Ampeloviruses Associated with Grapevine Leafroll Disease: A New Group of Viruses in India

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Abstract

Ampeloviruses (family *Closteroviridae*) are filamentous monopartite, single-stranded, positive-sense RNA genome. They are transmitted by mealybugs in semi-persistent manner and vegetative propagating material remains the major route of spread. Ampeloviruses are recent addition to the plant viruses in India. *Grapevine leafroll-associated virus* 3 (GLRaV-3) was first ampelovirus to be recorded from India in the year 2012. Of the nine distinct species of the genus *Ampelovirus*, only three, *Grapevine leafroll-associated virus* 1 (GLRaV-1), GLRaV-3, GLRaV-4 infecting grapevine have been reported from India. The isolates of GLRaV-3 and GLRaV-4 are diverse, a few being the recombinant ones. This chapter describes the grapevine leafroll disease caused by different ampeloviruses, their geographical distribution, characterization, diversity, management strategies and also discusses about the future course of works to be taken.

Keywords

Ampeloviruses • Grapevine • Diversity • Diagnostics • India

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2.1 Introduction

The term *Ampelovirus* is derived from an ancient Greek word *ampelos* meaning grapevine, the host for the type species. It includes the virus species with flexuous filamentous particles of size 1400–2000 nm long, monopartite, single-stranded, positive-sense RNA genome of 13.0–18.5 kb size, transmitted by pseudococcid mealybugs and soft scale insects. *Ampelovirus* is one of the four genera of the virus family *Closteroviridae*, others three being *Closterovirus*, *Crinivirus* and *Velarivirus* (Fig. 2.1). Additionally, the family consists of five unassigned viruses. Despite being named after grapevine the genus *Ampelovirus* also includes non-grapevine infecting viruses. Majority of the ampleoviruses are recorded from woody plants such as grapevine, plum, fig and pineapple. The virus species list of the genus *Ampelovirus* recognized by International Committee on Taxonomy of Viruses

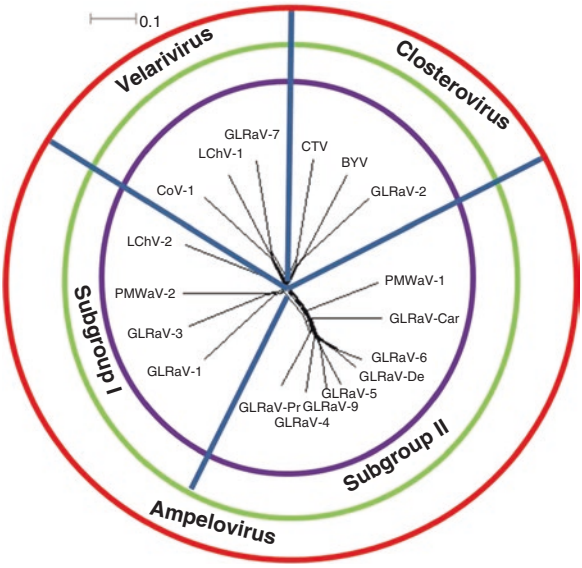

Fig. 2.1 Neighbour network reconstruction of the complete HSP70h genes of grapevine leafroll disease associated viruses. Nucleotide sequences were taken from GenBank and the network was constructed using SplitsTreev4 (Huson and Bryant 2006). Sequences used for constructing the network are: *GLRaV-1* Grapevine leafroll-associated virus 1, AF195822, *GLRaV-2* Grapevine leafroll-associated virus 2, AF039204, *GLRaV-3* Grapevine leafroll-associated virus 3, NC_004667, *GLRaV-4* Grapevine leafroll-associated virus 4, FJ467503, *GLRaV-5* Grapevine leafroll-associated virus 4 strain 5, NC_016081, *GLRaV-6* Grapevine leafroll-associated virus 4 strain 6, FJ467504, *GLRaV-9* Grapevine leafroll-associated virus 4 strain 9, AY297819, *GLRaV-De* Grapevine leafroll-associated virus 4 strain De, AM494935, *GLRaV-Car* Grapevine leafroll-associated virus 4 strain Car, FJ907331, *GLRaV-Pr* Grapevine leafroll-associated virus 4 strain Pr, AM182328, *GLRaV-7* Grapevine leafroll-associated virus 7, HE588185, *PMWaV-1* Pineapple mealybug wilt-associated virus 1, *PMWaV-2* Pineapple mealybug wilt-associated virus 2, *LChV-1* Little cherry virus 1, NC_001836, *LChV-2* Little cherry virus 2, AF416335, *CoV-1* Cordyline virus 1, HM588723, *CTV* Citrus tristeza virus, NC_001661, *BYV* Beet yellows virus

(ICTV) consists of nine species *Blackberry vein banding-associated virus* (BVBaV), *Grapevine leafroll-associated virus* 1 (GLRaV-1), *Grapevine leafroll-associated virus* 3 (GLRaV-3), *Grapevine leafroll-associated virus* 4 (GLRaV-4), *Little cherry virus* 2 (LChV-2), *Pineapple mealybug wilt-associated virus* 1 (PMWaV-1), *Pineapple mealybug wilt-associated virus* 2 (PMWaV-2), *Pineapple mealybug wilt-associated virus* 3 (PMWaV-3) and *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) (www.ictvonline.org/virusTaxonomy.asp).

Replication of ampeloviruses takes place in cytoplasm in association with membranous vesicles. The membranous vesicles may be derived either from endoplasmic reticulum or from peripheral vesiculation and disruption of mitochondria (GLRaV-1, GLRaV-3). The gene expression strategy happens to be ribosomal shifting for ORF1a and ORF1b. Other ORFs produces their respective proteins by translation of a set of nested 3′ co-terminal subgenomic RNAs (King et al. 2012).

