Chapter 31
Biotechnology Applications of Grapevine Viruses

V.V. Dolja and B. Meng

Abstract Plant virus genomes are engineered as vectors for functional genomics and production of foreign proteins. The application of plant virus vectors is of potential interest to the worldwide, multibillion dollar, grape and wine industries. These applications include grapevine functional genomics, pathogen control, and production of beneficial proteins such as vaccines and enzymes. However, grapevine virus biology exerts certain limitations on the utility of the virus-derived gene expression and RNA interference vectors. As is typical for viruses infecting woody plants, several grapevine viruses exhibit prolonged infection cycles and relatively low overall accumulation levels, mainly because of their phloem-specific pattern of systemic infection. Here we consider the biotechnology potential of grapevine virus vectors with a special emphasis on members of the families *Closteroviridae* and *Betaflexiviridae*.

Keywords Plant viruses • Gene expression vectors • RNAi • Functional genomics • Grapevine • *Closteroviridae* • *Betaflexiviridae*

Introduction

The decades-long history of transient gene expression vectors derived from plant viruses went through a period of initial exuberance followed by a more sober understanding and development of their practical applications (Dawson 2014; Gleba et al. 2014). A main promise of viral vectors is their abilities to replicate and to produce high levels of virus-derived mRNAs and proteins with no need for stable plant transformation resulting in genetically modified plants. These abilities are particularly

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strong in positive-strand RNA viruses that directly replicate mRNAs using their RNA-dependent RNA polymerase (RdRp) and RdRp recognition signals present in viral RNAs. In contrast, plant hosts, similar to all other cellular organisms, do not replicate their mRNAs, but rather transcribe them from their DNA genomes. The only known RNAs replicated by plants are small interfering RNAs or siRNAs that are derived from double-stranded RNA produced by the host RdRp that is nonhomologous to viral RdRps (Shabalina and Koonin 2008).

The major applications of plant virus vectors are epitomized by *Tobacco mosaic virus* (TMV), *Tobacco rattle virus* (TRV), and *Potato virus X* (PVX) (Kumagai et al. 1995; Ratcliff et al. 2001; Burton et al. 2000), which are the most widely used in protein expression and functional genomics. The TMV vector is characterized by a fast infection cycle and extremely high levels of virus-encoded protein accumulation in infected cells and plants. The major drawback of this vector is a relatively low genetic stability that results in a rapid loss of the inserted foreign genes. To mitigate this drawback, streamlined/deconstructed TMV vectors delivered by agro-inoculation were designed (Gleba et al. 2014). By avoiding genetic bottlenecks of the virus systemic transport that favor deletion of foreign inserts, massive direct agro-infection of whole plants has deflected the virus infection cycle toward rapid protein production. By deleting the TMV capsid protein gene required for systemic infection, the host cell resources were redirected to mass production of the recombinant protein, thus elevating the yield to 80% of the total soluble protein in leaf tissue or 5 g per kg of the "wet" leaf biomass (Gleba et al. 2014).

The RNA interference (RNAi) machinery is one of the molecular signatures of eukaryotes that has likely emerged as an antiviral host defense response and has subsequently diversified to fulfill a multitude of additional functions (Shabalina and Koonin 2008). Unlike animals, plants do still rely on RNAi as a major way to withstand viral infections. In a counter-defense response, a variety of diverse RNAi suppressors have evolved in plant viruses (Csorba et al. 2015). As a result, modulation of virus infection process by the interplay between plant RNAi and viral suppressors results in a spectrum of virus infection cycle scenarios ranging from host immunity to extreme susceptibility, depending on specific virus-host combinations and environmental conditions. One of the rather counter-intuitive outcomes of such interplay is virus-induced gene silencing (VIGS), a process whereby insertion of a recombinant nucleic acid fragment into virus genome triggers RNAi-mediated degradation of the RNAs possessing identical sequence. The VIGS approach is now widely used in functional genomics via transient silencing of the endogenous plant genes and assessing the resulting phenotypes. Although several plant viruses infecting both dicots and monocots were developed into VIGS vectors (Lacomme 2014), one derived from TRV appears to be the most potent and most widely used in such studies (Bachan and Dinesh-Kumar 2012).

This review focuses on the biotechnological potential of grapevine viruses (such as its applications in protein expression and functional genomics) that depends primarily on knowledge of their replication and genome expression mechanisms and particular features of their host biology.

