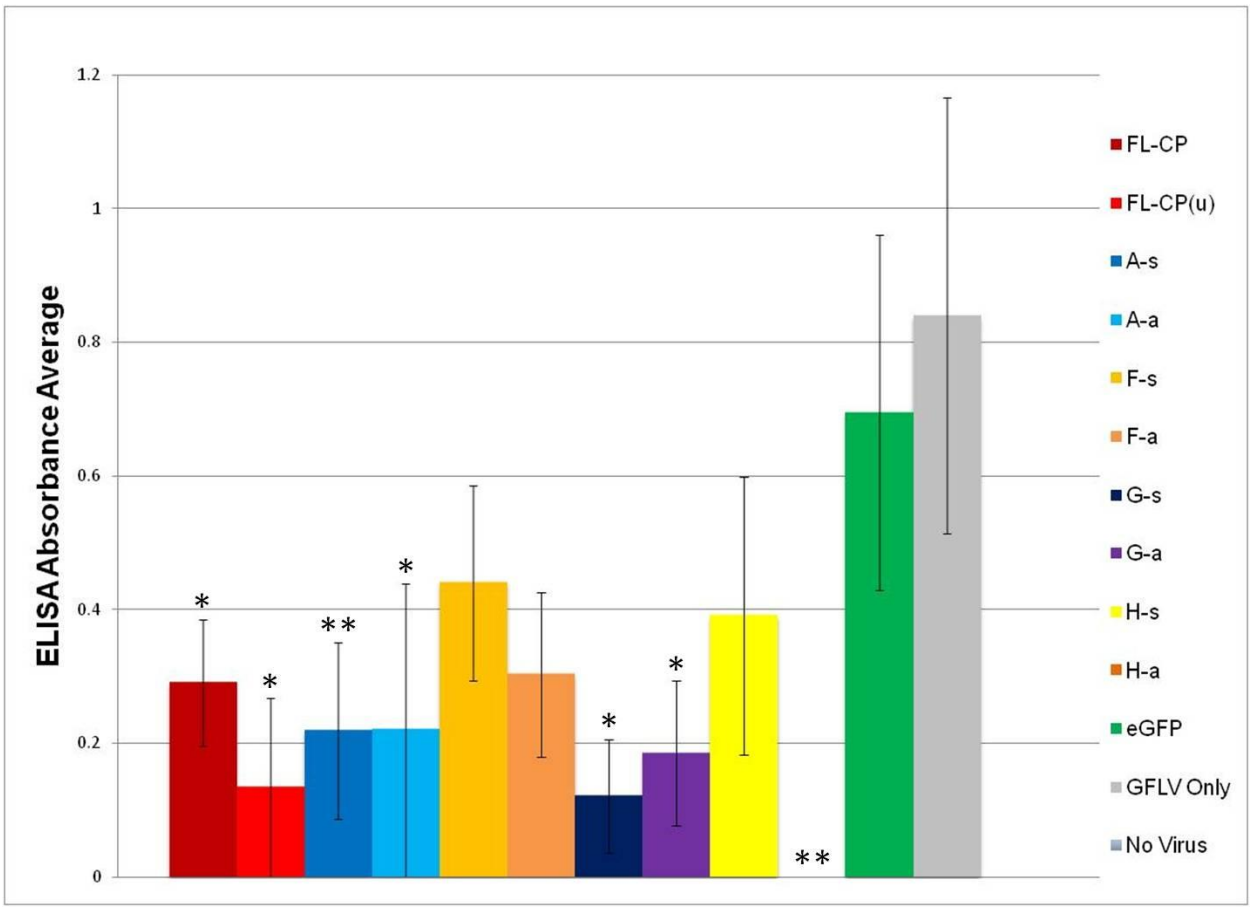

Figure 3.4. Relative GFLV titer measured by ELISA at six days post-inoculation in leaves agroinfiltrated with varied constructs. Absorbance value averages obtained across four experiments for 10 of the constructs [A-s, A-a, F-s, F-a, G-s, G-a, H-s, H-a, FL-CP and FL-CP(u)] that were examined together are shown. Data shown is from between-plant comparisons. Absorbance value averages significantly lower than the *eGFP* control (by pairwise t-test) are indicated at $p<0.05$ (**) and $p<0.10$ (*).

These results were consistent with the fact that some of the constructs suppressed virus accumulation in agroinfiltrated leaf patches in independent experiments. The results of the transient assay experiments indicated some of the newly designed constructs including A-s, A-a, G-a, G-s, H-a, I-a, and construct FL-CP(u) were better than the other constructs at reducing virus titers, while constructs E-a, F-a, F-s, and H-s performed poorly and were not significantly different than the *Agrobacterium* (*eGFP*) control treatment (in terms of relative virus titer) in most experiments (Figure 3.4, data not

shown). Other constructs, including B-s, C-s, C-a, D-s, E-s, I-s, and FL-CP, generally performed intermediately between these two groups (Figure 3.4, data not shown). As expected, the plants that were not infiltrated with *A. tumefaciens*, but infected with GFLV, had the highest virus titers in all experiments. The next highest relative virus titers were observed in leaves receiving the eGFP control treatment, as expected. In contrast, several constructs showed relatively lower virus titers versus control treatments, while one construct (H-a) unexpectedly showed no detectable virus in the construct infiltrated leaves after GFLV inoculation in any of the plants in all four experiments.

Of note, transient assay results from some constructs consisting of the same concatenated fragments, but in different orientations, such as H-s and H-a, substantially differed in their suppression of virus accumulation, while other concatenate construct pairs such as G-s & G-a and A-s & A-a did not (Figure 3.4).

Semi-quantitative RT-PCR on total RNA extracted from leaf disks both inside and outside the agroinfiltrated zones indicated reduced GFLV RNA2 abundance in leaves that received GFLV-derived constructs as compared to eGFP-infiltrated leaves from the same plant (Figure 3.3, other data not shown). These results, indicating reduced accumulation of viral RNAs in agroinfiltrated leaf patches, confirmed the trend observed from the DAS-ELISA testing.

It should be noted that primers used to detect GFLV virus titers were designed to bind within GFLV RNA2 in such a way that they do not yield a product in RT-PCR from the transgene constructs, allowing for specific detection of viral transcripts only. Furthermore, systemic infection of mechanically inoculated *N. benthamiana* was

observed by 6 days post-inoculation with GFLV in most cases and by 13 dpi in all mechanically-inoculated plants, as shown by DAS-ELISA and RT-PCR (data not shown).

Data from semi-quantitative RT-PCR also indicated the presence of construct mRNA outside of the infiltrated leaves (Figure 3.3), though at relatively lower amounts versus those observed within the infiltrated zones, indicating that while systemic movement outside the agroinfiltrated zone did occur, the extent was limited. Probably due to this systemic movement of construct RNA and presumably siRNA signals within single plants (Dunoyer et al. 2010, Kehr and Buhtz 2008, Molnar et al. 2010), the within-plant comparisons (*eGFP* control agroinfiltrated leaves versus construct agroinfiltrated leaves) were somewhat less consistent than between-plant comparisons (plants where both lower leaves where agroinfiltrated with *eGFP* versus construct treated plants) in DAS-ELISA.

Resistance evaluation in stable *N. benthamiana* transformants

ELISA testing of T_0 *N. benthamiana* plants produced following *A. tumefaciens*-mediated transformation indicated that over 90% of transformed plants were confirmed to have detectable NptII protein expression (data not shown). Transgenic T_0 plants were tested for their ability to resist GFLV infection by mechanical inoculation. Most of the T_0 transgenic plants were susceptible to infection by either GFLV-GHu or GFLV-F13 and had detectable infection at 7 dpi, with some appearing to remain uninfected through 14 dpi (data not shown). Based upon partial results from testing of the T_0 plants, constructs with relatively higher proportions of plants showing delayed infections or no

apparent infections include: D-s, G-s, G-a, and I-a (data not shown). Plants with no detectable infection were symptomless while infected plants exhibited the typical mosaic symptoms of GFLV-GHu.

Resistance was further tested in T_1 *N. benthamiana* plants derived from T_0 plants that showed a delay phenotype or no apparent infection based on DAS-ELISA results. Prior to inoculation, no evidence of seed-borne transmission of GFLV was found by ELISA in any of the T_1 plants derived from T_0 parents (data not shown). T_1 plants derived from T_0 plants transformed with constructs A-s, B-s, and D-s, were mechanically inoculated with GFLV-F13. Approximately 23.7% of the T_1 transgenic plants showed no detectable infection by GFLV-F13 at 7 dpi, with 8.8% appearing to remain uninfected through 14 dpi (Table 3.5). In general, transgenic lines from the A-s construct appeared to have a somewhat larger number of plants showing the delayed infection or resistance phenotype versus the B-s or D-s lines.

Table 3.5. Resistance to GFLV infection in T_1 transgenic *Nicotiana benthamiana* plants expressing constructs A-s, B-s and D-s for resistance to GFLV-F13. Plant reaction to virus infection was determined by ELISA testing at 7 and 14 dpi. The total number of plants tested as well as the number showing susceptible, delay, and resistant phenotypes are indicated.

GFLV Construct	Transgenic Line	Number Tested	Susceptible[1]	Delay[2]	Resistant[3]
A-s	A-41B	20	15	1	4
A-s	A-61F	20	14	5	1
A-s	A-76	18	13	1	4
A-s	A-98	10	6	2	2
B-s	B-4	20	17	2	1
B-s	B-12	13	7	3	3
B-s	B-14	20	17	2	1
B-s	B-15	20	13	7	0
D-s	D-3	20	18	2	0
D-s	D-5	20	18	2	0

[1]Susceptible indicates the number of plants infected at 7 dpi and 14 dpi.
[2]Delay indicates the number of plants infected by 14 dpi but not at 7 dpi.
[3]Resistant indicates the number of plants with no infection at 7 dpi or 14 dpi.

Testing of other constructs is underway as well as screening of T_2 plants. The fact that T_1 plants transformed with construct A-s showed a greater tendency toward delayed infection and/or no infection relative to those transformed with constructs B-s or D-s was in agreement with the results of the transient assay that indicated this construct was more capable of reducing virus titers relative to B-s or D-s.

DISCUSSION

Transgenic resistance to GFLV is highly desirable due to the destructive effects of GFLV on grapevine growth, yield, and fruit quality as well as the lack of natural resistance to this pathogen. Several attempts to develop constructs that could confer GFLV resistance in their host have been made with varying levels of success (Bardonnet et al. 1994, Gambino et al. 2005, 2010, Jardak-Jamoussi et al. 2009, Maghuly et al. 2006, Winterhagen et al. 2009, Xue et al. 1999). Most of these studies have utilized the coat protein ($2C^{CP}$) gene of the virus as the resistance construct, with this gene being cloned from a single isolate of GFLV. Evidence from transgenic papaya that express the *Papaya ringspot virus* coat protein gene suggests that resistance based on a single gene from a single viral isolate may not hold up to an encounter with a population of divergent isolates (Tennant et al. 2001) due to the specificity of the antiviral pathway of RNA silencing. RNA silencing is known to act in a sequence specific manner, and significant divergence from the transgene can allow for virus isolates to overcome transgenic resistance (Prins et al. 2008). Recent evidence examining GFLV populations in naturally infected vineyards (Oliver et al. 2010)

suggests that GFLV possesses great potential for genetic variation, which makes it doubtful that a single transgene is likely to confer adequate resistance against the variants of GFLV that naturally exist in vineyards. Therefore, we sought to develop transgenes which might confer more durable and broad-spectrum resistance by utilizing sequences within several different GFLV genes that are conserved across diverse isolates of GFLV.

Our search for conserved stretches of sequence from within the viral genome yielded numerous smaller regions that matched our search criteria (Figure 3.2). For ease of cloning and development of transgenic constructs, several of these regions which were in close proximity to one another were lumped together to yield eight larger conserved regions (Figure 3.2, Table 3.3) including portions from all four viral genes examined in our analysis, i.e. the RNA1-encoded $1E^{Pol}$ gene and the RNA2- encoded $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes. It should be acknowledged that only six RNA1 sequences were available for analysis at the time we carried out our study relative to the considerably greater amount of sequence information available for RNA2 (over 200 sequences) (Table 3.1). The 5' end of the $1E^{Pol}$ gene of GFLV is known to code for conserved protein domains that are common across the RdRp's of numerous virus species (Koonin 1991). The regions within the $1E^{Pol}$ gene of GFLV that we identified in our sequence analysis are from the 5' end of this gene, where the codons corresponding to these conserved domains are located. The RNA2 portion spanning genes $2B^{MP}$ and $2C^{CP}$ that we identified in our analysis as being within the conserved region that we cloned as fragment number 5 corresponds to the RG cleavage site (which is necessary for viral proteinase mediated cleavage of the $2B^{MP}$ and $2C^{CP}$) as

well as the coding sites for other amino acids that when changed, abolish systemic spread of the virus (Belin et al. 1999). Additionally, the conserved region that we identified in our analysis and cloned as fragment number 6 within the $2C^{CP}$ includes the nucleotides coding for amino acids within domain B, particularly those suggested to be involved in the $2B^{MP}$ and $2C^{CP}$ interactions necessary for cell-to-cell movement of GFLV (Schellenberger et al. 2010). The 3' untranslated region of RNA2, which we identified in our analysis and cloned as fragment number 8, has also been described previously as being relatively conserved among nepoviruses at the nucleotide level possibly due to interactions with protein $1E^{Pol}$ during genome replication (Serghini et al. 1990). Four of these regions that we identified were found in the $2B^{MP}$ and $2C^{CP}$, two genes which are under relatively strong negative selection pressure (Oliver et al. 2010). The strong negative selection at work on these two genes – which are essential for cell-to-cell movement and specific transmission by the nematode vector – may account for their high conservation.

It was believed that by generating constructs targeting multiple regions of the viral genome, any transgenic resistance would be more likely to act in a broad spectrum and durable manner – as a viral isolate which differed from the transgene in only one or two of these regions would still be vulnerable to transgenic silencing activated against the remaining similar region of the transgene.

Relative to perennial plants, model herbaceous hosts such *Nicotiana benthamiana* can be useful for assessing the effectiveness of transgene constructs, among other roles (Goodin et al. 2008). GFLV induces a systemic infection in *N. benthamiana,* and *N. benthamiana* transformed with the GFLV coat protein gene have

been used to evaluate transgene effectiveness against this pathogen (Bardonnet et al. 1994). Though GFLV and other nepoviruses have been reported to be seed transmissible in some model hosts (Cory and Hewitt 1968, Brückbauer and Rüdel 1962, Dias 1963), our results did not indicate that GFLV is capable of being seed transmitted in *N. benthamiana*, further validating the use of this host in resistance screening assays. While the use of stable transformants of model hosts can provide evidence on the effectiveness of transgenes in a relatively timely manner as compared to their perennial counterparts, this approach is still burdened by the necessity to generate and test numerous transgenic lines to evaluate the resistance conferred by the transgene. An approach that does not have these limitations would be desirable to streamline the identification of constructs with high potential for virus suppression. For this reason, in this study we developed a transient assay system utilizing agroinfiltration.

Agroinfiltration is an extremely useful technique to express novel proteins or RNAs in a transient manner in a local leaf area of transformed cells. Among the numerous applications of this technique (Vaghchhipawala et al. 2011), localized silencing following the infiltration of transgenes designed to silence specific proteins within the plant leaf has been observed (Johansen and Carrington 2001, Kościańska et al. 2005). Additionally, agroinfiltration of constructs designed to produce siRNA have been shown to interfere with subsequent viral infection of infiltrated plants with *Pepper mild mottle virus*, *Tobacco mosaic virus*, *Tobacco etch virus*, and *Alfalfa mosaic virus* (Tenllado et al. 2004, Tenllado and Díaz-Ruíz 2001, Zhao et al. 2006), and has been used to test the relative efficiency of constructs at inducing RNA silencing (Xiaoping et al. 2007).