2.2 Subgroups of *Ampelovirus*

Viruses belonging to the genus *Ampelovirus* show wide and distinct variations in genome size and organization. Accordingly they are grouped in two subgroups (Fig. 2.1). The subgroup I includes viruses with large (in excess of 17,000 nt) and complex (9–12 ORFs) genome *viz.* GLRaV-3, GLRaV-1, PMWaV-2, LChV-2 and BVBaV (Martelli et al. 2012; King et al. 2012; Naidu et al. 2015). GLRaV-3, the type species of the genus, has the largest genome in the genus comprising 12 ORFs (13 genes). The difference in genome size between isolates depends on the length of 5′ NTR (Naidu et al. 2015; Jarugula et al. 2010; Maree et al. 2008). Contrastingly, 3′ NTR of all isolates of GLRaV-3 is comparatively shorter in length having a consistent length of 277 nt and remain more conserved. The subgroup II comprises of smaller (approximately 13,000–14,000 nts) and simpler (6 ORFs, 7 genes) genome viral species *viz.* GLRaV-4, PMWaV-1, PMWaV-3 and PBNSPaV. One of the salient features of this subgroup is that they lack CPm. PMWaV-1 of the subgroup has a genome length of 13,071. Its seven ORFs (including ORF 1a and ORF 1b) express the replication related proteins, a 6 kDa hydrophobic protein, the HSP70h, the ~60 kDa protein, the CP and a 24 kDa protein, respectively (Fig. 2.2) (Martelli et al. 2012; King et al. 2012).

2.3 Symptoms and Transmission

Ampeloviruses cause a range of symptoms such as rolling, yellowing and reddening of the leaves (grapevine), stem pitting (plum), wilting and produce no symptom in pineapple. In natural condition these viruses are transmitted by mealy bugs (family *Pseudococcidae*) and scale insects (family *Coccidae*) in a semipersistent manner. The vector species and its range vary from virus to virus. Pineapple infecting ampeloviruses are transmitted by two species of the genus *Dysmicoccus* while LChV-2 is vectored by *Phenacoccus aceris*. None of the ampeloviruses is reported

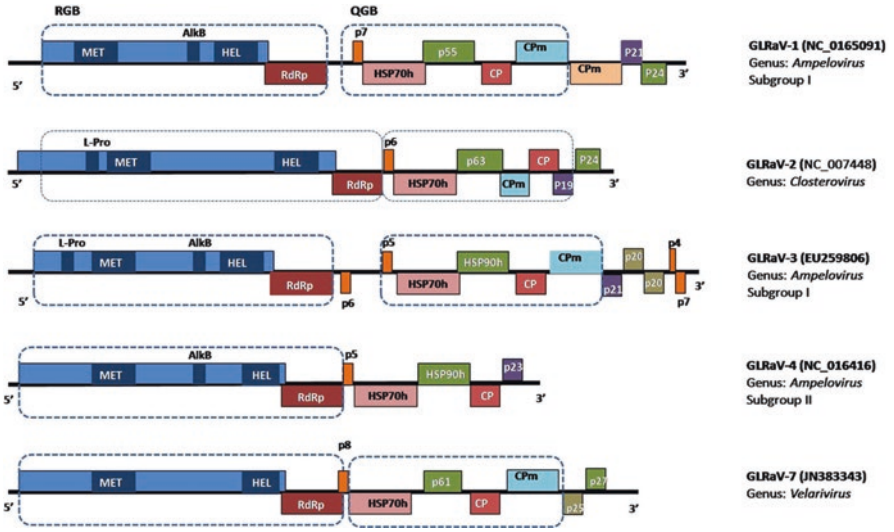

Fig. 2.2 Schematic representation of the genome organizations of grapevine leafroll disease associated viruses. *GLRaV-1* Grapevine leafroll-associated virus 1, NC_0165091, *GLRaV-2* Grapevine leafroll-associated virus 2, NC_007448, *GLRaV-3* Grapevine leafroll-associated virus 3, EU259806, *GLRaV-4* Grapevine leafroll-associated virus 4, NC_016416, *GLRaV-7* Grapevine leafroll-associated virus 7, JN383343. Corresponding genera, subgroups and accession numbers are indicated to the right side of the genome maps. The open reading frames (ORFs) are shown as *boxes* with designated protein domains such as *L-Pro* papain-like leader protease, *AlkB* AlkB domain, *MET* methyltransferase, *HEL* RNA helicase and *POL* RNA dependent RNA polymerase domains of the replicase. Conserved ORFs form the replication gene block (*RGB*) and quintuple gene block (*QGB*) and they are denoted by *dotted line boxes*. Abbreviations indicating ORFs are: *CP* coat protein, *CPm* minor coat protein, *RdRp* RNA-dependent RNA polymerase. The other ORFs are designated with approximate molecular weight and a common "p" designator. Figures drawn are not to the scale

to be transmitted through seed or by mechanical means (King et al. 2012). These viruses can be carried over in the vegetative cuttings used for propagation of their respective host plants and thus vegetative propagating materials become the primary source of virus spread over long distance (Kumar 2013; King et al. 2012).