Arguably, the most significant impediment to developing virus vector technologies for grapevine is the recalcitrance of this woody plant host to both mechanical inoculation and agro-infiltration, two techniques broadly used to launch virus vectors into host plants. In vineyard settings, most of the grapevine viruses are transmitted either by invertebrate vectors, such as mites, mealybugs, or nematodes, or by grafting and top working, processes that are prohibitive for the use of engineered virus vectors. Furthermore, the systemic spread of the viruses in grapevine upon initial infection is slow, on a scale of months, another obstacle to facile development of useful virus vectors. Here, we will consider the most promising approaches to overcoming these problems and paving the way to broader implementation of the virus vector technologies suited for grapevine.

Although in theory any of the grapevine-infecting viruses can be engineered into transient gene expression or VIGS vector, in practice, only one of them, the filamentous *Grapevine leafroll-associated virus-2* (GLRaV-2) from the genus *Closterovirus* (family *Closteroviridae*), was demonstrated to fulfill these roles (Dolja and Koonin 2013; Kurth et al. 2012). Ongoing work will likely result in successful development of additional vectors derived from other members of the family *Closteroviridae*. Certain progress has also been made toward developing vectors based on representatives of the genera *Vitivirus* and *Foveavirus* of the family *Betaflexiviridae* that are also filamentous, positive-strand RNA viruses (Muruganantham et al. 2009; Meng et al. 2012). Additional attractive opportunities in this field include *Grapevine fanleaf virus* (GFLV), an icosahedral, positive-strand RNA nepovirus capable of expressing a recombinant protein (Amari et al. 2010), and a single-strand DNA, geminivirus-like *Grapevine red blotch-associated virus* (Sudarshana et al. 2015).

Closterovirus-Derived Gene Expression and VIGS Vectors

The *Closteroviridae* is a large and economically important family of positive-strand RNA viruses that infect a variety of crop plants including grapevine, citrus, small fruits, and vegetables. The genomes of closterovirids are the largest among plant RNA viruses and come second to only those of the family *Coronaviridae* of animal viruses (Dolja et al. 2006). Based on phylogenetic analysis, genome architecture, and transmission by distinct insect vectors, this family is classified into four virus genera: the aphid-transmitted *Closterovirus*, the mealybug-transmitted *Ampelovirus*, the whitefly-transmitted *Crinivirus* (Dolja et al. 2006; Karasev 2000), and *Velarivirus* for which no insect vector is known (Al Rwahnih et al. 2012; Martelli et al. 2012). Each of these genera except for the genus *Crinivirus* contains grapevine-infecting viruses, most of which are associated with the leafroll disease complex. Among these viruses, only GLRaV-2, a closterovirus, has been so far engineered into a vector capable of systemic infection of grapevine that either produces recombinant protein or elicits VIGS response (Kurth et al. 2012). The most important aspects of *Closterovirus* research that enabled this development are considered below.

The first prerequisite of generating an RNA virus-based vector is a fully biologically active cDNA clone of the virus genome. Originally, such a clone has been developed for *Beet yellows virus* (BYV), a prototype closterovirus (Dolja 2003; Peremyslov and Dolja 2007). Due to the large size of BYV genome (15.5 kb), this development has been done in three steps. First, a full-length cDNA clone was generated and demonstrated to be replication-competent upon protoplast transfection with in vitro transcripts, allowing mapping of the replication-associated genes (Peremyslov et al. 1998). At the time, it was the largest cDNA clone available for any RNA virus. However, because this clone was defective in virus cell-to-cell movement, screening of additional BYV cDNA clones has been done, yielding variants competent in cell-to-cell movement (Peremyslov et al. 1999). Finally, due to relatively low infectivity of RNA transcripts upon mechanical inoculation, the viral cDNA has been cloned into a binary plasmid useful for agro-inoculation, a more efficient inoculation technique aided by agrobacteria that launch viral cDNA into plant cell nuclei, jump-starting its transcription followed by RNA translation and replication (Prokhnevsky et al. 2002).