A. *tumefaciens* strain C58Z707 transformed with our construct of interest was used in both the transient assay and the stable transformation of *N. benthamiana* so no additional cloning work was necessary to test constructs via both methods. The transient assay system we developed (Figure 3.1) was used to examine the effectiveness of each of the constructs tested in reducing GFLV titers in a localized area of the infiltrated leaves, and differences between constructs were observed as described. *N. benthamiana* transformed in a stable manner with a few constructs generated to evaluate the effectiveness of the transient assay as a predictor of antiviral transgene effectiveness appeared to support the conclusions regarding constructs from the transient assay results (Table 3.5). This indicates the usefulness of this transient assay system to evaluate the effectiveness of anti-GFLV constructs. Based on this finding, it would be interesting to use this transient assays system to test antiviral resistance constructs targeted towards other viruses of which *N. benthamiana* is a systemic host to determine its usefulness in developing and testing other antiviral constructs. However, it should be noted that if a similar transient system were applied to other virus-host systems, differences in rates of virus spread may make it necessary to alter the timetable used in this study. In addition, other delivery methods for viruses such as vector-mediated delivery or agroinoculation might be used, however, these are likely to have an impact on the rate of virus spread as older or more rapidly growing plants (such as during summer months and longer daylight hours) appeared to show faster viral systemic spread. In any case, variations from experiment to experiment can significantly affect reproducibility of results, so minimizing these variations is key.

As described previously, within plant comparisons were somewhat less consistent than between plant comparisons suggesting that between plant comparisons may be a more useful comparison for the purposes of determining the antiviral activity of individual constructs via the transient assay.

Curiously, transient assay results from some constructs consisting of the same concatenated fragments but in different orientations, such as H-s and H-a, demonstrated differential potential at suppressing virus accumulation, while other concatenate constructs such as G-s & G-a and A-s & A-a performed comparably (Figure 3.4). The reasons for these differences are unclear but could indicate that the orientation of some constructs may have a greater effect on their ability to trigger antiviral activity than others.

Overall, the transient assay allowed for relatively faster testing of transgenic constructs (~3 weeks) as compared to at least 3 months for *N. benthamiana* testing and 3 years for transgenic grapes in naturally infected vineyards. Potentially this system is high-throughput, in that fewer additional plants would be needed to test additional constructs, and very versatile with respect to the ease of using different GFLV isolates to challenge constructs. Challenging with divergent GFLV isolates would be readily straightforward using this system, whereas in grapevines which have to be infected using nematode vectors or graft inoculations, challenging with diverse isolates can be quite cumbersome. Finally, use of such a transient system can allow for the least promising constructs to be weeded out before tissue culture begins, potentially saving a great deal of time, money and effort that would otherwise be spent screening numerous transgenic lines for resistant individuals.

The relative percentage of stable transformants showing no infection in our study (Table 3.5) was in the range (5-20%) of those reported in other transformations with sense and antisense antiviral constructs (Prins et al. 2008) and also similar to some lines from other studies where transformants were generated using inverted repeat constructs (Jardak-Jamoussi et al. 2009). Since previous studies have indicated that, following agroinfiltration, siRNAs may be produced at a substantially higher concentration within the agroinfiltrated zone than would be normally produced during systemic silencing in a stable transformant (Kościańska et al. 2005), it is possible that reconfiguring the constructs into inverted repeat or hairpin forms that favor increased siRNA production (Prins et al. 2008) would increase the relative proportion of resistant stable transformants, and may also positively affect the results of the transient assay.

CONCLUSIONS

Overall, we identified eight conserved regions within the GFLV genome that may ultimately be useful in developing transgenic resistance to GFLV in grapevines with the potential to be both durable and broad-spectrum. These regions were developed into constructs which are capable of being used in transformation of grapevines or model hosts or in transient agroexpression assays. We developed a transient agroinfiltration assay system which indicated that some of these constructs are capable of interfering with virus multiplication in infiltrated zones in *N. benthamiana*. The results of our transient assay appeared to have some support from our tests of a large number of stable *N. benthamiana* transformants for virus resistance. This transient assay system

may be useful for testing transgenic constructs in an efficient manner that is both high-throughput and fast.

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CHAPTER 4

Virus-Resistant Transgenic Horticultural Crops: Safety Issues and Lessons from Risk Assessment Studies[3]

INTRODUCTION

Horticultural crops were the first transgenic crops commercialized in the United States in the mid 1990s. Among the first of these were virus-resistant transgenic summer squash (*Cucurbita pepo* ssp. *ovifera* var. *ovifera* L.). The first transgenic squash cultivars, deregulated in 1994, were resistant to *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV) (Fuchs and Gonsalves 1995, 2007, Tricoli et al. 1995). Other transgenic summer squash cultivars resistant to ZYMV, WMV, and *Cucumber mosaic virus* (CMV) were released in 1996 (Fuchs et al. 1998, Fuchs and Gonsalves 2007, Tricoli et al. 1995) followed by papaya (*Carica papaya* L.) genetically modified for resistance to *Papaya ringspot virus* (PRSV) in 1998 (Ferreira et al. 2002, Fuchs and Gonsalves 2007, Gonsalves 1998). To date, virus-resistant summer squash and papaya are the only transgenic horticultural crops that are commercially released in the United States, along with *Bt* sweet corn (*Zea mays* L.) (James 2008). Potato (*Solanum tuberosum* L.) resistant to *Potato virus Y* (PVY), *Potato leafroll virus* (PLRV) and the Colorado potato beetle were released in 1998, but were withdrawn from the market

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almost immediately thereafter due to anti-biotechnology campaigns and international trade barriers (Kaniewski and Thomas 2004). In the People's Republic of China, transgenic tomato (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum annuum* L.) resistant to CMV and papaya resistant to PRSV have also been released (Stone 2008).

The virus-resistant transgenic horticultural crops commercially available have been developed by applying the concept of pathogen-derived resistance (Sanford and Johnston 1985). Pathogen-derived resistance refers to the use of a pathogen's own genes to confer resistance in a host to that pathogen. In the case of the horticultural crops released to date in the United States, engineered virus resistance has been achieved through expression of viral coat protein genes (Gonsalves 1998, Tricoli et al. 1995).

The development and release of transgenic horticultural crops, in particular those engineered for virus resistance, have raised potential safety issues regarding their impact on the environment and human health (Fuchs and Gonsalves 2007, Hammond et al. 1999, Robinson 1996, Tepfer 2002). Similarly, concerns have been expressed over the release of horticultural crops expressing *cry* toxin genes from the bacterium, *Bacillus thuringiensis* (Bt) (Romeis et al. 2008, Shelton et al. 2002). Over the years, a significant amount of research has been done to address safety issues and examine potential risks. In this chapter, we provide a synopsis of transgenic horticultural crops, in particular virus-resistant transgenic crops with the major emphasis on those that are currently available in commerce. We also examine the mechanisms underlying engineered virus resistance and discuss potential safety issues with this technology. We then examine risk assessment research by focusing on commercial crops for which

data from realistic field studies are available, thereby using each released crop as a case study in our examination of the safety issues associated with transgenic horticultural crops. Finally, we summarize lessons from risk assessment research and evaluate whether safety issues account for the limited number of horticultural transgenic crops released to date.

Pathogen-Derived Resistance and Engineered Resistance to Viruses in Plants

The first report on engineered resistance to viruses in plants was published in 1986 (Abel et al. 1986). These researchers noted that tobacco plants expressing the coat protein gene of *Tobacco mosaic virus* (TMV) exhibited delayed infection following mechanical inoculation with TMV. This observation conformed to the concept of pathogen-derived resistance that had been postulated earlier (Sanford and Johnston 1985). Following the initial breakthrough by Abel et al. (1986), viral coat protein genes from various viruses were introduced into numerous economically important crop species in hopes of achieving resistance (Fuchs 2008). It was initially believed that resistance was provided by the viral protein itself via a mechanism involving excess plant-expressed coat protein that interfered with the uncoating step in viral replication (Lindbo and Dougherty 2005). However, it soon became apparent that resistance could be achieved in transgenic plants producing low or undetectable levels of coat protein (Lindbo et al. 1993). Further observations indicated that the mechanism involved degradation of the transgene-derived messenger ribonucleic acid (mRNA) into small fragments in a sequence-specific manner (Dietzen and Mitter 2006, Eamens et al. 2008, Lin et al. 2007, Voinnet 2005).

RNA Silencing and Engineered Resistance to Viruses in Plants

Extensive research has shown that engineered virus resistance in transgenic plants works primarily through the antiviral pathways of the mechanism known as RNA silencing. This mechanism regulates the expression of genes at the RNA level following RNA transcription by the host plant. RNA silencing is triggered by the replication of genomic viral RNA within the host cell (Eamens et al. 2008, Lindbo and Dougherty 2005, Voinnet 2008). Following entry into the cell, most viruses form double-stranded RNA (dsRNA) intermediates during their replication. These dsRNA are recognized by host RNase III Dicer-like enzymes and cleaved into short fragments called small interfering (si) RNA (~21 nts). These fragments then associate with the RNA-induced silencing complex (RISC), which proceeds to target homologous RNA sequences within the cell. Once identified by the RNA silencing machinery, these target RNA sequences are similarly cleaved (Eamens et al. 2008, Lindbo and Dougherty 2005, Voinnet 2005, 2008). In this way, the host cell severely limits or entirely prevents viral replication, resulting in resistance. Though originally identified in plant–virus interaction studies, RNA silencing has been shown to be present in a wide range of organisms including humans, plants, nematodes, and fruit flies and is believed to function as a sort of primitive immune system (Prins et al. 2008).

The activation of these cellular viral defenses within host plants is believed to explain the long-recognized phenomenon of recovery from viral symptoms seen in the upper leaves of some infected plants, as it has been shown that a silencing signal can move systemically within the plant, activating silencing ahead of the viral replication advance (Kehr and Buhtz 2008).

Lending further support to the importance of the RNA silencing system in plants is the discovery that most virus families encode one or more proteins that suppress silencing (Díaz-Pendón and Ding 2008, Ding and Voinnet 2007, Eamens et al. 2008, Moissiard and Voinnet 2004, Voinnet 2008). Viral suppressors have been shown to interact in multiple ways with the RNA silencing machinery to prevent effective control of the expression of the viral genome within the host. One such example is the HC-Pro protein from PVY. This protein binds the siRNAs produced by the cell's RNA silencing machinery, thereby squelching the signal, which effectively suppresses RNA silencing within the host cell (Lakatos et al. 2006). This not only can allow for continued PVY replication, but has also been shown to be primarily responsible for the observed phenomenon of viral synergism where two unrelated viruses, when co-infecting the host plant, can exhibit more severe symptoms than either virus on their own. Experiments with PVY and *Potato virus X* (PVX) have shown that HC-Pro of PVY can suppress host defenses and allow for PVX, which very weakly suppresses silencing on its own, to replicate and produce much more severe symptoms than either PVX or PVY cause on their own (Tepfer 2002). The fact that RNA silencing is a nucleotide sequence-based resistance mechanism has unique implications for risk assessment and the development of new virus-resistant transgenic plants. This feature is discussed in the next section.

SAFETY ISSUES AND RISKS

Due to the expression of viral gene constructs to provide resistance to viruses in transgenic plants, there are unique safety issues associated with this technology (Fuchs and Gonsalves 2007, Hammond et al. 1999, Robinson 1996, Tepfer 2002) versus many of the other commercialized transgenic crop plants. Among these are the risks of viral recombination and transencapsidation. However, not all of the potential risks associated with this technology are unique to virus-resistant transgenic plants. Risks associated with other transgenic plant technology including gene flow to free-living relatives, allergenicity and other human health effects, breakdown of resistance, and effects on nontarget organisms also need to be assessed with regard to engineered resistance against viruses. In the following section, we examine each of these areas of concern and assess their scientific merits.

Transencapsidation

One of the concerns associated with virus-resistant transgenic crops is the potential that viral capsid proteins, when produced in a transgenic host plant, may transencapsidate the genome of a challenge virus (Fuchs and Gonsalves 2007, Hammond et al. 1999, Robinson 1996, Tepfer 2002). Since viral capsid proteins play roles in such diverse processes as movement within the host, replication, suppression of gene silencing, and vector transmission specificity (Callaway et al. 2001), it is plausible that a transencapsidated virus may have altered properties. If the coat protein of a virus vectored by aphids, for example, were transgenically produced within a host

plant, it is conceivable that an aphid non-transmissible virus could be encapsidated within the coat protein derived from the transgene. This transencapsidated virus (with the coat protein from the aphid-borne virus and the genome of the aphid non-transmissible virus) might then acquire the characteristics of an aphid-borne virus, potentially allowing it to move more expeditiously into new host plants—possibly even to plant species that the parent viruses heretofore had not come into contact with. Transencapsidation has been shown experimentally (Hammond et al. 1999). However, many of the concerns with respect to transencapsidation have been alleviated by our current understanding of RNA silencing. This is true because when RNA silencing is active (as is expected if the transgenic plants exhibit viral resistance), the expression of viral-derived proteins is regulated posttranscriptionally, with little to no detectable capsid protein produced. In the case of untranslatable coat protein transgenes, this concern is alleviated altogether. Also, since the interactions between viral proteins or between capsid proteins and viral genomes is often very specific (Callaway et al. 2001), it seems unlikely that interactions necessary for a viral coat protein to aid in the replication or movement of its corresponding viral genome would take place in coordination with genomic material or proteins from a heterologous virus. Finally, it is unclear how these risks are substantially different from the risks already present when a host plant is infected with two distinct viruses—which has been shown to lead to transencapsidation (Hammond et al. 1999) —and how transencapsidation would be any more likely to occur in the transgenic case than in the non-transgenic multiple infection scenario (Fuchs and Gonsalves 2007, Hammond et al. 1999, Robinson 1996).

Even if transencapsidation did occur, it is questionable how it could result in viruses with permanently altered properties, since it is not conceivable how the movement of a transencapsidated virus to a new host would be anything other than a so-called dead end, since (due to the lack of a coat protein from the heterologous virus in the new host) all new viruses produced in the new host plant would be encapsidated within their own capsid protein (Fuchs and Gonsalves 2007, Hammond et al. 1999, Robinson 1996, Tepfer 2002). An exception would be if the new host were transgenic and expressing the coat protein gene of a closely related heterologous virus. In any event, if problems with transencapsidation did arise, the problem could be eliminated by ending the cultivation of the transgenic crop in question (Prins et al. 2008).