2.4 Ampeloviruses in India

The occurrence of ampeloviruses in India is reported recently. Before 2012, there was no authentic information on virus or virus like diseases of grapevine in India. A news report appeared in a daily *The Indian Express* (4th November, 2007) indicated the presence of grapevine leafroll disease (GLD) in the vineyards of Maharashtra, which accounts for 94% of country's wine production. It further mentioned how this disease has started a debate and blame game among the various stakeholders of viticulture and related industries (Jadhav and Sonawane 2007). This disease had started creating havoc among famers and wine and raisin industries. Few farmers

had already removed their vineyards because of GLD. Subsequently in the year 2012, Indian Agricultural Research Institute (IARI), New Delhi in collaboration with National Research Centre for Grapes (NRCG), Pune found the association of GLRaV-1 and GLRaV-3 in the vineyards of Nashik and Pune regions of Maharashtra (Kumar et al. 2012a, b). Till date, out of nine ICTV recognized ampeloviruses, only three viral species have been reported from India, all associated with grapevine leafroll disease. In this chapter a comprehensive account of work done on ampeloviruses in India *vis-a-vis* their global stand has been discussed and a way forward for the work has also been outlined.

2.5 Disease and Virus Description

2.5.1 Grapevine Leafroll Disease (GLD)

Globally, the first descriptions of grapevine leafroll date back to the mid nineteenth century. It got several synonyms in different languages such as White Emperor disease (English), Rollkranheit and Blattrollkranheit (German), Rugeau and Enrolument (French), Rossore and Accartocciamento fogliare (Italian), enrollamiento de la hoja and enrollado (Spanish), Enrolamento de la folha (Portuguese) (Martelli and Boudon-Padieu 2006). Scheu (1935) demonstrated the graft transmission of leafroll from diseased to healthy vines and hypothesized the viral origin of the disease. However, Harold Olmo, a viticulturist of University of California, Davis and his colleagues in 1943 reported that the concerned problem was perpetuated by vegetative propagation and proposed that a virus was involved with the disease (Olmo and Rizzi 1943). Further, scientists demonstrated that the disease was also transmissible via grafts, which in turn provided strong evidence that a virus is the causal organism (Alley and Golino 2000; Harmon and Snyder 1946). In India, though said to be present since 2002, the first authentic report of the disease appeared in 2012 (Kumar 2013; Kumar et al. 2012a, b; Jadhav and Sonawane 2007).

2.5.2 Symptoms

GLD is said to be a complex disease with asymptomatic and symptomatic phases. It is unique in its symptomatology as the exhibition of symptoms begins on mature leaves which is in contradiction to many virus diseases where the exhibition of symptoms take place on newly developing parts (Naidu et al. 2015). Expression of symptoms is highly variable from cultivar to cultivar and from season to season. Exhibition of red and reddish-purple discolourations in the interveinal areas of mature leaves at the basal part of the shoots in late spring or summer, depending on the climate and geographic location, is one of the early sign in dark-berried cultivars. In Indian condition the typical symptoms have been observed from November–December to February. Symptoms are more expressive in dark-fruited/red-fruited cultivars than in light-fruited/white-fruited cultivars. As the season advances, in dark-fruited

cultivars the red to reddish-purple colour in interveinal lamina become prominent, leaf blades become thick, brittle and the margins of the infected leaves roll downward (Fig. 2.3). In severe cases, the whole leaf surface becomes deep purple (Martelli and Boudon-Padieu 2006; Rayapati et al. 2008). The symptoms are similar in light-fruited cultivars, but the leaves become chlorotic to yellowish, instead of reddish to reddish-purple (Fig. 2.3). Some white-fruited cultivars show no visual sign of infection (i.e. latent infection). In advanced stages of infection, the margins of the leaves of both kinds of cultivars roll downward, expressing the symptom that gives the