All of the three incarnations of the BYV cDNA were tagged with either β-glucuronidase (GUS) or green fluorescent protein (GFP) reporters to facilitate measurements of virus replication and to visualize infected cells. Using these vectors was paramount to the identification of genes contributing to genome replication (Peng and Dolja 2000; Peremyslov et al. 1998), virus cell-to-cell movement (Alzhanova et al. 2000; Peremyslov et al. 1999, 2004b), virion assembly (Napuli et al. 2000, 2003; Peremyslov et al. 2004a), as well as the interdependence of the latter two processes (Alzhanova et al. 2001, 2007). Finally, two genes contributing to BYV systemic transport (Peng et al. 2003; Prokhnevsky et al. 2002) and a gene coding for a strong RNAi suppressor (Reed et al. 2003) were also identified, thus completing the functional characterization of the BYV genome (Dolja 2003). It was later found that co-expression of strong suppressors of RNAi with the BYV cDNA increased the number of primarily infected cells upon agro-inoculation by up to three orders of magnitude, thus boosting the efficiency of this process (Chiba et al. 2006). This phenomenon emphasized a critical role of a host RNAi defense in the virus invasiveness, that is, the ability to establish infection in the primarily inoculated cells. In addition, mapping of the transcription start sites of the BYV subgenomic RNAs and characterization of the dynamics of their accumulation provided critical information on the mechanisms of BYV genome expression (Agranovsky et al. 1994; Hagiwara et al. 1999; Peremyslov and Dolja 2002; Vitushkina et al. 2007).

Concurrently, important work on other closterovirids, *Citrus tristeza virus* (CTV) (Dawson et al. 2015) and *Lettuce infectious yellows virus* (LIYV, genus *Crinivirus*) (Tian et al. 1999), provided synergistic contributions to understanding of their molecular biology and functional genomics. In particular, a replication-competent CTV cDNA clone, even larger than that of BYV, has been generated and tagged with reporter genes (Folimonov et al. 2007; Satyanarayana et al. 1999). The more recently developed CTV-based gene expression vectors were shown to be not only capable of systemic infection in the natural citrus hosts but also exhibited remark-

able genetic stability in regard to retention of the inserted recombinant gene, as well as VIGS capability (Dawson et al. 2015; Hajeri et al. 2014).

The studies of BYV provided the bulk of knowledge on the engineering of the closterovirus genome required to generate an analogous cDNA clone of GLRaV-2. There were, however, several features that distinguish the two viruses and had to be investigated before this task could be successfully accomplished. Unlike BYV, which has one papain-like leader proteinase that is required for efficient genome amplification and systemic infection (Peng et al. 2003; Peng and Dolja 2000), GLRaV-2 has two such proteases that have likely evolved via gene duplication and functional divergence (Meng et al. 2005; Peng et al. 2001). Similar to other members of the family *Closteroviridae*, BYV is transmitted by aphids, whereas no aphid or other insect vectors are known for GLRaV-2, which, in agricultural settings, is transmitted exclusively by grafting or top working (Angelini et al., this book). This latter feature of GLRaV-2 is a positive attribute in regard to its vector potential, because it mitigates regulatory concerns for the uncontrolled spread of the modified virus via biological vectors. Perhaps, the most prominent biological differences between the two viruses is that BYV naturally infects several herbaceous hosts and is capable of exiting the phloem into surrounding tissues, whereas GLRaV-2 is only found in grapevine, where it is limited to phloem, similar to other grapevine viruses from the genera *Ampelovirus* and *Velarivirus* (Martelli et al. 2012). Importantly, grapevine is a perennial woody host that is recalcitrant to mechanical inoculation by viruses, likely due to leaf hardiness defined both in physical and chemical terms. It should be emphasized, however, that unlike other closteroviruses of the leafroll disease complex, GLRaV-2 can be mechanically transmitted (albeit with difficulty) to a herbaceous plant, *Nicotiana benthamiana*, a promiscuous experimental host for a vast variety of plant viruses including BYV.