Recombination

Another potential risk of virus-resistant transgenic crop plants expressing viral genes is that of recombination between viral-derived transgene mRNA and the genomic RNA of an infecting virus (Fuchs and Gonsalves 2007, Hammond et al. 1999, Robinson 1996, Tepfer 2002). It is thought that this type of recombination could potentially give rise to a new virus strain or new virus species possessing characteristics different from those of the parent viruses. Recombination may involve, for example, an incoming virus containing a viral coat protein gene whose product is defective in its ability to be vectored, and transcripts of a homologous viral transgene sequence possessing a functional copy of the coat protein gene. Recombination between these sequences may then restore vector transmissibility. Unlike the risk of transencapsidation, recombination cannot be as easily dismissed as an evolutionary dead end, since viral

progeny identical to the recombined strain could be produced in a new host (Fuchs and Gonsalves 2007, Robinson 1996, Tepfer 2002). Recombination between transcripts of a viral gene construct in a transgenic plant and an incoming virus has been shown by several groups (Fuchs 2007). If resistance were effective through RNA silencing, the occurrence of recombination is less likely unless the incoming virus were a related, but divergent, isolate of the virus from which the transgene is derived. In that case, it is conceivable that the incoming virus would replicate to the same extent as in a susceptible plant, providing opportunities for recombination. Nevertheless, it is unclear how recombination would be more likely to occur between a viral transgene mRNA and an infecting virus than between two viruses that are coinfecting the same host (a common situation) (Falk and Bruening 1994, Fuchs and Gonsalves 2007, Rubio et al. 1999, Turturo et al. 2008). It is likewise unclear how recombination is any more likely to occur in the transgenic case than in the case of cross-protection, an accepted method used in controlling viral diseases. Cross-protection relies on the use of mild virus strains to protect plants from economic damage caused by closely related severe virus strains (Fuchs et al. 1997, Lecoq 1998). Therefore, RNA molecules of distinct viral strains have ample opportunities to recombine in cross-protected plants. Though not shown to have emerged in cross-protected plants, recombinant viral strains resulting from recombination between *Arabis mosaic virus* (ArMV) and *Grapevine fanleaf virus* (GFLV) have been observed (Vigne et al. 2008). The use of recombinant mild strains of ArMV and GFLV to cross-protect against GFLV is an accepted experimental control method for GFLV (Komar et al. 2008) despite their deliberate dissemination in the environment.

Gene Flow to Free-Living Relatives

Another concern, not unique to virus-resistant transgenic crops, is the risk of transgene flow (Fuchs and Gonsalves 2007, Robinson 1996, Tepfer 2002). Transgene movement from a transgenic crop species to a free-living relative, through pollen flow, can be a significant concern. If transgenes provide a selective advantage, it is conceivable that hybrids between transgenic and free-living compatible species might acquire a fitness benefit and eventually a competitive edge over free-living plants (Ellstrand et al. 1999). In an extreme scenario, a transgenic hybrid may outcompete free-living plants and thereby lead to an elimination of entire species, land races, or varieties of non-transgenic plants. In the case of virus-resistant transgenic plants, it is postulated that a hybrid possessing a transgene conferring virus resistance might outcompete compatible free-living plants and become established in the natural environment. Though this is unlikely to be due to an increase in weediness potential of the transgenic crop itself, such a scenario may have far-reaching environmental consequences in the case of free-living species (Fuchs and Gonsalves 2007).

The likelihood of outcrossing between transgenic crop plants and free-living relatives depends on numerous factors, including pollen phenology, pollen compatibility, and spatial proximity (Ellstrand et al. 1999). Each of these factors could vary significantly between any two given transgenic crop species and environments. Therefore, evaluations of the likelihood of gene flow (and its effects) must be carried out on a case-by-case basis. Even if gene flow from a virus-resistant transgenic crop to free-living relatives could readily occur, it is not obvious what effect this would have. Gene flow from domesticated crop species developed through traditional breeding

practices has also been shown to occur (Ellstrand et al. 1999, Hoc et al. 2006, Kirkpatrick and Wilson 1988, Martínez-Castillo et al. 2007, Wilson 1990), but in the case of traditional breeding, problems arising from gene flow to free-living relatives have not been seen, and it is unclear how the effect of transgenic virus resistance genes would be substantially different from those of resistance genes derived from traditional breeding.

Effects on Nontarget Organisms

An additional concern regarding transgenic horticultural crops is their potential to have negative effects on nontarget organisms (Fuchs and Gonsalves 2007, Keese 2008), for example, organisms that are not intentionally targeted by the disease or pest management strategy. Effects on nontarget organisms can be difficult to evaluate, and even when an effect is seen, it can be difficult to assess the significance of this observation. With respect to virus-resistant transgenic crops, it is not easy to identify a mechanism which might result in an effect on nontargets, because the titer of the transgene protein product is likely many fold lower than the amount of the corresponding viral protein in a non-transgenic virus-infected plant (Tricoli 1995, Vigne et al. 2004). In addition, given the fact that the resistance mechanism is likely to be RNA silencing, in many cases little to no protein is likely produced at all, especially in the case of untranslatable transgenes. Furthermore, crop plants derived through traditional breeding practices have been shown to produce a wide range of allergens and toxins with clear effects on animals, plants, insects, and nematodes that may come into contact with these plants during their lifetime (Lemaux 2008).

Allergenicity and Human Health Effects

Another area of concern regarding transgenic horticultural crops is the possibility of the introduction of allergenic proteins into the food supply and the introduction or increase in the production of toxic compounds (Atherton 2002, Kuiper and Kleter 2003, Lack 2002, Lemaux 2008, Mills et al. 2003). Although this concern also applies to crop varieties developed using conventional breeding methods, transgenic products have received strict scrutiny presumably because of the nature of the transgene proteins. The underlying concept of safety evaluations of genetically modified foods was proposed in the early 1990s (OECD 1993) and is based on comparative analyses of the transgenic crop with the conventionally bred parent that has a history of safe use (i.e., substantial equivalence) (Constable et al. 2007). Additionally, safety testing of whole foods in animals is used to determine toxicity and allergenicity of genetically modified foods as well as toxicity testing of individual proteins. The latter tests, in combination with nutritional analysis, are regarded as more sensitive and accurate (Chassy et al. 2004, Deng et al. 2008). While postmarket monitoring of transgenic food crops provides data on patterns of human nutritional exposure and may be useful in confirming premarket risk assessment and the detection of rare unintended effects on health, the evaluation is not regarded as a component of risk assessment and is not a substitute for thorough premarket risk assessment (European Food Safety Agency 2008).

Based on this approach, a number of transgenic crops expressing protein products, such as those derived from *Bt*-derived toxins (*cry*) and marker transgenes (*npt*II, *uid*A), have been found to present little to no risk to food or feed safety (Craig et al. 2008, Fuchs et al. 1993, Fuchs and Gonsalves 2007, Gilissen et al. 1998, Ramessar

et al. 2007, Shelton et al. 2008). Further, analysis of potential pleiotropic effects on inherent plant toxins and antinutrients of transgenic plants and their progenitor cultivars (e.g., maize, rape, tomato, potato, and soybean) has shown minor to perceptible variations, albeit within the ranges documented in literature, in the contents of these compounds (Novak and Haslberger 2000). Natural biological variation, including nutrient variation, of individual plants grown under the same conditions is expected, given the influences of differences in plant development, metabolism, and biotic factors (Koenig et al. 2004, Novak and Haslberger 2000). However, the differences between the transgenic and the progenitor cultivar can also be attributed to somaclonal variation, given that the transformation of many crops, including papaya, involves an adventitious regeneration protocol, and in some cases, 2,4-dichlorophenoxyacetic acid (Cai et al. 1999, Chen et al. 2001, Fitch et al. 1990, Tennant et al. 2002), a plant growth regulator known to introduce genetic mutations, is used. Nonetheless, backcrossing to the original parental variety (and selecting progeny with appropriate traits) effectively eliminates composition alterations caused by tissue culture methods (Dahleen and Manoharan 2007). It is important to also note that variation in composition is not limited to transgenic crops generated by recombinant DNA technologies and tissue culture methods. Nutritional variation has been reported for a number of plant products derived from conventionally bred varieties, and ranges for most of the compositional variables are available in the literature (Shewfelt 1990). Variation in conventional crops is attributed to genetics as well as preharvest conditions, maturity at harvest, harvesting methods, postharvest handling, and storage conditions.

Although numerous animal studies have been conducted with transgenic crops carrying *cry* insecticidal, cowpea trypsin inhibitor, phytase, and snowdrop lectin genes (Craig et al. 2008), there are only a few published studies on the safety assessment of whole foods derived from transgenic crop plants transformed with viral coat protein genes. Presumably viral coat proteins are not regarded as potential allergens or toxins given the physicochemical and structural properties of the proteins and the low exposure levels due to low or undetectable transgene protein expression (because of RNA silencing). For transgenic viral proteins expressed in commercialized horticultural crops, sequence relatedness of 35% (or higher) or a continuous stretch of eight amino acids is not shared with known allergens (Hileman et al. 2002). Moreover, resistance to digestion under acidic conditions has not been demonstrated (Fuchs and Gonsalves 2007, Herman et al. 2006, Roberts et al. 2008). It is important to bear in mind that many of the crop plants available in commerce contain natural toxins and allergens (Lemaux 2008). Peanuts, tomatoes, soybeans, kiwi, and potatoes are a few examples.

Durability and Specificity of Engineered Resistance to Viruses in Plants

The issues of broad-spectrum, durable resistance with regard to virus-resistant transgenic horticultural crops do not conceivably have any impact on the environment and human health. If the engineered resistance to viruses were to show limitations in terms of durability and specificity, it would likely only create an agronomic problem and affect growers (Fuchs and Gonsalves 2007). However, these issues should be considered in light of an effective management of the technology.

Breakdown of Engineered Virus Resistance

The risk that virus resistance may break down or not prove durable is not unique to virus-resistant transgenic crops. It is a risk shared by conventional crops and other transgenic technologies including the pest resistance of *Bt* crops. The durability of resistance refers to the ability of a gene conferring resistance to hold up over time after being widely deployed. In the case of virus resistance, however, the potential mechanisms for resistance breakdown are different. As alluded to previously, one potential breakdown of resistance could occur if virus isolates that are genetically divergent enough, at the nucleotide sequence level, from the transgene are not recognized by the RNA silencing machinery and are subsequently capable of infecting the genetically modified host plant. Another potential mechanism of resistance breakdown centers on the virus' ability to mutate in such a way as to overcome the resistance triggered by the transgene. A third potential mechanism for resistance breakdown involves infection of the resistant host plant with a heterologous virus (Bau et al. 2008, Zagrai et al. 2008) encoding a strong suppressor of gene silencing. This heterologous virus might suppress the host resistance provided by the host transgene, thereby allowing the host to be infected by the original virus toward which their resistance had been targeted.

Though each of these mechanisms could lead to a potential breakdown in host resistance, it is important to note that the risk of resistance breakdown is not unique to virus-resistant transgenic crops nor is it unique to transgenic crops. Resistance breakdown is also an issue associated with the deployment of resistance genes derived via traditional breeding. Pathogens capable of overcoming deployed resistance genes

have been extensively documented (McDonald and Linde 2002); likewise, the deployment of new resistance genes has been shown to alter pathogen populations to overcome that resistance (McDonald and Linde 2002). Initial infection by a virus not targeted by a traditional breeding-derived resistance gene has also been shown to lead to a breakdown of the resistance to the virus targeted by the resistance gene (García-Cano et al. 2006)—analogous to the breakdown of resistance due to a co-suppressor of RNA silencing. Therefore, it is unclear how the risks of breakdown (with transgenic virus resistance) are substantially different from those associated with the use of resistance genes in traditional breeding.

Specificity of Engineered Virus Resistance

Another concern unique to virus-resistant transgenic technology is the so-called specificity of resistance provided by the transgene. As has been shown previously (Tennant et al. 2001), the resistance provided by the transgene might only be specific to the virus isolate from which it was derived and a few closely related isolates. Though previously not well understood, the current understanding of RNA silencing suggests that this may be due to the sequence specificity of the resistance mechanism itself. Since the RNA silencing mechanism relies on the alignment of cleaved fragments of the target RNA in the search for invading RNA sequences, divergence at the sequence level (over ~10%) can lead to an apparent breakdown in resistance (De Haan et al. 1992). However, this has not been shown to be the case with all of the virus-resistant transgenic plants (Tripathi et al. 2004), as a single transgene is able to confer resistance to challenge from numerous isolates of the same virus (Bau et al. 2003).

Also, it is important to note that similar specificity has been shown with resistance genes derived from traditional breeding (Thakur 2007), and it is unclear how the resistance provided in the transgenic case is substantially more specific than the resistance provided by the resistance genes derived by traditional breeding.

EXAMINATION OF RISKS ASSOCIATED WITH COMMERICIALIZED TRANSGENIC HORTICULTURAL CROPS

Once safety issues associated with transgenic horticultural crops are identified, how are risks assessed? How does one examine the significance of risk assessment data? When is there enough evidence to start drawing conclusions on the safety of transgenic horticultural crops? From risk assessment conclusions, are there any safety issues that need to be examined further? Or, are there any that can be put to rest, so to speak? In the following section, we address these questions with regard to virus-resistant transgenic horticultural crops.

Given the tremendous amount of transgenes that have been engineered to provide virus resistance and the numerous crops that these genes have been introduced into, there is a staggering amount of scientific literature on resistance to viruses in transgenic crop plants (Fuchs 2008).

To focus our efforts on findings that we believe to be the most relevant, we have chosen to examine risk assessment of virus-resistant transgenic horticultural crops already commercialized (i.e., papaya, summer squash, tomato, and sweet pepper) or awaiting deregulation (i.e., plum). In our examinations, we will rely primarily on realistic

field studies, which may provide the most accurate reflection of risks, and to a lesser extent on laboratory and greenhouse studies. We will look at the conclusions that can be made about the safety of virus-resistant transgenic horticultural crops by analyzing the significance of risk assessment studies, as well as at identifying gaps in knowledge where further experimental evidence may be needed before conclusions can be drawn with respect to the safety of these crops.

Squash Resistant to *Cucumber Mosaic Virus*, *Zucchini Yellow Mosaic Virus*, and *Watermelon Mosaic Virus*

Background

The first disease-resistant transgenic crop to be commercialized in the United States was transgenic summer squash. This squash, which possesses resistance to ZYMV and WMV, was deregulated in 1994 (Fuchs and Gonsalves 2007, Tricoli et al. 1995). Another summer squash cultivar resistant to CMV, ZYMV, and WMV was later released in 1996 (Fuchs et al. 1998, Fuchs and Gonsalves 2007, Tricoli et al. 1995). Virus-resistant transgenic squash possess the coat protein genes from each virus. Early testing indicated that they provide high resistance to viral infection (Fuchs et al. 1998, Fuchs and Gonsalves 1995, Tricoli et al. 1995) and prevent viral epidemics by reducing secondary plant-to-plant spread (Klas et al. 2006). No similar resistance to multiple viruses is available in traditionally bred commercial summer squash (Fuchs and Gonsalves 2007).

Squash is unique among commercial transgenic plants in that it is monoecious and readily outcrosses (Wilson 1990), emphasizing the significance of gene flow issues

for this crop. Since the center of origin for many squash species is in the southern United States and Mexico (Kirkpatrick and Wilson 1988), the commercialization of virus-resistant transgenic squash marks the first transgenic crop to be released within its center of origin.

Risk Studies

As summer squash was the first virus-resistant crop with a coat protein transgene to be commercialized, the potential allergenicity and impacts on human health were considered extensively (Tricoli 1995). No significant difference in protein, total fat, dietary fiber, carbohydrate, calories, vitamins A (and its precursor, β-carotene) and C, calcium, iron, sodium, ash, moisture, and sugar profiles (fructose, glucose, sucrose, maltose, and lactose) was found between transgenic and non-transgenic squash (Tricoli 1995). Also, an examination into human consumption of virus-infected non-transgenic summer squash fruits led to the conclusion that there was likely to be no significant negative impact on human health beyond those of virus-infected traditionally bred squash cultivars with which consumers have a long history of exposure to without any clear hazards arising (Tricoli 1995).