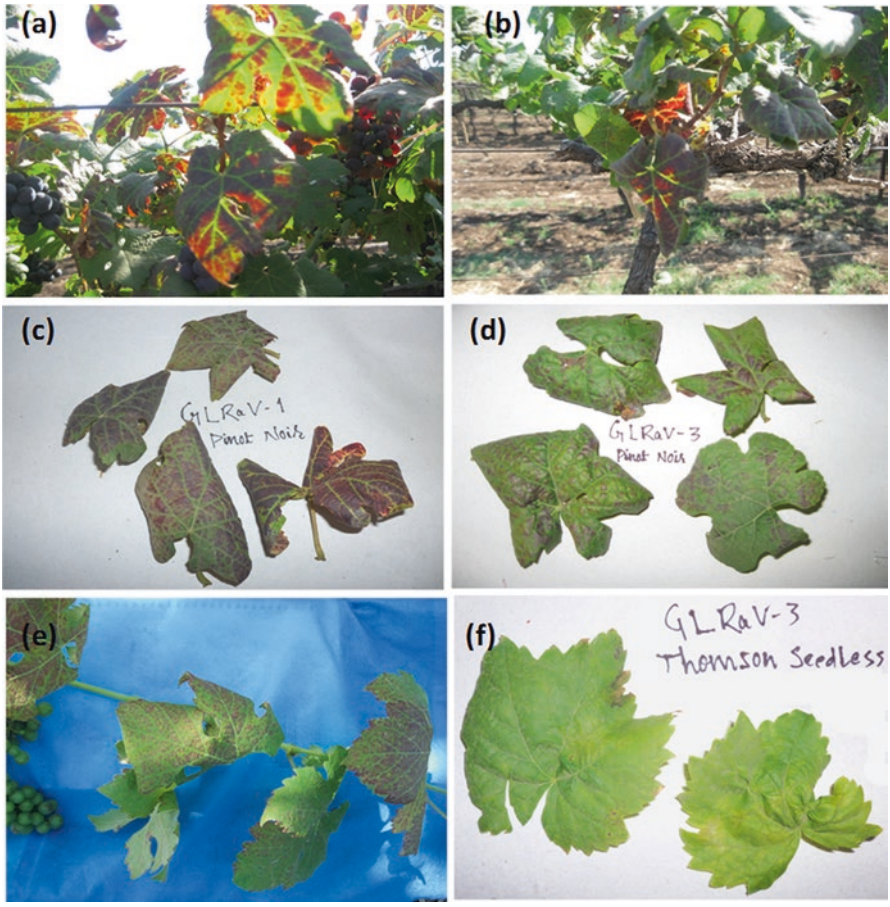

Fig. 2.3 Symptoms of grapevine leafroll disease (*GLD*) observed during the survey conducted for the study. (**a**) Vines of cultivar Cabernet Souvignon in a vineyard of Nashik; (**b**) Vine of a cultivar Pinot Noir at experimental farm of ICAR-National Research Centre for Grapes (ICAR-NRCG), Pune; (**c**, **d**) The close-up views of the leaves of two different vines of cultivar Pinot Noir (from ICAR-NRCG, Pune) found to be positive for GLRaV-1 and GLRaV-3, respectively; (**e**) Close up view of leaves of a vine of cultivar Shiraj from Nashik found to be positive for both GLRaV-1 and GLRaV-3; (**f**) Close up view of leaves of a vine of light-fruited cultivar Thompson Seedless (from ICAR-NRCG, Pune) found to be positive for GLRaV-3

disease its common name, i.e. "leafroll" (Rayapati et al. 2008; Martelli and Boudon-Padieu 2006). Most grape rootstocks, particularly American hybrids, do not show symptoms of leafroll even though they may carry the virus (Kovacs et al. 2001; Pietersen 2004). GLRaV-4 and related viruses elicit milder symptomatology compared to GLRaV-1 and GLRaV-3 (Martelli et al. 2012). Some strains of GLRaV-2 and -7 cause asymptomatic infection. Association of different GLRaVs and their strains with the disease further amplifies the complexity in symptomatology. Additionally, mixed infections among GLRaVs and with other viruses and viroids could be one of the factors in many intrigues of the disease (Naidu et al. 2015). Synthesis of two classes of anthocynins namely, *cyanidin-3-glucoside* and *malvidin-3-glucoside* has been reported to contribute in the expression of reddish-purple colour of virus-infected leaves of dark-fruit grapevine (Gutha et al. 2010).

2.5.3 Impacts of the Disease

The disease reduces yields, delays fruit ripening, reduces soluble solids, delays crop maturity, reduces berry anthocyanin & berry weight, and increases titratable acidity in fruit juice ultimately resulting in reduced wine quality (Atallah et al. 2012; Rayapati et al. 2008; Charles et al. 2006; Mannini et al. 1998). Degeneration of the phloem vessels and loss of photosynthetic potential of the leaves of infected vines are the major reason for decrease in quantity and quality (Freeborough and Burger 2008). As reviewed by Kumar (2013), GLRaV-3 reduces photosynthesis by 25–65 % depending upon the cultivar and environment. Bertamini et al. (2004) carried out a well designed research work showing the impact of disease on photosynthetic aspects of the host. In this study the virus-infected leaves showed reduced level of total chlorophyll (Chl), carotenoids (Car), soluble proteins and RuBP activity. An increase of Chl/Car ratio and a reduction of Chl a/Chl b ratio (ratio between chlorophyll a and chlorophyll b) were observed which could be due to the relatively faster decrease of Chl than Car. Photosynthetic study conducted in isolated thylakoids showed that because of leafroll infection there was marked inhibition of whole chain and photosystem (PS) II activity but only minimal inhibition of PS I activity was observed. It was inferred that the marked loss of PS II activity in infected leaves could be due to the loss of 47, 43, 33, 28–25, 23 and 17 kDa polypeptides as demonstrated by decrease in the amount of these polypeptides in SDS-PAGE analysis. The inhibition of donor side of PS II was also confirmed by immunological studies showing the significantly diminished content of 33 kDa protein of the water-splitting complex in infected leaves (Bertamini et al. 2004). Based on sensory descriptive analysis of 2010 wines it was suggested that GLD significantly affects the colour, aroma and astringency of wines. The study further suggested the influence of host × environment interactions on overall impact of the disease, causing maximum impact during cooler seasons (Alabi et al. 2016).

Globally, GLD is considered as the most economically destructive disease amongst the virus and virus like diseases of grapevines. Yield reductions due to GLD may vary, but reductions of around 50 % (or ≥60 % if the disease is severe)

are commonly reported on a worldwide basis (Rayapati et al. 2008). As per several reports, reduction in quantity produced of grapevines may be in the tune of 30–68 % (Atallah et al. 2012). Practically, even a small decrease in annual yields due to GLD has a cumulative impact on the long-term viability and profitability of a vineyard (Rayapati et al. 2008). The estimated economic impact of GLD ranges from approximately $25,000 to $40,000 per hectare in the absence of any control measure (Atallah et al. 2012).

2.5.4 Causal Agents: A Chronological Perspective

Despite confirmation of the nature of the disease as of viral origin by California based scientist Harmon and Snyder (1946), the causal agent remained unknown until the late 1970s. Namba et al. (1979) found closterovirus like particles in Japanese vines with leafroll symptoms, and reported the association of ampelovirus with the disease. A few years afterwards, two serologically different viruses from Switzerland were partially characterized and referred as "type I" and "type II" (Gugerli et al. 1984). Later, a number of new putative closteroviruses identified from vines with leafroll symptoms in Europe and USA. After 1995, Roman numerals were replaced by Arabic numerals to differentiate the different viruses (Martelli and Boudon-Padieu 2006). Till 2008, ten different viruses with filamentous particles, called grapevine leafroll-associated viruses (GLRaVs) were found associated with grapevine leafroll disease (GLD) and they were differentiated from one another by a number in increasing order as GLRaV-1 to -10 in the order of their discovery and were reported to be serologically distinct from each other (Martelli et al. 2002; Karthikeyan et al. 2008; Martelli 2009). By 2011, the number of GLRaVs had gone up to 12 but by the end of 2011, the number had been reduced to 11 due to withdrawing of GLRaV-8 from the ninth ICTV report because it proved to be the part of grapevine genome rather than being of viral origin (Martelli et al. 2012). The 11 filamentous viruses belonging to family *Closteroviridae* have been found associated with the leafroll disease of grapevines are GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-7, GLRaV-9, GLRaV-Pr (sequence originally deposited in GenBank under the name of GLRaV-10), GLRaV-De (sequence originally deposited in GenBank under the name of GLRaV-11) and GLRaV-Car (Martelli et al. 2012). Very recently, a novel ampelovirus has been detected in grapevines showing typical symptoms of GLD from Japan and it has been tentatively named as GLRaV-13 (Ito and Nakaune 2016). It showed closest but significantly distant relationship to GLRaV-1 in the subgroup I cluster of the genus *Ampelovirus*. But the name of GLRaV-13 might be controversial as its pathogenicity remains unclear; therefore, further study is needed in this regard (Ito and Nakaune 2016).