Because GLRaV-2 accumulates in *N. benthamiana* to higher levels than in grapevine, this convenient herbaceous host was used to isolate the virus, to sequence its genome and to engineer the first-generation full-length GLRaV-2 cDNA clone (Liu et al. 2009). Similar to the most advanced BYV clones, this GLRaV-2 clone possessed a strong 35S promoter derived from *Cauliflower mosaic virus* for viral cDNA transcription upon agro-inoculation (Fig. 31.1). A ribozyme sequence has been inserted downstream from the 3′-terminal nucleotide of the viral cDNA to facilitate release of the authentic viral RNA from a primary transcript, the termination of which was directed by the nopaline synthase terminator. This clone was tagged by insertion of the GFP open reading frame (ORF) downstream from the translation initiation codon of the viral capsid protein gene. Thus, a GFP-encoding recombinant subgenomic (sg) RNA has been produced under control of the native GLRaV-2 sgRNA promoter to direct GFP expression (Fig. 31.1). To restore expression of the GLRaV-2 capsid protein, a BYV sgRNA promoter that directs capsid protein expression in this closely related virus has been inserted downstream from the GFP stop codon (Liu et al. 2009). As was originally demonstrated for TMV (Donson et al. 1991), the use of heterologous sgRNA promoters from similar viruses to express recombinant genes is superior to duplication of homologous promoters

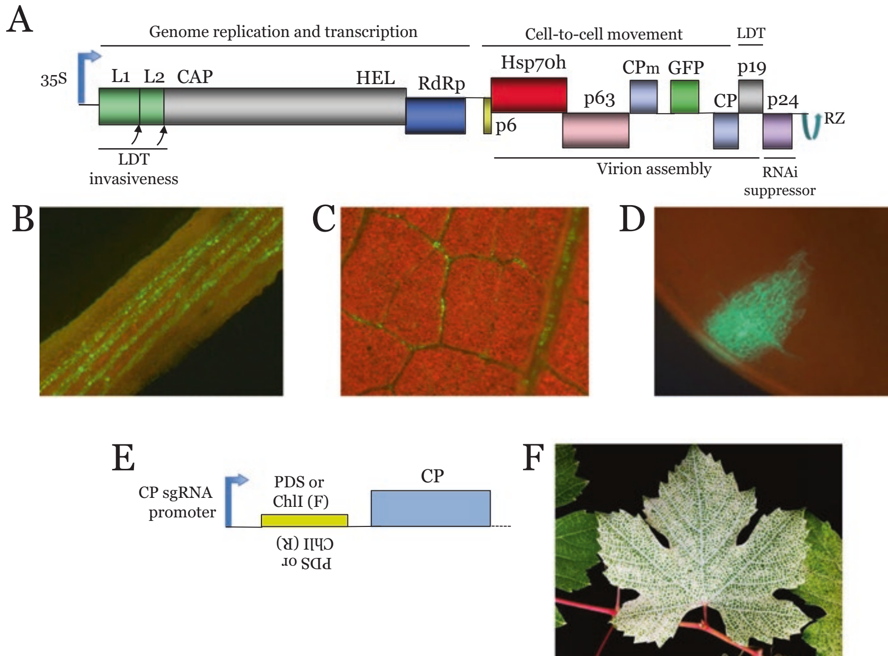

Fig. 31.1 The GLRaV-2-derived gene expression and VIGS vector dubbed vLR2 and engineered to express GFP (**a**–**d**) or a fragment of grapevine endogenous genes (**e**, **f**). (**a**) Genome map of vLR2-GFP. L1 and L2, papain-like leader proteases; CAP, capping enzyme, HEL, RNA helicase; RdRp, RNA-dependent RNA polymerase; p6, 6-kDa movement protein; Hsp70h, heat shock protein, 70-kDa, homolog; p63, 63-kDa virion protein; CPm, minor capsid protein; CP, major capsid protein; p19, 19-kDa protein; p24, 24-kDa RNAi suppressor. Gene functions inferred from BYV homologs are shown above and below diagram (**b**) Imaging of the vLR2-GFP in the inner bark of grapevine plants. (**c**) Imaging of the vLR2-GFP in the leaf veins. (**d**) Invasion of the vLR2-GFP into berry mesocarp. (**e**) Expression cassettes harboring RNAi-triggering gene fragments derived from the grapevine PDS or ChlI genes. Inserts were either in forward (*F*) or reverse (*R*) orientation. (**f**) Image of the leaf bleaching symptoms caused by VIGS of ChlI induced vLR2 infection. Note different bleaching levels in adjacent leaves

because the latter induce high rates of homologous recombination and rapid loss of the inserted genes.