Field trials relating to gene flow from virus-resistant transgenic summer squash to a free-living relative (*C. pepo* ssp. *ovifera* var. *texana*) and the persistence of transgenes among hybrids of transgenic and free-living *C. pepo* have been carried out. Gene flow occurred with sympatric populations (populations of related species existing in the same geographic area) under conditions of low disease pressure (Fuchs et al. 2004a). Hybrid plants containing transgenes were likely to produce more fruit, seed,

and be more vigorous than free-living *C. pepo* and non-transgenic hybrids under conditions of high disease pressure (Fuchs et al. 2004b). This was not true under conditions of low virus pressure, where free-living plants outperformed the transgenic hybrids (Fuchs et al. 2004b). These results clearly indicated the advantage that the transgenes might provide under conditions of high disease pressure, although it is not clear whether this poses a significant risk in terms of population dynamics. Surveys of free-living *C. pepo* for viruses in areas where transgenic summer squash had not yet been released showed an extremely low incidence of viruses, including CMV, ZYMV, and WMV (Quemada et al. 2008). These results suggested that viruses have a limited effect on the dynamics of free-living *C. pepo* populations (Quemada et al. 2008). Also, the studies on gene flow and its consequence in squash do not necessarily indicate any risk of engineered virus resistance beyond that of conventionally bred resistance genes, as far as free-living populations are concerned (Fuchs and Gonsalves 2007).

An additional study by Fuchs et al. (1998) investigated the likelihood of transencapsidation in transgenic squash, tomato, and melon, so as to allow the transmission of an aphid non-transmissible strain of CMV by aphids through interaction with the coat protein transgene derived from an aphid transmissible strain of CMV. The results of this study failed to demonstrate that transencapsidation could occur over two consecutive growing seasons (Fuchs et al. 1998). However, transencapsidation of an aphid nontransmissible strain of ZYMV likely occurred in transgenic squash expressing the coat protein gene of an aphid transmissible strain of WMV at a very low rate and without triggering an epidemic (Fuchs et al. 1999).

Similarly, virus-resistant transgenic summer squash had no effect on the genetic diversity of CMV strains, suggesting that these plants did not facilitate the emergence of recombinant viruses (Lin et al. 2003).

Papaya Resistant to *Papaya Ringspot Virus*

Background

An early success story in the development and commercialization of virus-resistant transgenic fruit crop plants is the case of papaya resistant to PRSV (Gonsalves 1998). This virus causes one of the most devastating viral diseases of papaya. There is no practical resistance known to PRSV in *Carica* germplasm; therefore, control of this virus has relied on exclusion, movement to new growing regions where PRSV is not found (often involving the destruction of native rainforest habitats), and the use of mild isolates of PRSV in attempts to control the disease via cross-protection (Gonsalves 1998). PRSV is an aphid-borne potyvirus and can readily spread over long distances by its vectors. Papaya is grown in tropical and semitropical regions and Hawaii is by far the largest producer of papaya in the United States. The production center for Hawaiian papaya is in the Puna district of the island of Hawaii. Production from this district comprises over 95% of the total Hawaiian papaya production. In 1992, PRSV was discovered in Hawaii's Puna district (Ferreira et al. 2002) and within 3 years nearly all of the plants in Puna were severely affected. By 1997, papaya production had declined by 36% from 21,800 tons (at the start of the outbreak) down to 14,000 tons (Gonsalves 1998).

Transgenic papaya cultivars, “SunUp” and “Rainbow,” resistant to PRSV were released in 1998 and widely planted in Hawaii providing effective virus control and allowing for increased papaya production (from the low in 1998) (Fuchs and Gonsalves 2007, Gonsalves 1998). Since the release of the PRSV-resistant transgenic papaya in Hawaii, additional papaya cultivars resistant to PRSV containing other PRSV coat protein transgenes have also been developed in Australia, Florida, Brazil, Taiwan, Jamaica, the Philippines, Thailand, and Venezuela (Bau et al. 2003, Davis and Ying 2004, Fermin et al. 2004, Hautea et al. 1999, Lines et al. 2002, Sakuanrungsirikul et al. 2005, Souza et al. 2005, Tennant et al. 2002). The transgenic varieties are at various stages of development and evaluation. Other transgenic papaya purportedly resistant to PRSV have also been developed utilizing the viral replicase gene (Chen et al. 2001) but the major focus here will be on PRSV-resistant papaya involving the coat protein gene.

Risk Studies

Since the development and commercial release of transgenic papaya expressing the coat protein gene of PRSV, numerous experiments and field trials have probed the potential risks associated with this transgenic technology.

As one of the first widely commercialized fruit crops, there has been much interest in the effects of the coat protein transgene on human health and potential allergenicity as well as investigations into compositional changes in fruit with respect to nutrient and antinutrient content. Descriptions of transgenic papayas developed in Hawaii report on percent soluble solids above the minimum required for commercial fruit

and yields of almost three times those of industry averages (Ferreira et al. 2002). Comparable values for vitamin C and minerals (potassium, phosphorus, calcium, magnesium, sodium, iron, copper, zinc, and boron) for the transgenic and non-transgenic cultivars have been published (Manshardt 1998, Mutsuga et al. 2001). Also, no evidence of ill effects has been linked to the consumption of transgenic papaya in the United States and Canada (Fuchs and Gonsalves 2007).

Another study on transgenic papaya from Thailand reported on comparable nutrient composition with the non-transgenic counterpart (Sakuanrungsirikul et al. 2005). Recently, the levels of nutrients (protein, fat, carbohydrate, minerals) and antinutrients (oxalates, hydrocyanic acid, and benzyl isothiocyanate) in three transgenic papaya lines expressing a PRSV coat protein gene construct, which are not currently available on the market, were compared to those of the commercial papaya cultivar “Sunrise solo” grown under the same conditions in an experimental plot in Jamaica (Roberts et al. 2008). Since papaya is a climacteric fruit, three stages of maturity were considered to facilitate an evaluation of the changes in various parameters that accompany the ripening process after harvest. With the exception of one transgenic line, no significant differences were observed in selected nutrients and antinutrients between the control and test samples at three stages of maturity, although a few random variations were noted (Roberts et al. 2008). Overall, the compositional changes over the three maturities were as expected and comparable to those reported (Bari et al. 2006, Chan et al. 1979, Yamamoto 1964). Sugars, vitamin C, and carotenoids followed a general upward trend, whereas slight decreases in moisture, ash, and fat at the final stage of ripening were observed. Some variability in the concentrations of the

three antinutrients tested was observed, but the values were within the range of concentrations reported for the parental variety (Umoh 1998). A general trend of decreasing antinutrient levels was noted during ripening in transgenic and control fruits. Similar nonsignificant variations in selected horticultural traits (water, lipid, nitrogen, protein, reducing sugar, vitamin A [and its precursor], and vitamin C) were obtained with transgenic papaya expressing the replicase gene of a PRSV isolate from the People's Republic of China (Chen et al. 2001, Xiangdong et al. 2007).

Recently, Powell et al. (2008) evaluated the safety of transgenic papaya in a subchronic feeding study. A diet formulated with 10% transgenic papaya, the equivalent of twice the average daily human consumption of fresh papayas based on food consumption data from the Caribbean and Latin America (CFNI 2000, WHOGEMS 2003), was administered to rats for 90 consecutive days. For comparison, reference non-transgenic papaya "Sunrise solo," from which the transgenic papaya was derived, and a control laboratory rodent diet formulation were also evaluated. Markers of general health, including body weight, food intake, and activities of plasma, liver, and kidney function enzymes (acid and alkaline phosphatases and alanine and aspartate transaminases) were comparable for the test, reference, and control groups. No significant effects were observed in organ weights or histopathology (Powell et al. 2008). Changes in the liver and kidney, the sites of biotransformation and detoxification, and of excretion of metabolic waste products, respectively, were not observed (Powell et al. 2008). Overall, the plasma cholesterol levels, which are markers of cardiovascular risk, were similar to the control as were triglycerides, which are biomarkers for hepatotoxicity. Although not statistically relevant, variations in the values

of the parameters monitored with the control, reference, and test groups were observed (Powell et al. 2008). Based on literature ranges, the variations were attributed to natural biological fluctuations and were not regarded as reflecting a toxicologically meaningful effect.

In addition to looking at the effect of transgenes on papaya fruit, other researchers have focused on the effects of transgenic papaya on nontarget organisms including soil microbial organisms. Hsieh and Pan (2006), looking at populations of fungi, bacteria, and actinomycetes present in the soil from field plantings of both transgenic and nontransgenic fields, found highly similar (>80%) populations in both soil environments (transgenic vs. non-transgenic) as well as in upper and lower soils within the environments. These authors concluded that the planting of transgenic papaya's effect on the soil microorganisms is limited (Hsieh and Pan 2006). Minor effects on nontargets have been observed (Wei et al. 2006), but these effects are varied and not consistent across all virus-resistant transgenic crops. Another study, also examining the effects of transgenic papaya on the soil, relied upon polymerase chain reaction to determine the persistence and availability of transgenic genes that may be released by transgenic papaya during growth (Lo et al. 2007). Though transgenic DNA was detected at low levels, no gene transfer events from soil DNA extracts to *Acinetobacter* (a bacterium well known for its ability to uptake foreign DNA) were observed (Lo et al. 2007).

Tomato and Sweet Pepper Resistant to *Cucumber Mosaic Virus*

Background

With regard to transgenic tomato and sweet pepper engineered for CMV resistance, the body of publications is much smaller than for the aforementioned transgenic crops. Transgenic tomato and sweet pepper containing the coat protein gene from a local isolate of CMV were released in the People's Republic of China (Chen et al. 2003). CMV is a cucumovirus that is transmitted in a nonpersistent manner by several aphid species (Garcia-Arenal and Palukaitis 2008). It has the widest host range of any known plant virus with 1,300 species in more than 500 genera of over 100 families. Control of CMV can be achieved by planting resistant crops but resistance in many crops species is often not available to a broad range of CMV strains (Garcia-Arenal and Palukaitis 2008).

Risk Studies

A study on gene flow was performed with transgenic sweet pepper and tomato by Ming et al. (1997). Seeds and pollen of many different plants at varying distances from fields of transgenic plants were examined to determine whether gene flow had occurred. Selection of seedlings on antibiotic-containing medium and polymerase chain reaction were used to monitor transgene movement, but transfer of transgenes was not detected (Ming et al. 1997).

For assurance of food safety, transgenic sweet peppers and tomatoes expressing the coat protein gene of CMV were evaluated in animal feeding studies (Chen et al. 2003). The animals received about 12,600 and 7,100 times the average

daily human consumption of sweet peppers and tomatoes, respectively. Comparable performance of rats fed transgenic and non-transgenic diets was demonstrated (Chen et al. 2003). Significant differences were not reported in mean weekly body weights, body weight gains, or food consumption of rats fed transgenic or non-transgenic sweet pepper and tomato diets. Similarly, significant differences were not observed with the hematological and blood biochemical parameters monitored (including cholesterol and triglyceride), although fluctuations in the values were observed (Chen et al. 2003). In another study, Cai et al. (2003) conducted 30-day acute toxicity (LD^{50}) experiments with male and female rats and mice using gavage administration of a series of doses ranging from 1 to 10 g/kg body weight of dry pepper fruit containing the coat protein gene of TMV and CMV. Abnormalities in body weights, organ weights, histopathology, and hematology were not observed (Cai et al. 2003).

Examination of Risks Associated With Transgenic Plum Which is Under Consideration for Deregulation

Background

Though not yet approved for commercialization, transgenic plum cultivar "Honey sweet" (aka C5) resistant to *Plum pox virus* (PPV) has been deregulated by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS), the U.S. Food and Drug Administration (FDA), and the U.S. Environmental Protection Agency (EPA) in the United States and is being widely tested in Europe under varying growing conditions. PPV is a potyvirus that is considered the most important pathogen in *Prunus* by the U.S. and E.C. agencies (Capote et al. 2006). Conventional breeding

has not been able to produce any trees of commercially acceptable varieties with high resistance to PPV. Therefore, control of PPV has relied on prevention via certified planting material, quarantine measures, and eradication (Capote et al. 2006). PPV is spread by multiple aphid species, but control of the vector is not feasible for both efficacy and environmental impact reasons (Ravelonandro et al. 2000).

Transgenic plum trees containing multiple copies of the PPV coat protein gene were developed (Scorza et al. 1994) and line C5 was shown to be highly resistant to PPV infection (Ravelonandro et al. 2000). The involvement of the post-transcriptional RNA silencing mechanism in the resistant line C5 was confirmed (Kundu et al. 2008, Scorza et al. 2001). Clone C5 was tested extensively in the field in the Czech Republic, Poland, Romania, and Spain to demonstrate the effectiveness of the engineered resistance. Transgenic C5 trees all remained free from PPV infection even six (Hily et al. 2004, Polák et al. 2008) and eight years after transfer to the field (Malinowski et al. 2006), while 100% of the control, non-transgenic trees were infected with PPV after these periods. Though these data indicate the durability of the engineered resistance to PPV, trees inoculated with PPV via chip budding exhibited very mild symptoms after several years, though these symptoms did not progress to severe symptoms (Malinowski et al. 2006, Polák et al. 2008).

Risk Studies

Fruit compositional analyses indicated that PPV-resistant transgenic C5 is typical for *P. domestica* plums in terms of protein, total fat, antioxidant capacity, phenolics, starch, dietary fiber, ash, moisture, acidity, carbohydrates, sugar profiles (glucose,

sucrose, lactose, maltose, and fructose), calcium, magnesium, sodium, potassium, iron, and vitamins A, B_1, B_2, B_3, and C (Scorza 2004).

The potential for viral recombination between transgene transcripts and incoming PPV RNA was examined in transgenic plums as well as the effects of PPV-resistant transgenic plums on aphid vector populations (Capote et al. 2008). Utilizing transgenic European plum lines as well as non-transgenic plums from an experimental orchard and Japanese plums from an external control plot, 85 PPV isolates were collected from these three populations of trees and their genetic diversity was compared. Looking at variable regions of the PPV genome including the coat protein gene, no significant differences in genetic variability were found among isolates from the three populations, indicating that the PPV populations were not being selectively altered in the transgenic trees (Capote et al. 2008). Subsequent analysis of 12 PPV isolates showed no detectable recombinant virus (Capote et al. 2008). Of note in this experiment, C5 could not be used as a source of virus isolates for testing since it remained free from infection eight years after natural exposure to PPV populations. Recombination in C5 trees therefore would have been impossible due to lack of virus infection.

For assessing the impact of transgenic plums on nontarget organisms, the diversity of aphid populations visiting transgenic and non-transgenic plums was investigated (Capote et al. 2008). Aphids were captured, identified, counted, and their viruliferous potential was subsequently characterized. These comparisons found no significant differences between the aphid populations from either transgenic or control plums in terms of total aphid numbers, aphid species distributions, and viruliferous potential over the two year period of the study (Capote et al. 2008). To test the effect of

heterologous viruses on the stability of RNA silencing in transgenic plum line C5, trees were graft-inoculated with different combinations of PPV and either *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), or *Apple chlorotic leafspot virus* (ACLSV) (Zagrai et al. 2008). PNRSV, PDV, and ACLSV are common in *Prunus* sp. The engineered resistance to PPV was stable and was not suppressed by the presence of heterologous viruses during a 3 year field trial in Romania and Spain (Zagrai et al. 2008).