In ninth report of ICTV, out of eleven viruses associated with GLD, one (GLRaV-2) has been approved as the member of the genus *Closterovirus*, three (GLRaV-1, -3, and -5) have been placed in the genus *Ampelovirus* and six (GLRaV-4, -6, -9, GLRaV-Pr, GLRaV-De and GLRaV-Car) have been putatively assigned to the genus *Ampelovirus*, whereas one GLD causing virus (GLRaV-7) could not be

assigned to any genus of the family *Closteroviridae* (King et al. 2012). As per the studies of various researchers, ratification vote on taxonomic proposal of ICTV-2013 abolished the species GLRaV-5 and floated a new species GLRaV-4 which was earlier putatively assigned to the genus *Ampelovirus* (Adam et al. 2013). In the ratification vote on taxonomic proposal of ICTV-2014, a new genus *Velarivirus* was created and GLRaV-7, which earlier remained unassigned to any genus of the family *Closteroviridae*, has been given the status of type species of the genus *Velarivirus* (Adam et al. 2014). Recent studies based on genome size, structure and shared biological, epidemiological and serological characteristics suggested to consider GLRaV-5, GLRaV-6, GLRaV-9, GLRaV-Pr, GLRaV-De and GLRaV-Car as the strains of GLRaV-4 and thus they are written as GLRaV-4 strain 5, GLRaV-4 strain 6, GLRaV-4 strain Pr, GLRaV-4 strain De and GLRaV-4 strain Car, respectively. Together these viruses are known as GLRaV-4 like viruses i.e. GLRaV-4 LV (Naidu et al. 2015; Martelli et al. 2012). It can be noted that all grapevine infecting ampeloviruses can cause grapevine leafroll disease (GLD) whereas all GLD causing viruses cannot be ampeloviruses, such as GLRaV-2 (genus *Closterovirus*) and GLRaV-7 (genus *Velarivirus*). Therefore, the recent taxonomy, as available on ICTV website, has grouped GLD causing viruses into five species namely, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 (and its strains) and GLRaV-7 (www.ictvonline.org/virusTaxonomy.asp). Further studies on tentative GLRaV-13 may lead to minor alteration in the taxonomy of GLD causing viruses.

2.5.5 Genome Organization of GLRaVs

The genome size of GLRaVs range from 13, 626 nt in GLRaV-4 strain Car to 18, 671 nt in GLRaV-3 encoding six ORFs to 12 ORFs, respectively (Naidu et al. 2015). A major portion of 5′ end of genome of GLRaVs encoding a characteristic core of replication-associated genes is referred as replication gene block (RGB) (Fig. 2.2). The RGB constitutes ORF 1a and 1b encoding replication-associated proteins containing important domains such as methyltransferase (MET), RNA helicase (HEL) and RNA-dependent RNA polymerase (RdRp). Except GLRaV-7 and GLRaV-2 (i.e. ampeloviruses associated with GLD), ORF 1a of GLRaVs uniquely harbours an AlkB domain, which is a characteristic feature of many RNA viruses infecting woody plants. This domain has role in reversal of alkylation damage through RNA demethylation. ORFs located downstream to RGB are responsible for encoding structural and accessory proteins of GLRaVs. Unlike RGB, downstream ORFs are more variable and do not possess the same level of organizational conservation. In this portion of genome of GLRaVs (except GLRaV-4 LV), there occurs a block of five ORFs known as quintuple gene block (QGB), a hallmark of the family *Closteroviridae* (Fig. 2.2). In QGB the first ORF is a small transmembrane protein having role in cell-to-cell movement, second is homologous to cellular heat shock protein 70 (HSP70h), third in the QGB is ~60 kDa protein, sometimes denoted as HSP90h (as in GLRaV-3 and GLRaV-4 LV). Both second and third genes of QGB cooperate in cell-to-cell movement and virion head assembly. CP and coat protein

minor (CPm) are the last two genes of QGB. CP gene encodes for coat protein and gives the characteristic elongated morphology to the virion. CPm is responsible for the formation of main component of the virion head in other closteroviruses (Naidu et al. 2014, 2015; Martelli et al. 2012).