Upon agro-inoculation to *N. benthamiana* plants, the resulting GLRaV-2 cDNA clone was able to establish a systemic infection in the phloem tissue. This virus-host combination has been used to determine contributions of the GLRaV-2 leader proteinases to polyprotein processing, RNA amplification, and long-distance transport (Liu et al. 2009). It was also found that each of the leader proteinases is required for virus invasiveness defined as an ability to establish infection in the primarily inoculated cells in grapevine. However, LR-GFP failed to establish systemic infection in the virus' natural host, grapevine, suggesting that propagation in *N. benthamiana* might have resulted in selection of a virus variant fit to reproduce in this herbaceous host, but not in grapevine.

To test this possibility, the entire viral cDNA clone has been reassembled using cDNA fragments obtained by reverse transcription of the GLRaV-2 genomic RNA present in the infected grapevine. Only the fragments with a consensus sequences were used in this process to avoid incidental mutations that could emerge during error-prone virus replication or cDNA generation. The resulting reassembled cDNA clone vLR2-GFP contained as many as 75 single nucleotide differences compared to that of the *N. benthamiana*-propagated LR-GFP. Some of these differences could be due to natural variation between the two virus isolates used in this work, whereas others could have resulted from propagation of the original isolate in *N. benthamiana*. Strikingly, the vLR2-GFP was systemically infectious in grapevine upon vacuum agro-infiltration of the whole micropropagated plantlets that were transferred to soil following this process (Kurth et al. 2012).

Investigation of the vLR2-GFP infection dynamics showed that, starting at ~1 month upon agro-inoculation, the virus was initially detected in the stem phloem cells, then in leaf petioles, gradually invading leaf veins and later entering the root phloem. When the berry clusters emerge, vLR2-GFP was detected in some berries where it was present in phloem vasculature, later exiting into mesocarp (berry flesh) cells (Kurth et al. 2012). The visual symptoms of virus infection including leaf reddening appeared late in a season; typically, these symptoms induced by vLR2-GFP infection were milder compared to those of the wild type GLRaV-2.

One of the most common limitations of the plant virus-derived gene expression vector is their relatively low genetic stability that is particularly problematic in TMV-based vectors (Dawson 2014; Gleba et al. 2007). Even in potyvirus vectors, in which there is selection pressure for the maintenance of the polyprotein-encoding open reading frame, a few weeks long propagation of the vector infection results in consistent appearance of variants with truncation or total loss of the expression cassette (Dolja et al. 1992; 1993). This overall genetic instability was attributed to spontaneous nonhomologous recombination that shortens virus vector genome and gives the resulting variants competitive advantage over the intact vector genomes. The vLR2-GFP vector exhibited much greater genetic stability in a course of infection in grapevine. Only a fraction of vector-infected plants showed deletions within the expression cassette at 1 year postinoculation, providing an ample time window for using this vector for both research and applied purposes (Kurth et al. 2012).

Further boost to the utility of vLR2 vectors was their ability to elicit strong virus-induced gene silencing (VIGS) response in the infected grapevine. Given that BYV and GLRaV-2 possess strong suppressors of RNAi (Chiba et al. 2006; Reed et al. 2003), the VIGS capability of vLR2 was rather surprising. This capability was demonstrated via insertion into vLR2 expression cassette of the cDNA sequences derived from the endogenous grapevine genes encoding the enzymes required for chlorophyll biogenesis, phytoene desaturase (PDS), and subunit I of magnesium-protoporphyrin IX chelatase (ChlI) (Kurth et al. 2012). The few hundred nucleotides-long fragments of the PDS or ChlI ORFs were engineered into vLR2 in either forward or reverse orientation (Fig. 31.1e); each of the four resulting vector variants caused strong VIGS response upon grapevine infection. This response was manifested as yellow or white chlorosis due to chlorophyll photobleaching that initially

appeared along the leaf veins where virus replicated, gradually spreading into other leaf tissues (Fig. 31.1f). Upon growth of the vines, VIGS symptoms appeared in cyclical manner apparently reflecting complex pattern of virus spread, VIGS response, and plant growth and differentiation. Once again, VIGS was well pronounced in plants for long periods of time, in excess of 17 months postinoculation (Dolja and Koonin 2013). Although the mechanisms underlying unusual genetic stability of the vectors derived from closteroviruses including GLRaV-2 and CTV are not known, it seems possible that, similar to coronaviruses, closterovirus replication-associated polyproteins contain RNA-processing enzymatic domains with the proofreading activities (Denison et al. 2011). By reducing the number of mismatches, and/or the replicase-template dissociation rate, these very large replication complexes could therefore reduce the frequency of deletions via copy-choice mechanism.