OVERVIEW OF RISK LESSONS OF TRANSGENIC HORTICULTURAL CROPS

Based on the existing body of research into the potential risks posed by virus-resistant genetically modified horticultural crops, there appears to be a significant amount of evidence that these crops have little to no detrimental impact on the environment and human health beyond those of conventional horticultural crops.

The work on summer squash supports the aforementioned claim that transencapsidation is not a significant environmental risk beyond that already posed by multiple infected conventionally bred plants (Fuchs et al. 1998, 1999). This is also true for the risks of gene flow to free-living relatives (Fuchs et al. 2004a, 2004b, Quemada et al. 2008) and human health effects, specifically allergenicity (Tricoli 1995). Furthermore, there is a documented safe release of this transgenic crop over the past 12 years in the United States (Fuchs and Gonsalves 2007).

In the case of papaya, the results from the toxicity and nutritional experiments seem to very strongly refute risks to human health (Cai et al. 2003, Powell et al. 2008, Roberts et al. 2008, Sakuanrungsirikul et al. 2005). The threat of negative nontarget

effects appears to be lessened though the papers published on this topic are very limited in scope (Hsieh and Pan 2006, Lo et al. 2007, Wei et al. 2006). With respect to the other areas of risk, direct experimental evidence does not appear to exist in sufficient quantities to make any firm conclusions on transencapsidation, recombination, and gene flow. However, the safe commercial use of transgenic papaya over a decade and evidence from other transgenic crops may speak to some of these issues enough to be extrapolated to the PRSV papaya case (Fuchs and Gonsalves 2007).

The work on plums supports the fact that recombination is unlikely to facilitate the emergence of virus species with altered or new biological properties beyond the occurrence in conventional plums subjected to mixed virus infection (Capote et al. 2008). This also applies to the risks on nontarget organisms (Capote et al. 2008). It is doubtful that transencapsidation could occur to a meaningful level in transgenic plum line C5, because of undetectable levels of PPV coat protein (Hily et al. 2004, Kundu et al. 2008, Scorza et al. 2001). This also casts doubt on whether the coat protein is expressed at a meaningful level to have human health impacts differing significantly from those seen in PPV-infected non-transgenic plums.

Based on the information from the transgenic tomato and sweet pepper, there is further evidence to support the claim that gene flow is not a major concern in this case (Garcia-Arenal and Palukaitis 2008). Most significantly, the animal feeding studies give further support to the safety of virus-resistant transgenic crops expressing viral coat protein genes (Cai et al. 2003, Chen et al. 2003).

Similar conclusions on the risk assessment of other transgenic horticultural crops have been reported. The only *Bt* horticultural crop commercially available in the United

States is *Bt* sweet corn, although other *Bt* transgenic vegetables (i.e., cauliflower, cabbage, and eggplant) are being considered for commercialization in India (Shelton et al. 2008). Studies have shown that *Bt* sweet corn provides consistently excellent control of lepidopteran pests. This technology also substantially reduces insecticide applications and better preserves predators of the European corn borer than commonly used broad-spectrum insecticides (Shelton et al. 2008). *Bt* sweet corn, like virus-resistant summer squash and papaya, are consumed in the United States with no ill effects reported.

In summary, transgenic horticultural crops have become important components of disease and pest management programs in the United States and the People's Republic of China. Their adoption rate is constantly increasing since their first release in the mid 1990s. Safety issues have been expressed during their development and release, but most of these risks are the same or similar to those posed by traditionally bred plants with host resistance. Since the commercialization of virus-resistant transgenic squash in 1996, considerable data have been gathered from many parts of the world on the effects on the environment and human health of virus-resistant and *Bt* horticultural crops. From these studies, some general trends have emerged. Commercialized summer squash, tomato, sweet pepper, and papaya expressing viral coat protein genes, and *Bt* sweet corn have effectively controlled viruses and species of Lepidoptera, respectively. Also, extensive research has been published showing that these transgenic crops have little to no impact on the environment and human health beyond those of virus-infected plants in natural settings and in traditional agriculture,

and conventional pest management strategies (Capote et al. 2008, Fuchs and Gonsalves 2007, Keese 2008, Shelton et al. 2002, 2008, Widmer et al. 2007).

PERSPECTIVES

Introduction

Since the initial discovery of engineered resistance to viruses via expression of the TMV coat protein gene in plants (Abel et al. 1986), pathogen-derived resistance (Sanford and Johnston 1985) and coat protein-mediated resistance have proven to be effective tools to control viruses in horticultural crops (Fuchs 2008). As discussed, PRSV, ZYMV, CMV, and WMV have been effectively controlled in commercial settings of transgenic papaya, summer squash, tomato, and sweet pepper with the use of coat protein genes. In addition, in 2007 alone, more than 25 field trial permits for resistance against other viruses were granted by the USDA-APHIS (ISB 2008). Target viruses included *Tomato spotted wilt virus*, *Beet necrotic yellow vein virus*, *Sorghum mosaic virus*, *Grapevine fanleaf virus*, *Grapevine leafroll-associated virus 2*, *Grapevine leafroll-associated virus 3*, *Citrus tristeza virus*, *Cassava mosaic virus*, *Sugarcane mosaic virus*, *Sugarcane yellow leaf virus*, *Papaya leaf distortion mosaic virus*, PPV, and PVY (ISB 2008).

In recent years, new knowledge of the mechanism behind engineered virus resistance has been gained. This has greatly expanded the potential for utilizing the antiviral pathways of RNA silencing to control plant viral diseases. Though all the currently available commercial virus-resistant transgenic crops utilize the coat protein-mediated resistance, recent publications, patents, and field trial data reveal that there

are many alternative approaches currently in the pipeline to engineer virus resistance in plants. Transgenes incorporating short fused sequences derived from different viral strains have been engineered successfully to provide resistance to several virus species (Bucher et al. 2006). Other methods which, unlike the RNA silencing-based approaches, do seem to depend on protein production have also been shown to have some promise, including the use of defective viral movement protein and replicase genes (Prins et al. 2008). The risks that these gene constructs pose are not yet fully determined and are likely different from those risks already discussed with regard to the viral coat protein gene. Though the risk assessment of the coat protein technology has, as discussed, alleviated most of the concerns of this technology, questions remain about the new technologies that are becoming available. Do they alleviate any of the problems of the existing technology? Do they raise new safety issues that may pose their own potential risks? Do they alleviate a concern that was no longer seen as a problem only to raise new issues that are potentially more difficult to assess? In the following section, we will describe some of the latest approaches toward virus resistance in transgenic plants and discuss if and how these technologies alleviate some of the existing concerns.

Future Trends

Given the discovery and elucidation of the antiviral pathways of RNA silencing, many new approaches have been used to develop transgenes more likely to stimulate RNA silencing via the design of sophisticated transgenes. Since RNA silencing theoretically is stimulated in a plant cell by the presence of dsRNA, many of these

transgene constructs attempt to transcribe RNA molecules that are more likely to form dsRNA structures. One strategy for accomplishing this is the use of inverted repeats, which involves the creation of a transgene containing two copies of the viral target complementary DNA (cDNA) sequence separated by a spacer DNA of some length (Prins et al. 2008, Waterhouse et al. 1998). After transcription, it is thought that the resulting RNA will form a hairpin with the inverted RNA forming a double-stranded structure. The use of introns is a similar approach in that two inverted regions of viral-derived cDNA are separated by an intron (Smith et al. 2000). Once the intron is spliced by host machinery following transcription, a dsRNA structure is formed. Another approach consists of producing two complementary pieces of RNA which can then form a dsRNA from bidirectional promoters (Li et al. 2004). These new strategies have been shown to produce a significantly higher proportion of virus-resistant transgenic plants than the use of full-length coat protein transgenes (Prins et al. 2008, Smith et al. 2008, Waterhouse et al. 1998). In addition, they hold a seeming advantage over a full-length coat protein gene in the sense orientation as they are generally unable to produce a functional protein, alleviating concerns arising from the presence of the coat protein in plant material. Coat protein expression can also be prevented by using transgenic approaches involving a transgene that produces an RNA product which is untranslatable, either because it lacks the necessary translation start codon for ribosomal processing or because it is oriented in antisense directions. It should be noted that the use of introns, often derived from plants themselves, potentially poses the risk of silencing host genes from where the intron was derived, if the RNA silencing

machinery incorrectly processes the transgene RNA. A similar concern applies to the siRNA technology overall (Snove and Holen 2004).

Another approach utilizing the knowledge of viral silencing is to produce resistance by using modified plant microRNA (miRNA) cistrons to produce a range of artificial antiviral miRNAs (Niu et al. 2006, Qu et al. 2007). The durability of this approach compared to the use of longer dsRNA approaches has not been demonstrated (Garcia and Simón-Mateo 2006).

Some nonviral sources of virus resistance have also been investigated. These would theoretically alleviate concerns about synergism, recombination, and transencapsidation. These include the transfer of host resistance genes against viruses into other hosts via genetic engineering, or the silencing of host genes that are necessary for viral replication (Prins et al. 2008). The use of plant-generated antibodies against viruses, which failed to progress for many years though initially perceived as promising, has recently been shown to be effective in controlling viruses in plants expressing the transgenes for the production of these antibodies (Nölke et al. 2004). The potential risks of these technologies remain largely undiscussed and untested.

CONCLUSIONS

To date only a handful of horticultural transgenic crops, including vegetable crops (summer squash, sweet pepper, tomato, and sweet corn) and one fruit crop (papaya) are available commercially. Most of these crops have been engineered for virus resistance (summer squash, sweet pepper, tomato, and papaya), while sweet corn has been developed for insect tolerance. Noteworthy, the majority of virus-resistant

transgenic crops were released over a decade ago. Why haven't more transgenic horticultural crops been released? Though virus-resistant transgenic plum is considered for deregulation in the United States, why have transgenic horticultural crops not been released recently? Can safety issues be held accountable for the limited number of transgenic horticultural crops released commercially? As discussed in this chapter, extensive research on risk assessment of transgenic horticultural crops has been carried out in various environments and varied conditions of disease and pest pressure (Fuchs et al. 2007, Fuchs and Gonsalves 2007, Keese 2008, Shelton et al. 2002, 2008). This wealth of information implies that safety issues should not hinder the release of new horticultural crops that are engineered based on the identical or similar technologies to those used for the development of the transgenic horticultural crops already released. Factors other than safety issues (e.g., institutional, policy, and economical factors) are apparently playing more important roles worldwide in stymieing the adoption of transgenic horticultural crops (Davidson 2008, Fermin et al. 2005, Gonsalves et al. 2007).

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CHAPTER 5

Genetic Diversity of *Prunus necrotic ringspot virus* Isolates Within a Cherry Orchard in New York[4]

ABSTRACT

A survey for *Prunus necrotic ringspot virus* (PNRSV) in an orchard of *Prunus cerasus* cv. Montmorency and *Prunus avium* cv. Hedelfingen in New York by enzyme-linked immunosorbent assay indicated an eightfold higher infection rate in sour cherry (33%, 32 of 96) than in sweet cherry (4%, 6 of 136) trees. The presence of PNRSV was confirmed by reverse transcription-polymerase chain reaction and amplification of the coat protein (CP) gene in total RNA from infected leaf tissue. Latent infection was prevalent in the majority of trees infected (87%, 33 of 38), while a few of them exhibited shock symptoms or had severely reduced growth (13%, 5 of 38). Asymptomatic PNRSV-infected trees clustered in spatial proximity to symptomatic trees. Sequence analysis of the CP gene (675 bp) indicated a population structure consisting of one predominant molecular variant for 10 isolates and six minor molecular variants for seven isolates. A high sequence identity was found between the CP gene of PNRSV isolates from cherry trees and other isolates from diverse hosts and various geographic origins at the nucleotide and amino acid levels (88 to 100%). Phylogenetic analyses showed a

[4] Oliver, J.E., Freer, J., Andersen, R.L., Cox, K.D., Robinson, T.L. and Fuchs M. 2009. Genetic Diversity of *Prunus necrotic ringspot virus* Isolates Within a Cherry Orchard in New York. *Plant Dis.* 93:599-606.

clustering of PNRSV isolates from cherry trees in New York in the predominant group PV-96.

INTRODUCTION

Prunus necrotic ringspot virus (PNRSV) causes serious losses worldwide to cultivated *Prunus* spp., including cherry, peach, apricot, nectarine, plum, and almond. Reduced vigor (up to 30%), yield (20 to 56%), and fruit quality have been reported in infected trees (Parker et al. 1959, Scott et al. 1989, Uyemoto and Scott 1992). In cherry, PNRSV symptomatology can be latent or consist of foliar mosaics, rings, or chlorotic areas that may develop into severe necrotic spots with a shot hole appearance. The latter symptoms are referred to as shock syndrome (Mink 1992, Pscheidt 2007). PNRSV can also infect ornamentals, including roses (Moury et al. 2001, Wong et al. 1988).

PNRSV belongs to the genus *Ilarvirus* in the family *Bromoviridae* (Lang and Howell 2001). The viral genome is tripartite and consists of single-stranded positive-sense RNAs. Replicative functions are encoded by RNA1 and RNA2, while RNA3, which is bicistronic, encodes the movement and coat protein (CP) genes. The CP is expressed from a subgenomic RNA called RNA4 (Bol 2005). No arthropod, nematode, plasmodiophorid, or fungus vector is known for PNRSV. Instead, the virus spreads readily through budding and grafting (Howell and Lang 2001). It is also seed- and pollen-borne by the wind, pollinator insects, and sucking insects (Howell and Lang 2001, Milne and Walter 2003).

Many isolates of PNRSV with diverse biological properties have been identified (Crosslin and Mink 1992, Hammond 2003, Hammond and Crosslin 1998, Moury et al. 2001). Also, distinct serogroups of PNRSV have been characterized with polyclonal antisera and monoclonal antibodies (Mink 1992, Mink et al. 1987, Moury et al. 2001, Myrta et al. 2001, Spiegel et al. 1999). The sequence of RNA3, including the CP gene, has been determined for numerous isolates, and phylogenetic analyses have revealed a clustering of PNRSV isolates into three major groups, including PV-32, PE-5, and PV-96 (Aparicio et al. 1999, Hammond 2003, Scott et al. 1998, Vaskavám et al. 2000). An additional phylogroup named CH30 was recently proposed (Glasa et al. 2002).

Reports from the early 1960s documented the occurrence of PNRSV in New York, especially in sour (*Prunus cerasus* L.) and sweet (*Prunus avium* L.) cherry trees (Allen 1963a, 1963b), as well as in cherry rootstocks (Gilmer and Kamalsky 1962). PNRSV was also described in rose in New York in the late 1980s (Vaskavám et al. 2000). However, no information is available on the genetic variability of PNRSV isolates from New York. Similarly, little is known on the population structure of PNRSV isolates within an infected *Prunus* orchard. The objectives of our study were to (i) determine the incidence of PNRSV in an experimental orchard of sour (*P. cerasus* cv. Montmorency) and sweet (*P. avium* cv. Hedelfingen) cherry in which a few trees (2%, 5 of 232) exhibited typical shock symptoms in the spring of 2006, and (ii) provide some insights into the population structure and genetic diversity of PNRSV isolates. Individual trees were surveyed over two consecutive years for PNRSV by double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA), and the CP gene of infecting isolates was characterized by reverse transcription–polymerase chain reaction

(RT-PCR) and sequencing. We report here a higher incidence of PNRSV in sour cherry trees versus sweet cherry trees over the 2-year period of our study, and a clustering of these New York isolates into the predominant phylogenetic group PV-96.