There is a marked difference between the arrangement of CP and CPm genes in QGB between GLRaV-2 and GLRaVs-1, -3, and -7. Like other members of the genus *Closterovirus*, in GLRaV-2 CPm gene is followed by CP gene whereas in GLRaVs-1, -3, and -7 i.e. CP gene is followed by CPm gene. Interestingly, two divergent copies of CPm is found in GLRaV-1 whereas CPm is conspicuous by its absence in GLRaV-4 LV (Fig. 2.2). GLRaV-3 is unique by the presence of an additional ORF encoding 6 k-Da (ORF 2) protein and a GC-rich intergenic region between ORF 2 and ORF 3 which is unlike the other members of the family *Closteroviridae*. Presence of ORF 11 (p4) and ORF 12 (p7) further add to the uniqueness of GLRaV-3 as they are absent in other closteroviruses (Naidu et al. 2015; Martelli et al. 2012). ORFs proximal to 3′ end of GLRaVs are more versatile and their functions are yet to be known. Still, based on analogies it has been suggested that these ORFs could be responsible for suppression of the host RNA silencing and long distance transport of the virus (Naidu et al. 2015). Replication of ampeloviruses in general has been briefly discussed in the beginning of this chapter however; lack of universally conserved QGB in GLRaVs not only suggests the likely differences in replication but also indicates the possibility of different host-virus interactions between individual GLRaVs. Additionally, lack of a CPm in GLRaV-4 LV and its duplication in GLRaV-1 suggests the probable dissimilarities in head segmentation patterns among GLRaVs. As far as 5′ UTR is concerned GLRaVs stand unique because of remarkable diversity in its sequence and predicted secondary structure (Naidu et al. 2015).

2.5.6 Transmission and Host-Range

GLD, once thought to be only graft transmissible, was found to be spreading within vineyards and mealybugs were first shown to be responsible for transmitting associated viruses in 1990 (Tsai et al. 2010; Engelbrecht and Kasdorf 1990). Since then, some mealybug (family *Pseudococcidae*) and soft-scale (family *Coccidae*) species have been shown to transmit different GLRaVs (Tsai et al. 2010). Transmission of GLRaVs seems to occur in a semi-persistent modality (Tsai et al. 2008). So far, vectors of GLRaV-1, -3, -4, -5, -6, -9 and GLRaV-Pr have been identified (Martelli and Boudon-Padieu 2006; Martelli et al. 2012). GLRaV-1, -3, and -4 and its strains are transmitted by several species of mealybugs of the genera *Heliococcus* (GLRaV-1, and -3), *Phenacoccus* (GLRaV-1, and -3), *Pseudococcus* (GLRaV-1, and -3) and *Planococcus* (GLRaV-3, -4 and its strains) and scale insects of the genera *Pulvinaria* (GLRaV-1, and -3), *Neopulvinaria* (GLRaV-1, and -3), *Parthenolecanium* (GLRaV-1, and -3), *Coccus* (only GLRaV-3), *Saissetia* (only GLRaV-3), *Parasaissetia* (only GLRaV-3), *Ceroplastes* (GLRaV-3, −4 and its strains) (Naidu et al. 2014; Kumar 2013; King et al. 2012). There is very limited

knowledge of transmission biology of these viruses as far as scale insects are concerned and based on mealybugs transmission, lack of virus-vector specificity has been suggested. Further, till date no insect vector has been identified for GLRaV-2 and −7. Vegetative cuttings of grapevine are transient and can carry their virus payload along with them and because of this fact viruses associated with GLD are sometimes called as "suitcase" or "samsonite" viruses (Rayapati et al. 2008). Mechanical transmission of ampeloviruses is not reported but GLRaV-2 has been experimentally shown to be mechanically transmitted from grapevine tissues to *Nocotiana benthamiana* (Naidu et al. 2014). Use of infected plant materials, while establishing new vineyards or during replacing vines in an established vineyard is the principal means of spread of GLD. The associated viruses do not have any natural hosts other than *Vitis* species. However, very recently there has been a report of natural infection of GLRaV-1 to pomegranate trees in Turkey. Thus, pomegranate (*Punica granatum* L.) could be an alternate host for GLRaV-1 (Caglayan et al. 2016). Further studies in this regard may give an in-depth understanding of the host range of GLD associated viruses.

2.5.7 Geographical Distribution

GLD is new to India and found in all grape-growing regions of the world, including Europe, South and North America, Middle East, Africa and Oceania (Sharma et al. 2011). Because of its wide presence it has been said that wherever grapevines are grown, occurrence of grapevine leafroll disease can be seen (Goheen 1988). In India the disease was first reported from the vineyards of Nashik and Pune regions of Maharashtra. Kumar (2013) suggested the presence of disease in the vineyards of Nashik and Pune regions which eventually fall in hot-tropical agro-climate but the study could not find GLD in Koppal district of Karnataka (mild-tropical agro-climate) and in Jammu and Kashmir (temperate agro-climate). However, in the same year another group of researchers proved the presence of GLD in another part of temperate region of India i.e. in Himachal Pradesh (Kumar et al. 2013). In a recent study disease has also been found in Manipur, a North-Eastern state of India. The associated virus in Manipur has been detected to be as GLRaV-4 (Fig. 2.4). GLRaV-3 and GLRaV-1 are the two most common viruses associated with the leafroll disease of grapevine not only at Indian condition but also at global level (Kumar 2013; Fuchs et al. 2009).

2.5.8 Virus Characterization, Recombination and Selection Pressure Analyses

Nucleotide data of NCBI suggest the availability of 44 full genome sequences of GLRaVs and their isolates. But, till date the complete genome sequencing of any ampelovirus has not been done in India. Partial characterization of GLRaV-1, GLRaV-3 and GLRaV-4 from India has been attempted. GLRaV-1 and -3 have been