Obviously, with the significant progress in understanding molecular biology of the closteroviruses, any of these viruses infecting grapevine could be developed into gene expression and, potentially, VIGS vectors. However, the utility of such vectors could be limited by at least two important features related to the virus biology. One such feature is transmissibility by the insect vectors. For instance, GLRaV-1 and GLRaV-3 are transmitted by several mealybug and soft scale insect species raising a serious regulatory concern with the release of corresponding recombinant viruses into agricultural settings. Another problem is the relatively high pathogenicity of GLRaV-1 and GLRaV-3, each of which severely affects vine productivity and also results in gradual decline of infected plants (Maree et al. 2013). Thus, tagging each of these viruses with a reporter such as GFP could be useful for investigating molecular and cellular biology of virus infection, but practical utility of the corresponding vectors is questionable at best.

There is, however, a closterovirid, the biology of which appears even better suited for the purposes of virus vector development than that of GLRaV-2. This virus was traditionally designated GLRaV-7; however, at least by itself, it is not known to cause leafroll or any other detectable disease symptoms in grapevine. Furthermore, GLRaV-7 is not known to be transmitted by any vector organisms. Sequencing of the entire ~16.5 kb GLRaV-7 genome followed by phylogenetic analysis showed substantial divergence from each of the three previously established genera of the family *Closteroviridae* (Al Rwahnih et al. 2012). On the other hand, this analysis revealed a significant relatedness of GLRaV-7 with two other unclassified closterovirids also not known to elicit pronounced disease symptoms, *Little cherry virus 1* and *Cordyline virus-1* (Jelkmann et al. 1997; Melzer et al. 2011). Recently, these three viruses were classified by ICTV into a new genus termed *Velarivirus*. It seems all but certain that GLRaV-7 will be developed into a promising gene expression vector for grapevine, although its VIGS potential is yet to be determined.

Vector Potential of Vitiviruses and Foveaviruses

Betaflexiviridae is another important plant virus family that contains 11 genera of single-stranded, positive sense RNA viruses with filamentous virion morphology (King et al. 2012). Three of the genera contain viruses that naturally infect grapevine: *Vitivirus*, *Foveavirus*, and *Trichovirus*. The genomes of viruses in the family *Betaflexiviridae* range from 6.5 to 9.3 kb in size and encode between two and six ORFs, depending on the specific genus. For example, members of the genus *Vitivirus* have genomes of ~7.6 kb which encode five ORFs with a single movement protein. Like members of the family *Closteroviridae*, viruses of the genus *Vitivirus* are restricted to the phloem tissue (King et al. 2012). In contrast, members of the genus *Foveavirus* have larger genomes (8.7–9.3 kb) that encode five ORFs, three of which encode a set of three movement proteins collectively termed as the triple gene block (Martelli and Jelkmann 1998; King et al. 2012). The type member of the genus *Foveavirus*, *Apple stem pitting virus* (ASPV), is not phloem limited. However, it remains to be determined if other members of the genus are also not phloem limited. Members of the genus *Vitivirus* and GRSPaV, the only grapevine-infecting species of the genus *Foveavirus*, are involved in the rugose wood disease complex (Martelli et al. 2012; Meng and Gonsalves 2008; Chap. 12, Meng and Rowhani, this book).

Development of the members of the family *Betaflexiviridae* into vectors for protein expression and VIGS has begun only recently. *Grapevine virus A* (genus *Vitivirus*) was engineered as a vector in which the putative promoter responsible for the expression of the movement protein (MP) from a distinct strain of the virus was inserted into the viral genome (Haviv et al. 2006). This GVA-based vector successfully expressed several foreign genes, including those for GFP, GUS, and the capsid protein of CTV (Haviv et al. 2006). To further test the potential of GVA as a VIGS vector for use in the elucidation of gene functions, a 500-bp fragment derived from *PDS* of *N. benthamiana* was cloned into the GVA vector. When introduced into leaves of *N. benthamiana* through agro-infiltration, the resulting recombinant virus induced silencing of the endogenous *PDS,* as judged by the photobleaching phenotype, as well as reduced levels of the PDS mRNA (Muruganantham et al. 2009). As expected for a virus with tropism to the phloem tissue, the effects of gene silencing were confined to the vascular tissue.