MATERIALS AND METHODS

Cherry orchard and leaf sample collection. The cherry orchard surveyed in this study was an 8-year-old rootstock trial block established on the Research North Farm at the New York State Agricultural Experiment Station (NYSAES), Cornell University, Geneva.

Test trees consisted of *P. cerasus* cv. Montmorency and *P. avium* cv. Hedelfingen grafted onto dwarfing, semi-dwarfing, or full vigor rootstocks, including *P. cerasus* × *P. canescens* cvs. Gisela 3, Gisela 4, Gisela 5, Gisela 6, Gisela 7, Gisela 195/20, *P. cerasus* L. (Tabel Edabriz), *P. cerasus* L. (Weiroot 10, 13, 53, 72, and 158), and *P. mahaleb* L. (Mahaleb). For Hedelfingen, the full vigor rootstock *P. avium* L. (Mazzard) was also used. Replicates of eight trees were planted for most cultivar/rootstock combinations in a split-plot complete block design with random replicate allocation within blocks. A few *P. avium* cv. Hedelfingen trees were also on rootstocks P50, *P. avium* × *P. cerasus* (PHL-A), and *P. mahaleb* L. (Mahaleb). Trees were planted in four rows of 65 trees each, spaced 4.5 m apart within rows and 6.0 m between rows.

For high pollination rates of *P. avium* cv. Hedelfingen, compatible *P. avium* cultivars (i.e., Black Gold, Vandalay, and Emperor Francis) were planted in adjacent

rows as pollenizers. Also, honey bee and bumble bee colonies were placed in the orchard before and during bloom.

Leaf samples (10 to 12 per tree) were collected for PNRSV detection by DAS-ELISA in June 2006. Young leaves (2 to 5 per twig) were collected from the yearly growth at the tip of lower branches. Alternatively, in May 2007, young leaf tissue was collected from budwood that was removed prior to bloom from selected trees in the orchard and forced in a greenhouse, and was subsequently used for PNRSV detection by DAS-ELISA and RT-PCR. We considered a PNRSV isolate as a viral culture from a single tree. A few trees were also assayed for *Prune dwarf virus* (PDV) by DAS-ELISA in 2006.

Virus detection by DAS-ELISA. PNRSV and PDV were detected by DAS-ELISA in crude leaf extracts with specific antibodies (Bioreba, Reinach, Switzerland). A portion of 10 to 12 stacked leaves was torn and ground in 200 mM Tris-HCl, pH 8.2, 140 mM NaCl, 2% polyvinylpyrrolidone 40, and 0.05% Tween 20 at a 1:5 ratio (wt/vol) using a semi-automated ball bearing HOMEX tissue homogenizer (Bioreba). Test conditions were according to the manufacturer's instructions (Bioreba). Substrate hydrolysis was recorded at 405 nm with an absorbance BioTek ELx808 microplate reader (BioTek, Winooski, VT). Samples were considered positive if their optical density (OD 405 nm) readings were at least twice those of healthy controls ±20%. PNRSV incidence determined by DAS-ELISA was subject to analysis of variance (ANOVA) for a split-plot design using the General Linear Model (GLM) procedure of SAS (version 9.13; SAS Institute Inc., Cary, MC). All percentage data were subject to arcsine square root transformation prior to analysis.

Spatial distribution analysis of PNRSV-infected cherry trees in the experimental orchard. The spatial distribution of shot hole symptom development in 2006 and cumulated DAS-ELISA scores in 2006 and 2007 were mapped using the three dimensional contour mapping feature of Sigmaplot version 9.0 with a scatterplot overlay (Systat Software, Inc., San Jose, CA). Contour maps indicate the location of symptomatic trees by coordinates x, y, and z, with the x-axis being the row number, the y-axis being the within-row location (e.g., tree number), and the z-axis being the type of cherry tree. The scatterplot overlay indicates PNRSV symptoms and/or DAS-ELISA scores for each tree by color and shape, respectively.

Virus detection by RT-PCR. PNRSV was detected by RT-PCR in total RNA from leaf samples of sour and sweet cherry trees that tested positive in DAS-ELISA in 2007. For RNA extraction, leaf tissue (100 mg) was placed in a 2.0-ml microfuge tube, dipped in liquid nitrogen, and disrupted with a TissueLyser homogenizer (Qiagen, Valencia, CA) for 2 min at 30 MHz in the presence of one stainless steel bead (5 mm diameter). Total RNA was extracted from homogenized leaf material using the RNeasy Mini Plant Kit (Qiagen).

The PNRSV full-length CP gene was characterized by RT-PCR using primers described previously (Aparicio and Pallàs 2002). In addition, a primer pair specific to the 1,5-bisphosphate carboxylase chloroplast gene (*Rbcl*) of *P. persica* (GenBank accession no. AF206813) was used in standard and multiplex RT-PCR to assess the quality of total RNA preparations and evaluate the effectiveness of the detection assay (Sánchez-Navarro et al. 2005).

One-step RT-PCR was carried out using the Access System (Promega, Madison, WI) with *Avian myeloblastosis virus* RTase, *Tfl* DNA polymerase, and 50 pmoles of specific primers in a 50 µl final volume according to the manufacturer's protocol. Single-tube RT-PCR used a 45 min heating step at 45°C and a 2 min heating step at 94°C followed by 45 cycles of 1 min melting at 94°C, 1 min annealing at 55°C, and 2 min elongation at 68°C with a final extension of 7 min at 68°C. The reaction products were resolved by electrophoresis in 1.5% agarose gels in 90 mM Tris-borate, 2 mM EDTA, pH 8.0, stained with ethidium bromide, and subsequently visualized under UV light.

Viral sequence determination and analysis. DNA amplicons of the PNRSV CP gene obtained by RT-PCR were extracted from agarose gels with the QIAquick Gel Extraction Kit (Qiagen) and sequenced bidirectionally using the Big Dye Terminator Kit, AmpliTaq-FS DNA polymerase, and an Applied Biosystem Automated 3730xl DNA Analyzer at the Cornell University DNA Sequencing Facility in Ithaca, NY. Sequences were analyzed and compared using the DNASTAR Lasergene v7.2 software package. The program CLUSTAL W was used for alignment of nucleotide sequences (Thompson et al. 1994). Phylogenetic relationships were determined with the neighbor-joining method (Saitou and Nei 1987) using PNRSV isolate PE-5 (GenBank accession no. L38823) from peach, isolate PV-32 (GenBank accession no. U03857) from apple, isolate PV-96 (GenBank accession no. S78312) from *P. mahaleb*, and isolates CH30 (GenBank accession no. AF034994) or SW6 (GenBank accession no. AF013287) from sweet cherry as representative of their respective phylogenetic groups. The robustness of the inferred evolutionary relationships was assessed by 1,000 bootstrap replicates.

The algorithm SISCAN (Gibbs et al. 2000) was used to confirm the occurrence of recombination events suspected by phylogenetic analysis and sequence alignments.

RESULTS

Typical PNRSV shock symptoms, i.e., leaf necrosis and shot holing (Figure 5.1A), were observed on a limited number of trees (2%, 5 of 232) in an 8-year-old experimental cherry orchard on the Research North Farm at NYSAES, Geneva, NY in the spring of 2006. New leaves that developed later in the season remained symptomless. Delayed budbreak, retarded bloom, death of leaf and flower buds, slow fruit set, and reduced vigor (Figure 5.1B and C) were also early indications of the shock syndrome induced by PNRSV. Symptomatic trees were all sour cherry. They were located in the southwest corner (row 1, tree 11, and row 2, trees 7 and 15), north-central area (row 2, tree 46), and northeast corner (row 3, tree 56) of the orchard (Figure 5.2).

Figure 5.1. Reaction of *Prunus cerasus* cv. Montmorency to *Prunus necrotic ringspot virus* (PNRSV) infection. A, Foliar necrotic spot and shot holes, and B, differential leaf and flower bud development and reduced vigor of a PNRSV-infected tree in comparison to C, a healthy tree.

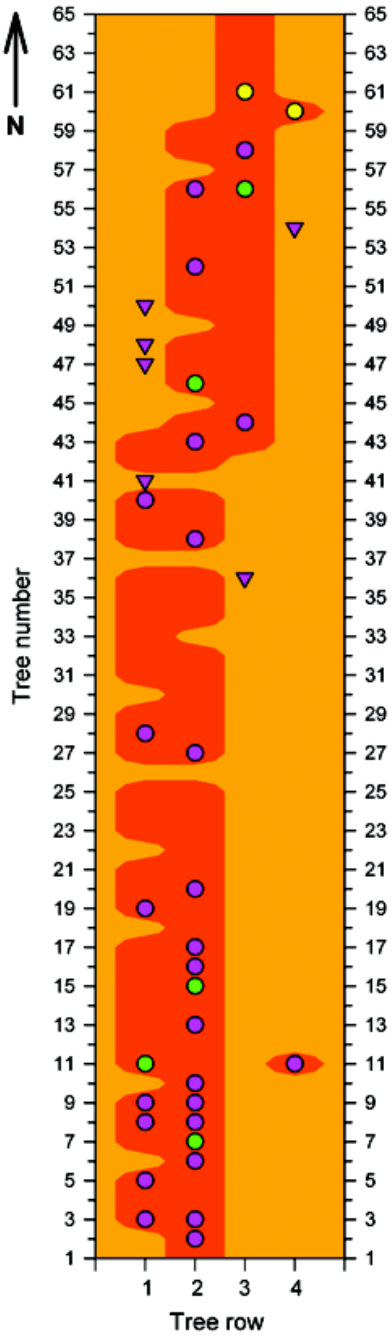

Figure 5.2. Spatial distribution of *Prunus necrotic ringspot virus* (PNRSV)-infected cherry trees in the experimental orchard surveyed. Tree rows are indicated on the x-axis and tree numbers are indicated on the y-axis. Sour cherry trees showing shot hole symptoms in 2006 are represented by green circles. Sweet and sour cherry trees reacting positively in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for PNRSV are shown in purple triangles and circles, respectively. Healthy sweet and sour cherry trees are in pale orange and orange, respectively. Sour cherry trees with the two most divergent PNRSV isolates (ChrT214 and ChrT224) are indicated by yellow circles. There were no trees where disease was observed without corresponding virus detection by DAS-ELISA. North (N) is indicated by an arrow.

One hundred thirty-six sweet cherry trees (*P. avium* cv. Hedelfingen) and 96 sour cherry trees (*P. cerasus* cv. Montmorency) grafted onto 16 dwarfing, semi-dwarfing, and full vigor rootstocks were surveyed for PNRSV in 2006 and 2007 (Table 5.1). Thirty-eight of the 232 trees (16%) tested were positive for PNRSV in DAS-ELISA.

Table 5.1. Incidence of *Prunus necrotic ringspot virus* (PNRSV) in cherry trees in an experimental orchard at the Research North Farm, NYSAES at Geneva, NY.

Scion		Rootstock	Infected/Tested[a]	%
Prunus cerasus L. cv. Montmorency		Gisela 3	1/8	12
		Gisela 5	1/8	12
		Gisela 6	5/8	63
		Gisela 7	3/8	38
		Gisela 195/20	1/8	13
		Weiroot 010	1/8	13
		Weiroot 013	2/8	25
		Weiroot 053	0/8	0
		Weiroot 072	5/9	56
		Weiroot 158	5/8	63
		Tabel Edabriz	4/8	50
		Mahaleb	4/7	57
		Subtotal	32/96	33
Prunus avium L. cv. Hedelfingen		Gisela 3	0/8	0
		Gisela 4	0/4	0
		Gisela 5	0/8	0
		Gisela 6	0/8	0
		Gisela 7	1/8	13
		Gisela 195/20	0/8	0
		Weiroot 010	0/8	0
		Weiroot 013	0/8	0
		Weiroot 053	0/8	0
		Weiroot 072	0/8	0
		Weiroot 158	1/8	13
		Tabel Edabriz	0/8	0
		Mahaleb	0/7	0
		P. avium Mazzard	0/8	0
		P-HL-A	1/2	50
		P50	3/7	43
		Subtotal	6/116	5
Prunus avium L. pollinizer	Black Gold	Mahaleb/Mazzard	0/13	0
	Emperor Francis	Mahaleb	0/1	0
	Vandalay	nd	0/6	0
		Subtotal	0/20	0
		Total	38/232	16

[a]Data represent the number of trees infected by PNRSV as shown by double-antibody sandwich enzyme-linked immunosorbent assay and reverse transcription – polymerase chain reaction over the total number of trees tested in 2006 and 2007; nd: not determined.

Absorbance values were clearly higher for infected compared to healthy leaf samples with OD 405 nm readings of 0.92 ± 0.16 versus 0.10 ± 0.01 after substrate hydrolysis for 1 h. PDV was not detected in any of the trees tested by DAS-ELISA.

The majority of PNRSV-infected trees (84%, 32 of 38) were sour cherry, while only a few of the infected trees were sweet cherry (16%, 6 of 38), although none of the pollenizer trees were infected (Table 5.1). Twenty-one infected trees were identified in 2006 and 17 additional trees in 2007, suggesting an increased incidence of PNRSV in the experimental orchard over two consecutive years—likely as a result of natural virus transmission via pollen movement. Taken together, our survey data were consistent with an eightfold higher incidence of PNRSV in sour cherry (33%, 32 of 96) than in sweet cherry (4%, 6 of 136) (Table 5.1). Analysis of DAS-ELISA results indicated that cherry species (*P. cerasus* vs. *P. avium*) was the primary factor influencing PNRSV incidence (df =1; $F = 31.17$; $P = 0.0008$). Rootstock (df =7; $F = 1.04$; $P = 0.4788$) and cultivar × rootstock interaction (df = 7; $F = 1.02$; $P = 0.4186$) were not significant.

Analysis of the spatial distribution of PNRSV-infected trees using the three-dimensional contour mapping feature of Sigmaplot indicated a clustering of the infected trees in close proximity to the five sour cherry trees that exhibited shock symptoms at the beginning of the survey (Figure 5.2). Infected trees clustered in the southwest corner (rows 1 to 2, trees 2 to 20), north-central area (rows 1 to 3, trees 36 to 52), and northeast corner (rows 2 to 4, trees 54 to 61) of the orchard. Infected sour cherry trees were dispersed throughout the orchard, whereas sweet cherry trees were located in the north-central area (rows 1 to 4, trees 36 to 54). These results were consistent with a

short distance spread of PNRSV within the orchard, likely from the initially infected, symptomatic sour cherry trees to adjacent trees.

Latent infection was observed for most of the infected trees (87%, 33 of 38), with a few sour cherry trees (13%, 5 of 38) exhibiting shock symptoms or with severely reduced vigor (Figure 5.1C). PNRSV-infected cherry trees were grafted onto rootstocks Gisela 3, Gisela 5, Gisela 6, Gisela 7, Gisela 195/20, *P. cerasus* L. (Tabel Edabriz), *P. cerasus* L. (Weiroot 10, 13, 72, and 158), *P. mahaleb* L. (Mahaleb), P50, and P-HL-A (Table 5.1). *P. cerasus* cv. Montmorency grafted onto *P. cerasus* L. Weiroot 53 remained PNRSV-free throughout the survey.

The CP genes of a subset of 23 PNRSV isolates (17 from sour cherry trees and six from sweet cherry trees) from the 2007 survey were characterized by RT-PCR. For each of the 23 isolates, a DNA amplicon of the expected size (approximately 675 bp in length) was obtained from total RNA of infected leaf tissue (Figure 5.3).