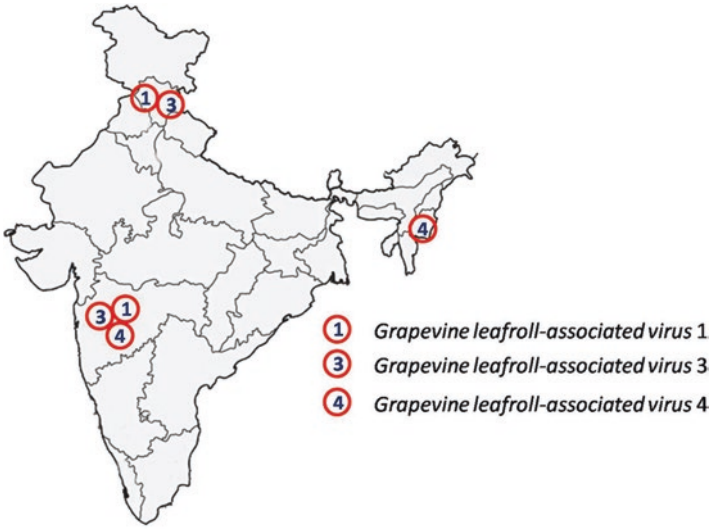

Fig. 2.4 Distribution of Grapevine leafroll-associated viruses (GLRaVs) in different states of India

characterized following one-step RT-PCR while GLRaV-4 has been characterized using two-step RT-PCR. The p24 gene of two isolates of GLRaV-1 was characterized to be of 630 base pairs (bp) and based on p24 gene phylogeny, the global isolates of GLRaV-1 segregated into three distinct groups. Two Indian isolates of GLRaV-1 clustered in group 1 with Claretvine and RRG isolates from USA (Kumar et al. 2012b). However, based on CP and HSP70h (heat shock protein 70 homologue) genes a recent study showed that global isolates of GLRaV-1 clustered into eight and seven groups, respectively (Fan et al. 2015). Partial HSP70h and entire p19.7 genes of 546 bp and 540 bp, respectively were characterized from the eleven isolates of GLRaV-3. The global isolates of GLRaV-3 segregated into eight clusters irrespective of their geographic origins (Naidu et al. 2015; Maree et al. 2015). Most of the Indian isolates clustered in group 2 of the global isolates but isolates Revella-4/12, Revella-4/14, KS-B-7 and Nashik showed discordant grouping behaviour based on different gene based phylogenies. Globally, this was the first such report of incongruent grouping patterns of isolates of GLRaV-3 based on different genes (Kumar 2013). On the basis of CP, HSP70h and p23 phylogenies, GLRaV-4 isolates from India grouped in group 1 with LR106 isolate of USA. In p23 phylogeny two isolates were closely related to LR106 isolate while other two isolates were distantly related to the same isolate.

Turturo et al. (2005) was the first to indicate the phenomenon of recombination in GLRaV-3 population. Later, Farooq et al. (2013) confirmed the recombination events in GLRaV-3 and proved that CP gene acts as one of the recombination hotspots in GLRaV-3 genome. However, based on p19.7 gene recombinant analysis, the Nashik isolate of GLRaV-3 from India was noted to be a recombinant isolate,

having parental sequences of 6–18 isolate from USA and Manjri- A2–38/36 isolate from India. It was also hypothesized that the recombination events could be the reason behind phylogenic incongruence and evolutionary process (Kumar 2013). The normalized values for the ratio of nonsynonymous substitutions per nonsynonymous site (*dN*) to synonymous substitutions per synonymous site (*dS*) indicated that HSP70h and p19.7, despite being under strong purifying selection pressures to preserve the amino acid sequences encoded by them and thereby retaining the biological functions, showed the contrasting patterns of evolution with their differential selection pressures. HSP70h gene (69.06 %) was under more purifying or negative selection pressure than p19.7 gene (49.16 %) and thus HSP70h gene of GLRaV-3 was subjected to stronger functional constraints which is nothing but the amount of intolerance towards nucleotide substitution. The relative higher value of normalized *dN-dS* for p19.7 indicates the comparatively flexible nature of the gene to accommodate the non-synonymous changes (Kumar 2013).

F-Pachore vani, GRP-G, GDR-I and GRP-G isolates of GLRaV-4 from India were observed to be the recombinant ones. Further, GDR-I, GRP-G and TS-N isolates from India contributed their genomic region either as major parents or minor parents in the evolution of some GLRaV-4 isolates from other countries. In case of GLRaV-4, 46 % of the codons in CP, 58.8 % of the codons in HSP70h genomic regions and 23.4 % of codons in the p23 genomic region were under purifying selection pressure. The HSP70h gene of GLRaV-4 isolates exhibited 1.5–2.7 times lower dN/dS values compared to the CP and p23 genes, indicating a stronger negative or purifying selection pressure acting upon HSP70h compared to CP and p23 genes.

2.5.9 Management

Because of the graft transmissibility nature of GLD, the best way of its management lies in the fact of employing the first line of defence i.e. to use the virus free propagating materials at the time of vineyard establishment or replacement of diseased vines. Screening for virus free vines at nursery stage is an essential step for producing the GLD free propagating materials. Robust diagnostics make the screening process easier. Globally different kinds of diagnostics have been developed and used for producing the disease free planting materials. Amongst them serology with ELISA has been remained the method of choice and thus it has been used widely. In India, polyclonal antisera using expressed fusion coat protein have been used to develop the sensitive diagnostics against GLRaV-3 and GLRaV-4. Such diagnostics can be used by certified nurseries for production of clonally selected and sanitized propagation material which is very effective and the only preventive method available for leafroll management (Martelli and Boudon-Padieu 2006; Rayapati et al. 2008). In recent years micrografting of shoot apices onto hypocotyls from Vialla seeds has been proved effective against seven grapevine viruses including GLRaV-1, -2, and -3 (Spilmont et al. 2012). Virus elimination from grapevine selections using tissue culture could be used for certification purpose (Sim et al. 2012).