To investigate the potential use of GVA as a VIGS vector for grapevine, a 304-bp fragment derived from the *PDS* gene of grapevine was amplified and introduced into the GVA vector. This vector, designated pGVA-vvPDS-377, induced photobleaching in leaves 2–3 weeks after inoculation through agro-drenching (Muruganantham et al. 2009) validating a potential of GVA-based vector for VIGS in grapevine. Interestingly, the photobleaching phenotype exhibited in grapevine differed considerably from that in *N. benthamiana*. The photobleaching was not confined to the vascular tissue but rather was observed uniformly at leaf margins and later on the entire leaf blade (Muruganantham et al. 2009). This is quite different from the photobleaching induced by the GLRaV-2-based vector (Kurth et al. 2012). In theory, both viruses are restricted to the phloem tissue and are expected to

exhibit similar phenotypes when used to silence the *PDS* gene in grapevines. It remains to be elucidated if the difference in the phenotype due to silencing of *PDS* between the two viral vector systems is a reflection of the inherent difference between the two viruses, the different delivery systems, or the grapevine cultivars that were used by the two research groups.

Another candidate to be developed as a vector for protein expression and VIGS is GRSPaV, which is the only grapevine-infecting member of the genus *Foveavirus* that was recently characterized (Meng et al. 1998; Zhang et al. 1998; Martelli and Jelkmann 1998). Several characteristics make GRSPaV an attractive candidate for this purpose. First, it is widely distributed in commercial grapevines and is not regulated in most grape-growing countries. Second, the genome structure and expression strategy of GRSPaV are similar to those of PVX, a virus that has been one of the most successful plant virus-based gene expression vectors. Third, GRSPaV has filamentous virions with a helical symmetry, an open structure that allows packaging of genome with a large insert. This offers significant advantage over viruses that have closed spherical structure. Lastly, infection with GRSPaV, at least with certain strains of the virus, causes no or very mild symptoms in most commercial grape cultivars. This is a very important consideration when choosing a virus as a vector because delivery of vectors derived from highly pathogenic viruses would lead to disease and symptoms that will interfere with the intended purpose of the vector (Zhang et al. 1998; Meng et al. 1998, 1999, 2005, 2006; Meng and Gonsalves 2007). For details on this virus, the reader is referred to Chap. 12 of this book.

Toward this end, a full-length cDNA clone of GRSPaV and its GFP-tagged variant were engineered into a binary vector. When launched through agro-inoculation, both constructs were infectious in *N. benthamiana* and the grapevine host (Meng et al. 2013). Importantly, the GFP-tagged variant successfully expressed GFP in both *N. benthamiana* and grapevine. Interestingly, the GFP-tagged clone was unable to move systemically in *N. benthamiana*. Perhaps GRSPaV has coevolved with and adapted to the grapevine host and as such is unable to move systemically in this herbaceous plant. This GFP-tagged variant was very slow at systemic movement in the grapevine, as demonstrated in a preliminary study (Meng et al. 2013). Evidently, further testing of this GFP-tagged variant and the wild-type clone in different grapevine cultivars is necessary before the potential of GRSPaV as a protein expression or VIGS vector can be clearly established.

Numerous other grapevine viruses with different genetic makeups, genome expression strategies, and classification in different taxonomic groups are potential candidates as vectors in grapevine. Examples include members of the genera *Nepovirus* (family *Secoviridae*), *Maculavirus*, and *Marafivirus* (both in the family *Tymoviridae*) and the recently identified geminivirus, *Grapevine red blotch-associated virus* (GRBaV). It should be noted that all these viruses have spherical virions with limited capacity of accommodating foreign sequences compared to viruses with helical symmetry such as those of the families *Closteroviridae* and *Betaflexiviridae* (Gleba et al. 2007).