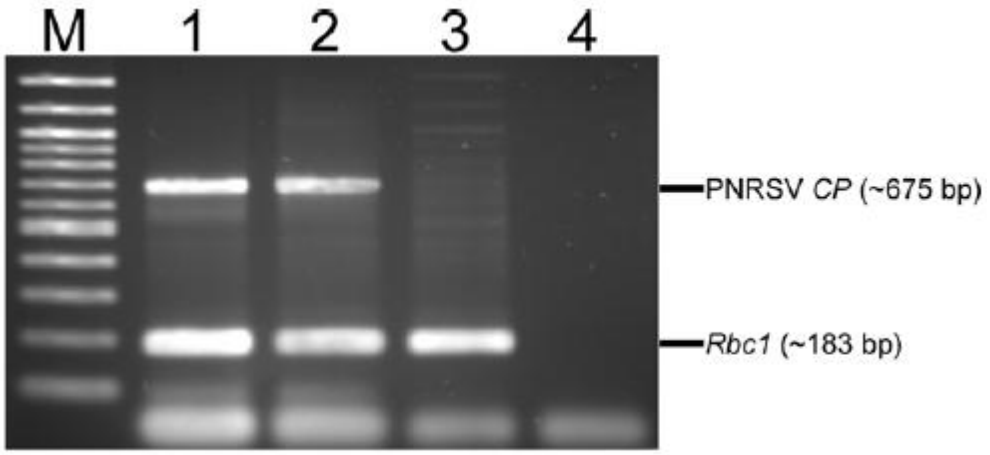

Figure 5.3. Agarose gel analysis of DNA amplicons obtained by multiplex reverse transcription–polymerase chain reaction (RT-PCR). Total RNA extracted from leaves of sour cherry trees was used, along with primer pairs specific to the *Prunus necrotic ringspot virus* (PNRSV) coat protein gene and the *Prunus Rbcl* gene. Lanes 1-2: trees 1.28 and 2.52 infected by PNRSV, respectively; lane 3: healthy tree; lane 4: RT-PCR control lacking total plant RNA; lane M: 100-bp DNA size standard (Promega). PNRSV coat protein gene amplicon is shown at ~675 bp and the *Rbc1* amplicon at ~183 bp.

As expected, no PNRSV DNA product was amplified from total RNA of healthy trees; however, a 183-bp-long fragment corresponding to the plant internal control *RbcI* was obtained (Figure 5.3).

The CP gene amplicons obtained by RT-PCR from 17 PNRSV isolates (13 from sour cherry trees and four from sweet cherry trees) were sequenced. Identical nucleotide sequences were obtained for the majority (59%, 10 of 17) of the isolates characterized (ChrT2, ChrT5, ChrT6, ChrT8, ChrT24, ChrT42, ChrT60, ChrT82, ChrT208, and ChrT225), suggesting a population structure with one predominant molecular variant. The remaining isolates (41%, 7 of 17) corresponded to six minor molecular variants, one for isolates ChrT77 and ChrT133, and one for each of the following isolates: ChrT43, ChrT50, ChrT54, ChrT214, and ChrT224. Nucleotide sequences of PNRSV isolates from sour and sweet cherry that were unique were deposited in GenBank with accession numbers FJ213730 to FJ231738.

The PNRSV isolates sequenced in this study each had a 675-bp-long CP gene with sequence identity ranging from 97 to 100% at the nucleotide level and from 98.2 to 100% at the amino acid level. Interestingly, the CP gene of all isolates, except ChrT214 and ChrT224 (88%, 15 of 17), had higher nucleotide and amino acid (99.6 to 100%) sequence identities. Isolates ChrT214 and ChrT224 were slightly more divergent, with 97 to 97.3% and 97.2 to 99.6% sequence identity at the nucleic acid level, respectively. However, their deduced CP amino acid sequence was highly similar (98.2 to 98.7% for ChrT214 and 99.1 to 99.6% for ChrT224) to that of the other PNRSV isolates, even at the N-terminus where most of the variability is contained (Aparicio et al. 1999, Aparicio and Pallàs 2002, Fiore et al. 2008, Gilmer and Kamalsky 1962, Hammond and Crosslin

Table 5.2. List of *Prunus necrotic ringspot virus* (PNRSV) isolates used in this study to determine phylogenetic relationships.

Host	Isolates	GenBank accession no.	Country
Sour cherry	ChrIt.mrs1	AJ133209	Italy
	PS 7/11	AF170161	Czech Republic
	PS 7/12	AF170162	Czech Republic
	UN	AF170163	Czech Republic
	PS 7/5a	AF170166	Czech Republic
	NRSiz6	AF332615	Poland
	NRSiz8	AF332617	Poland
	NRSiz9	AF332618	Poland
	JW	DQ983491	Poland
	Mk	EU368738	Poland
	KU	AY037791	Slovakia
	NT	AY037790	Slovakia
	ChrT2	This study	USA
	ChrT50	This study	USA
	ChrT54	This study	USA
	ChrT77	This study	USA
	ChrT214	This study	USA
	ChrT224	This study	USA
Sweet cherry	ChrC1.cor1	EF565248	Chile
	ChrC1.bin1	EF565249	Chile
	ChrC1.swe1	EF565250	Chile
	ChrC1.roy1	EF565251	Chile
	1/13	AF170156	Czech Republic
	21/1	AF170157	Czech Republic
	PS 12/16	AF170158	Czech Republic
	PS 14/22	AF170159	Czech Republic
	4/8	AF170165	Czech Republic
	7/20	AF170164	Czech Republic
	ChrIt.lam1	AJ133203	Italy
	ChrIt.bla1	AJ133210	Italy
	NRSiz1	AF332612	Poland
	cz2	DQ983494	Poland
	SW6	AF013287	USA
	ChrT42	This study	USA
	ChrT43	This study	USA
	ChrT133	This study	USA
Wild cherry	Palampur	AM920668	India
P. mahaleb	PV96	S78312	Germany
Flowering cherry	Beijing	DQ300178	P.R. China
Apricot	AprIt.cafl1	AJ133199	Italy
	AprIt.nap1	AJ133200	Italy
	AprIt.try1	AJ133201	Italy
	I-9	DQ983493	Poland
Nectarine	NctSp.mur1	AJ133208	Spain
	NctCl.ear1	EF565252	Chile
	Nct.avg1	EF565253	Chile
Peach	PchIt.may1	AJ133205	Italy
	PchTu.unk1	AJ133206	Tunisia
	PchIt.mry1	AJ133207	Italy
	Unknown1	AM408909	India
	Unknown2	AM408910	India

Table 5.2 (Continued)

	PchCl.pom1	EF565254	Chile
	PchCl.loa1	EF565255	Chile
	PchCl.loa2	EF565256	Chile
	PchCl.ric1	EF565257	Chile
	PchCl.aug1	EF565258	Chile
	PchCl.sum1	EF565259	Chile
	PchBr.unk1	EF565264	Brazil
	PchBr.unk2	EF565265	Brazil
	PchBr.unk3	EF565266	Brazil
	PlmUy.ear1	EF565268	Uruguay
	PchUy.jun1	EF565269	Uruguay
	B56	DQ983492	Poland
	PE-5	L38823	USA
	30/4	NC_004364	USA
Plum	NRSiz0	AF332611	Poland
	NRSiz2	AF332613	Poland
	NRSiz5	AF332614	Poland
	NRSiz7	AG332616	Poland
	PlmAl.unk1	AJ133211	Albania
	PlmIt.Clf1	AJ133212	Italy
	PlmIt.mrb1	AJ133213	Italy
	PlmCl.mrb1	EF565260	Chile
	PlmCl.bla1	EF565261	Chile
	PlmCl.fri1	EF565262	Chile
	PlmCl.dag1	EF565263	Chile
	PL38	EU368737	Italy
	PlmUy.go11	EF565267	Uruguay
	B1	DQ983495	Poland
	U0	DQ983496	Poland
	Emp	DQ983499	Poland
	Prune	AF013286	USA
Almond	Mission	AF013285	USA
	AlmIt.pre1	AJ133202	Italy
	AlmIt.cor1	AJ133204	Italy
	AlmCl.car1	EF565247	Chile
	AL1	EU368735	Australia
	AL17	EU368736	Italy
Apple	Unknown1	AM419814	India
	Unknown2	AM491772	India
	Unknown3	AM931161	India
	PV32	Y07568	Spain
Pelargonium	Pa1	AJ969110	India
Rose	Yunnan	AY684271	P.R. China
	RM-2	AY948440	India
	RM-5	AY948441	India
	E260	AJ619958	India
	Unknown	AJ969095	India
	I-23	DQ003584	Poland
	I1	DQ983497	Poland
	143	DQ983498	Poland

1998, Mink 1992, Spiegel et al. 1999, Ulubas and Ertunc 2004). Isolates ChrT214 and ChrT224 were from two sour cherry trees (trees 60 and 61 in rows 4 and 3, respectively) located in the northeast corner of the orchard (Figure 5.2).

A multiple sequence alignment of the full-length CP gene of 39 PNRSV isolates from cherry (Table 5.2), including those characterized in this study, indicated 88.3 to 100% and 90.2 to 100% sequence identity at the nucleotide and amino acid levels, respectively. This percentage was slightly lower (87.7 to 100% at the nucleotide level and 88 to 100% at the amino acid level) when the nucleotide sequences of the CP gene of 101 PNRSV isolates from *Prunus* and non-*Prunus* species (Table 5.2) were compared.

Phylogenetic analyses of the full-length CP gene sequence of PNRSV isolates from diverse hosts and various geographic origins indicated a clustering of cherry isolates from New York (675 bp) into the predominant group PV-96 together with other isolates (675 to 681 bp) from sour cherry, sweet cherry, peach, apricot, nectarine, plum, almond, and apple from Australia, Europe, South America, and the United States (Figure 5.4). The same clustering of PNRSV isolates was also observed when phylogroups were obtained from the deduced 224 to 226 CP amino acid sequences (data not shown). Furthermore, phylogenetic analyses did not indicate a clear relationship between genetic variability of PNRSV isolates and their geographical origin (Figure 5.4). Similarly, no relationship was found between genetic variability and host genotype (Figure 5.4). Our data on phylogenetic relationships confirmed previous reports (Aparicio et al. 1999, Aparicio et al. 2002, Fiore et al. 2008, Glasa et al. 2002, Ulubas and Ertunc 2004, Vaskavám 2000). Finally, PNRSV isolate ChrT214 was

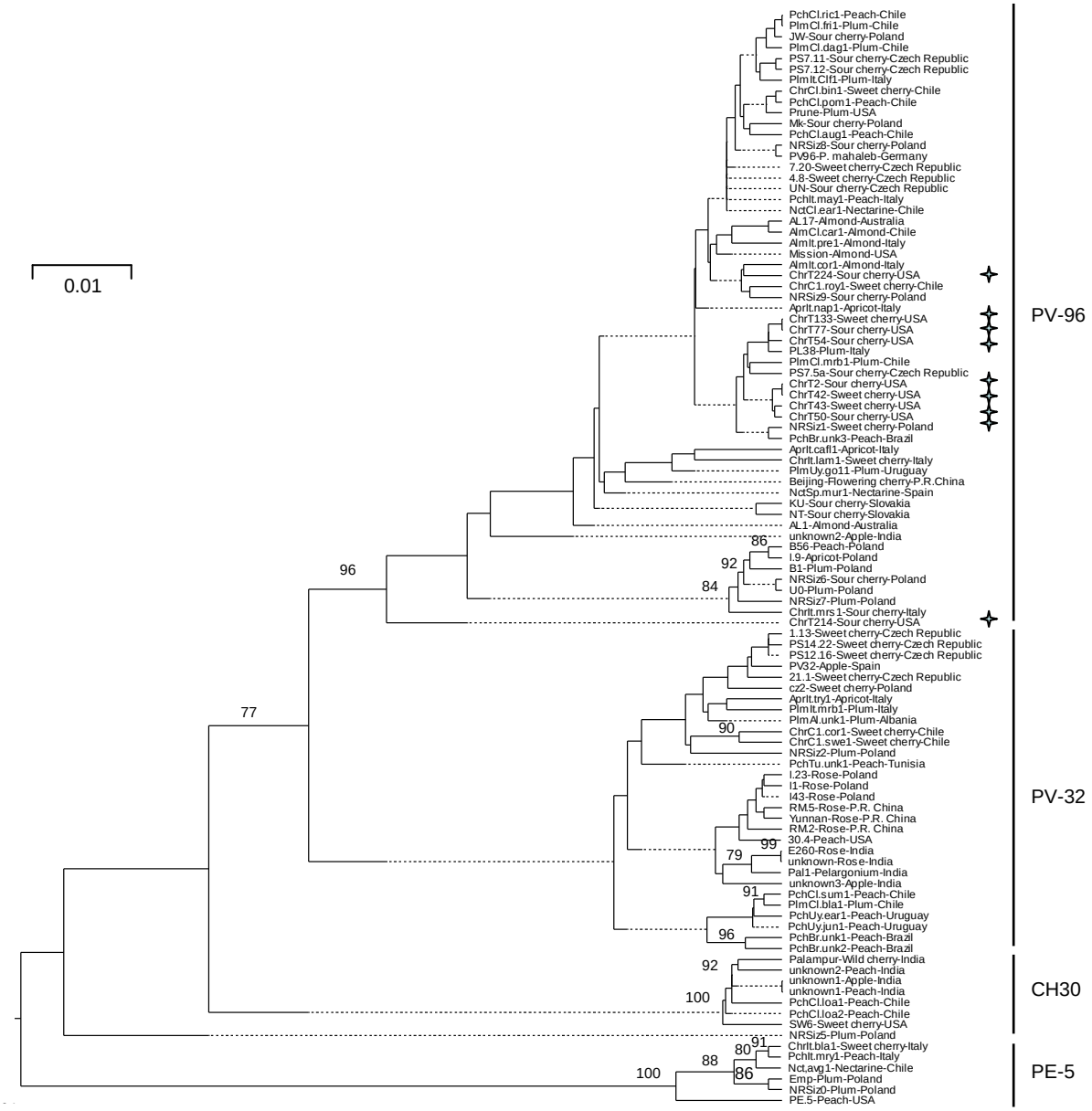

Figure 5.4. Phylogenetic tree reconstructed from complete nucleotide sequence of *Prunus necrotic ringspot virus* (PNRSV) coat protein gene by the neighbor-joining method with 1,000 bootstrap replicates. Branch lengths represent phylogenetic distances determined by distance matrices of nucleotide sequences. Numbers above critical branches are significant bootstrap values (>75%). Scale bar represents a relative genetic distance of 0.01. For each sequence, corresponding isolate and country of origin are indicated. PNRSV isolates sequenced in this study are indicated by stars.

suspected to have resulted from recombination events, based upon multiple nucleotide sequence alignments and phylogenetic analyses (Figure 5.4). However, SISCAN analysis (Gibbs et al. 2000) did not provide strong support for recombination within the CP gene of isolate ChrT214 (data not shown).

DISCUSSION

The incidence of PNRSV was determined by visual symptom monitoring, DAS-ELISA, and RT-PCR in an experimental cherry orchard in which a few trees initially exhibited typical shock symptoms. PNRSV was found mainly in sour cherry trees and to a lesser extent in sweet cherry trees. Latent infection occurred in the majority of infected trees, and severely reduced vigor was observed in a few sour cherry trees. Symptoms ranging from none to mild or severe are typical reactions of cherry to PNRSV infection (Allen 1963a, Gilmer 1961, Gilmer and Kamalsky 1962, Hauck et al. 2002, Mink 1992, Parker et al. 1959, Pscheidt 2007).