The various tissue culture techniques either alone or in combination with others have been used to eliminate several viruses from different plants. Meristem tip culture has been used to eliminate GLRaV-1 along with GFLV (Fayek et al. 2009; Youssef et al. 2009). Somatic embryogenesis has also been used to eliminate several phloem limited grapevine viruses including GLRaV-1 and GLRaV-3 (Gambino et al. 2006). Efforts are also being made to develop resistance against GLD using transgenic approach but till date no transgenic has been released for cultivation purpose (Ling et al. 2008; Gouveia and Nolasco 2012; Kumar 2013).

Roguing i.e. selective removal of infected vines is the least costly method to manage the GLD. Level of infection, timing of removal in relation to age of the vineyard, and the cost-benefit ratio of replanting are the factors which must be taken into account while selectively removing the infected vines. But, in general "roguing and replanting" the individual vines is more effective in the formative years of vineyards i.e. much before the establishment of infection at large scale (Rayapati et al. 2008). Sensitive diagnostic assay based annual rouging would always be better (Naidu et al. 2014). It has been suggested that rouging can give an additional benefit of $17,000–$22,000/ha to the growers (Atallah et al. 2012). Further, Fuller et al. (2013) has suggested that the economical benefits from using certified virus-free planting materials is more than $50 million per year for the North Coast region of California. Vector management is another important strategy to manage the leafroll diseases of grapevines especially when vineyards are susceptible to sustained immigration of mealybugs (Charles et al. 2006). Managing grape mealybug is most effective when the insects are in their crawling stage. Chloronicotinyl insecticides such as imidacloprid can be used as along with irrigation water. Chemigation with thiamethoxam and dinotefuran has shown their effectiveness in deficit irrigation situations. Foliar sprays of chloropyriphos can also be used for dormant applications (Rayapati et al. 2008). Using a combination of systemic and contact insecticides would be better strategy for vector management (Tsai et al. 2008). Wallingord et al. (2015) tested the efficacy of horticultural oil and two classes of insecticides namely, acetamiprid and spirotetramat on grape mealy bug (*Pseudococcus maritimus*), primary vector for GLRaVs in North America and they found that the tested materials slowed the spread of vector with varied efficacy. Following the hygienic practices by the workers and use of sanitized equipments would also reduce the spread of mealybugs and scale insects which in turn will check the spread of the disease (Pietersen et al. 2013; Naidu et al. 2014).

2.6 Concluding Remarks

Ampeloviruses are group of viruses named after grapevine but the group also includes non-grapevine infecting viruses. Out of nine ampeloviruses reported worldwide, only three grapevine infecting viruses i.e. GLRaV-1, GLRaV-3 and GLRaV-4 have been recently reported from India. Grapevine leafroll disease is an important and complex disease of grapevine. Further investigation is needed to look for other associated viruses in India. It is also needed to explore the other grapevine

growing areas of the country for the associated viruses. Partial characterization of the viruses discovered from Indian vineyards has been carried out but complete sequence of any ampelovirus from India has not been done so far. Thus, there is a need to go for complete sequencing of these viruses so that we can have a broader understanding of viruses and the disease in Indian scenario. The scope of diversity study can be widened to include more number of isolates which in turn will lead towards a better understanding of genetic diversity, population structure and evolution of these viruses. The elucidation of biological and epidemiological implications of knowledge generated from such diversity studies will help in improving the sanitary status of grapevine planting materials. It will finally provide the avenues for development of robust strategies for mitigating the negative impacts of the disease.

In India the study of GLD is of recent origin but globally the disease has been discovered in mid-nineteenth century and mid-twentieth century in Europe and United States, respectively. Despite the fact of having a long history of its discovery at global level our knowledge on various aspects of the diseases and the associated viruses is quite limited (Naidu et al. 2014). A multidisciplinary system biology approach using modern tools of molecular biology, -omics, cell biology and other related disciplines along with the available genome sequence of the grapevine can shed more light on the disease, associated viruses and unparalleled complexity of the disease. Further investigations should be focussed to decipher the unknown functional genomics, host-pathogen interactome, gap between genomics and phenomics of the disease and transmission specificity of GLRaVs with their specific vectors (Naidu et al. 2014, 2015). Viral suppressors of RNA silencing (VSR) of GLRaV-3 (ORF 10) from India has been studied by Kumar (2013) but there is a need to widen the study as the detailed research into VSRs of GLRaVs will lead towards deciphering the mechanisms of silencing suppression (Naidu et al. 2015).

Further research is needed to decide the situations under which chemical control of vectors either alone or in combination with other measures such as rouging can be recommended to manage GLD (Wallingford et al. 2015). Additionally, research is also needed to have a deeper understanding of ecology and epidemiology of GLD. The discovery of pomegranate being as a natural alternate host of GLRaV-1 in Turkey (Caglayan et al. 2016) has added another dimension of complexity in the disease. Further investigation is required to see the implications of alternate host in the ecology and epidemiology of the GLD. In coming years a due vigilance is anticipated from the growers of the regions where both pomegranate and grapevine are cultivated in neighbourhood of each other. Proper hygienic condition and sanitary measures would also be required from the nurseries while producing the planting materials for pomegranate trees and grapevines both. In Indian condition there is a dire need to make efforts so that the knowledge generated from research can be translated for practical purpose which requires a powerful and enduring togetherness between research and extension personnel. The diagnostics develop in laboratories must help in producing the certified virus-free planting materials by recognized nurseries. Quarantine is an important aspect for disease like GLD as it has been suggested that the disease has been introduced to India through imported planting materials (Kumar 2013). Sensitive diagnostics can help in quarantine certification

of imported planting materials and thus will check the further introduction other associated viruses and their strains. Therefore, in India the researchers should also strive to keep on developing more sensitive diagnostics against GLRaVs. As suggested by Naidu et al. (2014), use of certified virus-free planting materials in combination of roguing and sanitation on regular basis along with environmentally safe vector management strategies would lead towards sustainable management of GLD.

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