Applications of Gene Expression Vectors Derived from Grapevine Viruses

The most immediate applications of recombinant virus vectors are in molecular virology. An ability to tag virus genomes with reporter genes that has been pioneered in the potexvirus and potyvirus models (Chapman et al. 1992; Dolja et al. 1992) facilitated investigation of the virus infection cycle, including virus transport and interactions with the host. There is, however, an area of plant virology that is poorly explored due to difficulties of launching infections of the woody plants using viruses engineered to express reporter proteins. It is not known how these viruses manage to sustain multiyear infections in the voluminous and hostile environment of these plants. Are there aspects of virus-host interactions that are unique to woody and perennial plants compared to annual herbaceous plants? Rather intriguing initial insights to this question were provided using comparative genomics and the best developed models of woody plant viruses, CTV and GLRaV-2. In a CTV-citrus model, visualization of infection using the GFP reporter revealed complex and host species-specific patterns of virus-host interactions mostly reflected in the ability of the virus to spread systemically and from cell-to-cell in distinct phloem tissues. These patterns, as well as virus pathogenicity, are defined, in a large degree, by the CTV-specific genes that are dispensable for successful infection in some host species, but not in others (Dawson et al. 2013). Although more limited, similar studies using GFP-tagged GLRaV-2 showed that the tandem leader proteinases L1 and L2 play grapevine host-specific roles in virus invasiveness (Liu et al. 2009); a likely function of these proteins in virus systemic spread in its natural host is yet to be explored.

Other questions that could be answered using tagged viruses are seasonal changes in tissue-specific infection patterns including dormancy, mechanisms of virus transmission by insect vectors, and functions of the genes that are specific to particular viruses infecting woody plants. Sometimes, as is the case for CTV, such genes are found in only a single virus (see above). In other instances exemplified by AlkB (a gene encoding RNA demethylase), such genes are more broadly, but not universally, distributed among diverse viruses infecting woody or perennial plants (Martelli et al. 2007; van den Born et al. 2008). Although it is assumed that AlkB could play a role in protecting viral RNA from methylation by enzymes that may constitute a host defense from long-term virus infections, the exact function of AlkB is yet to be addressed by means of reverse genetics. Although not all of the grapevine-infecting viruses possess AlkB, some of them, including GLRaV-3, GVA, and GRSPaV, do. Even though most of these questions pertain to fundamental research, answering them will facilitate both the practical application of virus-derived vectors and control of viral diseases.

The second major field where virus gene expression vectors could find immediate and broad application is functional genomics of grapevine. As has been shown, the vLR2 vector has powerful, systemic VIGS capability that efficiently shuts down expression of the endogenous grapevine genes PDS and ChlI (Kurth et al. 2012).

Thus, this vector holds strong potential for mapping gene functions in grapevine including those involved in metabolic and biosynthetic pathways that determine nutritional, medicinal, and winemaking qualities of this crop plant. It should be emphasized that the major advantages of VIGS over stable plant transformation are more rapid implementation and relative ease of obtaining desired phenotypes. An additional potential benefit is that gene silencing triggered by VIGS is applicable to mature plants thus allowing the targeting of genes that could induce embryonic lethality if shut off permanently. As discussed above, the remarkable genetic stability of vLR2 VIGS variants provides years-long experimental window sufficient to determine phenotypes associated with seasonal development, e.g., flowering or berry ripening (Dolja and Koonin 2013). On the other hand, it appears that the vLR2 has only a limited utility for producing beneficial proteins, e.g., "edible vaccines," in grapevine, due to relatively low levels of recombinant protein expression and patchy distribution patterns throughout the plant, especially in berry clusters (Kurth et al. 2012).

The third potential application of the vLR2 is for the control of pathogens and herbivores. A VIGS capability of this vector could be used to map grapevine genes responsible for pathogen resistance: downregulation of the candidate resistance gene via RNAi will result in increased disease susceptibility of infected plants. When identified, novel resistance genes can be introduced to grape cultivars that lack such genes, either via stable transformation or by expression from a virus vector.

Reciprocally, VIGS can be used to downregulate pathogen susceptibility genes, particularly those specifically expressed in the phloem. In this case, the expected phenotype is a reduced pathogen invasiveness and disease attenuation or complete immunity of the plants to infection. In this case, VIGS itself will control a target pathogen, streamlining the use of a viral vector for practical application.

Another direct and potentially powerful VIGS application is targeting of the RNAi-susceptible pathogens and herbivores themselves. A practical potential of such applications has been demonstrated for citrus plants infected by a CTV vector that was able to induce RNAi in an insect that transmits the bacterial citrus greening disease (Hajeri et al. 2014).

It should be recognized that, despite a broad spectrum and a great potential applicability of viral vectors in grapevine, the utility of these vectors is yet to be tapped into. This apparent paradox may depend on a variety of circumstances with a lack of proper and focused investment being among the most important.

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