The population structure of 17 PNRSV isolates was determined by analysis and comparison of the CP gene sequences. It consisted of one predominant molecular variant and six minor variants. These data are in agreement with the population structure of other plant viruses (Garcia-Arenal et al. 2001). To the best of our knowledge, this is the first report on the population structure of PNRSV isolates within a *Prunus* orchard. We analyzed only a subset of the PNRSV isolates identified in the experimental cherry orchard (50%, 17 of 34) rather than the entire population given the high genetic conservation obtained. Indeed, analysis of the genetic diversity of PNRSV isolates provided insights into the limited variability within the CP gene at the nucleotide

level (0.000 to 0.004), except for isolates ChrT214 (0.030 to 0.037) and ChrT224 (0.004 to 0.028). Also, phylogenetic analysis indicated a clustering of PNRSV isolates from sour and sweet cherry trees in this New York study into group PV-96, in which the majority of PNRSV isolates characterized to date cluster (Aparicio and Pallàs 2002, Fiore et al. 2008, Glasa et al. 2002, Hammond 2003). This is the first report on the genetic variability of PNRSV isolates from sour cherry in North America.

Although relatively limited in number, the PNRSV isolates sequenced in this study were distributed throughout the experimental orchard (data not shown). Thus, our findings on genetic variability enabled us to speculate on the introduction of the virus into the experimental orchard and its subsequent spread between trees. In spite of the fact that the source of PNRSV from which the virus was introduced into the experimental cherry orchard is unclear, only a few trees (16%, 38 of 232) were infected, suggesting that the virus was likely not disseminated by grafting. If grafting were involved, it would have been reasonable to expect a higher number of infected trees. Also, in this case, infection would likely have been detected earlier, instead of 8 years post-planting. Rather, our findings suggest that pollen movement may account for the introduction of PNRSV into the experimental orchard. If pollen movement was responsible, it is likely that the pollen of the virus source host was highly compatible with *P. cerasus* cv. Montmorency since sour cherry trees were predominantly infected. Furthermore, the extremely high nucleotide sequence identity (99.6 to 100%) within the CP gene of most of the isolates (88%, 15 of 17) characterized was consistent with the introduction of a single PNRSV strain. Therefore, we hypothesize that introduction and dissemination of that strain into the experimental cherry orchard occurred through pollen

flow from infected cultivated or wild cherry trees grown in the vicinity of the experimental orchard.

Introduction of PNRSV could have been a single event and, once infection of a few cherry trees (mainly sour cherry) had occurred, the virus could have subsequently continued spreading within the orchard by pollen transfer. Another possibility is that the virus may have been introduced into the experimental orchard by successive, independent pollen transfers. Regardless of the sequence of pollen transfer events from outside sources, PNRSV was introduced into the experimental cherry orchard mainly from a single virus source. The fact that the CP gene sequence of isolates ChrT214 and ChrT224 was slightly divergent at the nucleotide level compared to the other 15 PNRSV isolates characterized in this study suggests that secondary virus sources may exist within the vicinity of the northeast corner of the experimental orchard.

In this study, PNRSV was found to mainly infect sour cherry trees and to a lesser extent sweet cherry trees. These findings are consistent with a slower rate of PNRSV spread in sweet relative to sour cherry, as reported previously in New York orchards (Gilmer 1961). There are at least three plausible explanations for the higher incidence of PNRSV in sour cherry despite the presence of more sweet cherry trees within our experimental orchard. These include potential differences between cherry genotypes in terms of virus susceptibility, flowering phenology, and pollen biology. Since differential susceptibility to PNRSV between sweet and sour cherry is not known (Pscheidt 2007), flower phenology and pollen biology could account for the higher incidence of PNRSV in sour cherry. Sweet cherry is out-crossing while sour cherry is usually self-pollinating (Hauck et al. 2002). Thus, sweet cherry pollen must come from another compatible

cultivar, and a high degree of bee activity on and between sweet cherry trees is required to adequately pollinate the crop during bloom. Pollination of sweet cherry was achieved in this way in the experimental orchard, and sweet cherry production was high throughout the survey for PNRSV (R. L. Andersen, *unpublished*). Sour and sweet cherry do not readily pollinate each other, although cross-pollination can occur to a limited extent (Hauck et al. 2002). Therefore, if PNRSV had infected sour cherry first in our experimental orchard, it is conceivable that pollen production and movement from PNRSV-infected *P. cerasus* cv. Montmorency occurred more readily within sour cherry trees than from sour cherry to sweet cherry trees. Furthermore, although sweet cherry trees start blooming before sour cherry trees, bloom times often overlap. However, if pollen movement in sour cherry trees was not in synchrony with stigma development in *P. avium* cv. Hedelfingen, this may have also favored PNRSV movement within sour cherries and hindered movement from sour cherry to sweet cherry trees. As a consequence of both of these scenarios, more sour cherry trees would be expected to become infected with PNRSV, explaining the higher incidence of the virus among sour cherry trees seen in this study.

Our findings on the genetic variability of PNRSV isolates provide further evidence of pollen movement and virus spread within the experimental orchard. The nucleotide sequences of the CP gene of several isolates from sour cherry (ChrT2, ChrT5, ChrT6, ChrT8, ChrT24, ChrT42, ChrT60, ChrT82, and ChrT225) and isolate ChrT208 from sweet cherry are identical, suggesting movement of a single PNRSV isolate from sour to sweet cherry trees. Similarly, the nucleotide sequences of the CP gene of isolates ChrT77 from sour cherry and ChrT133 from sweet cherry are identical. These data are

consistent with limited pollen-mediated transfer of PNRSV from sour cherry to sweet cherry trees.

Previously, the susceptibility of cherry rootstocks to PNRSV was investigated by graft-inoculation of *P. avium* cv. Bing trees (Howell and Lang 2001, Lang and Howell 2001). The following rootstocks were classified as sensitive: Gisela 7, Gisela 195/20, and Weiroot 10, 13, and 53. Gisela 3, Gisela 5, Gisela 6, Tabel Edabriz, Mahaleb, and Weiroot 72 and 158 were classified as tolerant (Howell and Lang 2001, Lang and Howell 2001). Tree reaction to graft-inoculation of PNRSV was evaluated based on symptom severity, i.e., gum exudation at the graft union, inhibition of lateral shoot elongation, foliar symptoms, and rapidity of tree death (Howell and Lang 2001, Lang and Howell 2001). Our survey data indicate moderate to high infection rates of *P. cerasus* cv. Montmorency grafted onto Gisela 6, Gisela 7, Tabel Edabriz, Mahaleb, and Weiroot 72 and 158, and low infection rates of trees grafted onto Gisela 3, Gisela 5, and Weiroot 10, 13, and 53. Although our work did not focus on cherry rootstock susceptibility to PNRSV, there seems to be a differential reaction of cherry trees to PNRSV infection following graft-inoculation (Howell and Lang 2001, Lang and Howell 2001) and pollen transfer (this study) for Gisela 6, Gisela 195/20, Tabel Edabriz, Mahaleb, and Weiroot 10, 53, 72, and 158. One plausible explanation for this apparent discrepancy is the fact that cherry tree reaction to graft-inoculation in previous studies (Howell and Lang 2001, Lang and Howell 2001) was scored based on disease severity, whereas in this study, reaction to pollen infection was evaluated based on monitoring symptom development and PNRSV detection in the canopy by DAS-ELISA and RT-PCR. Differences in virus strains, environmental conditions, and scion genotype (*P.*

avium cv. Bing versus *P. cerasus* cv. Montmorency) could also account for these differential reactions of scions grafted onto different rootstocks (Howell and Lang 2001, Lang and Howell 2001 versus our study).

Host resistance would be an ideal strategy to manage PNRSV. Since resistance to PNRSV is not known in *Prunus* spp., including sour and sweet cherry (Mink 1992, Pscheidt 2007, Scott et al. 1989, Uyemoto and Scott 1992), the foremost control strategy in an orchard is exclusion. This approach consists of identifying infected trees at a very early stage of infection followed by diligent removal and destruction in order to limit secondary virus spread to healthy trees or to new orchards. Since latent infection is common for PNRSV, virus spread can readily occur through pollen transmission even when trees are asymptomatic. Therefore, until resistant trees can be developed, surveying cherry orchards by DAS-ELISA and RT-PCR, as shown in our study, is critical for optimal PNRSV detection and subsequent elimination of infected trees.

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CHAPTER 6

Implications and Future Work

The devastating effects of *Grapevine fanleaf virus* (GFLV) on vine vigor and fruit yield cannot be easily avoided once GFLV and its nematode vector, *Xiphinema index*, become established in a vineyard setting (Andret-Link et al. 2004). Currently, environmentally consequential nematicides, extended fallow periods and the use of rootstocks tolerant to *X. index* are the only control options against GFLV - since there are no commercially available grapevine materials with resistance to this virus (Andret-Link et al. 2004, Oliver and Fuchs 2011). Efforts to develop transgenic grapevine rootstocks to control GFLV have shown promise in some cases, including a three-year field trial in France (Vigne et al. 2004). However, the long-term effectiveness of these transgenic grapevines for GFLV management has not yet been determined. Based upon evidence from studies of other virus-resistant transgenic plants (Oliver et al. 2011), including grapevines (Fuchs and Gonsalves 2007), this technology is safe for use in crop plants, and the durability of this type of resistance is comparable to traditionally developed resistant varieties (Oliver et al. 2011). However, since the existing transgenic grapevines for GFLV resistance have not been tested over an extended period, and, in addition, were developed using a single GFLV gene from a single GFLV isolate, it is possible that a resistance breakdown could occur - due to the sequence specificity of the RNA silencing process (Prins et al. 2008) - if these

grapevines were to encounter a GFLV isolate that was divergent from the transgene at the nucleotide sequence level.

In order to develop transgenic grapevines with resistance that might be more effective, we analyzed the genomic variability of GFLV to identify regions that are conserved among GFLV isolates. By choosing conserved genomic regions for the design of GFLV resistance transgenes, as well as by combining conserved fragments from multiple parts of the viral genome into a single construct, we believe that the resulting transgenes will be more likely to confer resistance to GFLV in a broad-spectrum and durable manner.

Since the development and testing of transgenic grapevine materials can be a time-consuming, expensive, and laborious process - due at least in part to the limited means available to infect grapevines with GFLV (nematode-mediated or graft-inoculation only), to the grapevine's perennial nature, and to its relative large size which makes greenhouse trials unfeasible (Oliver and Fuchs 2011) - we developed a transient assay system for testing these constructs' ability to reduce GFLV multiplication in the systemic host, *Nicotiana benthamiana*. This transient assay system has the advantage of being both high-throughput (able to easily test numerous constructs) and fast (a relatively quick testing turnaround). In addition, it can quite easily be adapted for challenging constructs with diverse viral variants of GFLV. The use of this transient assay allowed for the most promising GFLV resistance constructs to be identified and used for grapevine transformation – potentially saving significant time and money related to the development and field testing of transgenic grapevine rootstock lines for each construct.

Ultimately, the usefulness of the transgenic constructs that we developed (and the transient assay's ability to identify those that are most promising in terms of resistance) has not yet been proven. Only through the transformation and testing of grapevine material in realistic field settings, where the transgenic grapevine materials encounter many GFLV viral variants via nematode-based inoculations, will we be able to prove the usefulness of this transient assay system and these constructs. This work remains to be completed with grapevine rootstocks developed from our transgenic constructs. Field testing in multiple locations where transgenic grapevines might be grown is important, but we believe that the testing that is already underway for some of these rootstocks in the very same vineyard where our sequencing study was carried out (Oliver et al. 2010) is highly justified based on the population diversity identified.

One potential concern regarding the constructs that we developed relates to the somewhat inconsistent and low numbers of stably transformed *N. benthamiana* that have shown resistance upon GFLV challenge so far. Though the reason for these low numbers is unclear, it may relate to the relative abundance of siRNA produced from these constructs *in planta*. While *Agrobacterium* transient expression in general results in high levels of transgene transcription within the infiltrated cells, often siRNA production in stable transformants may vary depending on transgene copy number, insertion loci, degree of methylation, effectiveness of suppressors of silencing, and other variables (Prins et al. 2008). Agroinfiltration of constructs designed to trigger RNA silencing has been reported to result in much higher concentrations of siRNA within the agroinfiltrated zone versus those seen during systemic silencing (Kościańska et al. 2005). These factors may account for the results seen in our studies. For this reason it

would be informative if the relative amounts of siRNAs produced from the transgenes could be compared in the stable transformants and in leaves in which transient expression is taking place. Observed differences might explain some of the apparent inconsistencies between the transient assay and the stable transformation results. However, it should be acknowledged that relatively few of the constructs have been tested past the T_0 generation, and testing of the corresponding T_1 or T_2 lines may yet indicate a greater proportion of resistant individuals. If, however, these generations also show a relatively low number of resistant individuals (and especially if this can be connected with low siRNA production from the transgene), then it might be possible to clone the constructs into hairpin, inverted repeat, or intron-separated orientations which have been shown to increase the proportions of transformants exhibiting the siRNA production necessary to confer resistance to viral challenge (Prins et al. 2008, Wesley et al. 2001).

Relating to the transient assay itself, since the reduction in virus multiplication is only observable within the agroinfiltrated leaf area, it might also be interesting to use a transient means to stimulate systemic silencing within the plant, perhaps by using the Virus Induced Gene Silencing (VIGS) system developed by Dinesh-Kumar et al. (2003). This possibility was of interest to us during this work, and each of the constructs we developed was cloned into the pTRV2 plasmid for VIGS expression (Dinesh-Kumar et al. 2003). However, these plasmids containing our constructs of interest have not yet been tested, so their usefulness is unclear at this time.

Also, with respect to the transient assay system, though it was developed with the idea of being amenable to challenging constructs with divergent GFLV isolates,

currently only two isolates of GFLV have been used to challenge constructs: GFLV-F13 and GFLV-GHu. These two isolates are more than 15% different from one another at the nucleotide level. In order to test whether any of our constructs are capable of conferring broad-spectrum resistance against numerous potential viral variants, the use of additional isolates may be informative, and reveal as yet unobserved isolate-specific differences between constructs.

Ultimately, the goal of this work has been to develop useful transgenic resistance to GFLV for grapevine rootstocks. If transgenic grapevine rootstocks exhibiting high levels of resistance to GFLV can be deployed in naturally infected vineyards, it would provide growers with a valuable means of controlling this pathogen. In addition, if the constructs that we have developed work as expected, these vines would exhibit durable and broad-spectrum resistance to GFLV and protect scions grafted onto them from GFLV infection. Also, since the resistance would be provided by the rootstocks, the popular scion varieties that are currently grown could continue to be used by growers - and as most grapevines are currently grafted anyway, this would minimally impact current grower practices. The fact that the grafted scion varieties would not be transgenic may also make the use of transgenic grapevine rootstocks more widely acceptable by consumers who – despite the evidence of the safety of virus-resistant transgenic plants – remain hesitant to consume transgenic plant products. Also, with respect to the environment, the use of transgenic GFLV-resistant rootstocks would provide a means of virus control without the use of the harsh nematicides currently used to control this disease problem. Furthermore, with respect to a perceived environmental risk of transgenic crop plants relating to gene flow to free-living relatives, the risk of

gene flow from rootstocks, which are not allowed to flower in vineyard settings under current practices, would be comparatively low.

It is my hope that my work presented in this dissertation may play a part in helping these potential benefits of virus-resistant transgenic grapevines to be realized.

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