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Genetic variability and pathological properties of *Grapevine Leafroll-associated Virus 2* isolates

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Abstract The genetic variability among *Grapevine leafroll-associated virus 2* (GLRaV-2) isolates was investigated in several grapevine accessions from various geographic origins in three genomic fragments, which encode the 70 kDa heat-shock protein homologue, the coat protein and the ~60 kDa protein. The majority of the isolates were identical or only slightly different from one another and formed a monophyletic group. Several other variants were found which differed greatly from the main cluster and were mostly present in autochthonous or specific vine cultivars. Phylogenetic analyses on the CP gene sequences, including the isolates analyzed in this work and some others from related literature, allowed five clades to be identified (PN, H4, RG, BD and PV20). Biological trials for graft incompatibility and leafroll symptoms revealed that GLRaV-2 divergent variants had different pathological properties, mainly according to their phylogenetic grouping. The BD isolate seemed to be a mild variant of the virus, because it was unable to induce graft incompatibility and it rarely caused leafroll symptoms. The RG isolate appeared to be a more virulent variant, given the strong decrease in rooted grafted grapevines obtained in nursery, in particular with the use of certain rootstocks; however, it does not induce leafroll symptoms. The variants belonging to the PN group, which were the most widespread, caused both graft incompatibility and leafroll symptoms, according to the viral variant. Graft incompatibility also proved to depend strongly on the rootstock used.

Keywords Closterovirus · GLRaV-2 · Incompatibility · Leafroll

Abbreviations

BT	Bootstrap
CP	Coat protein
GLRaV-2	Grapevine leafroll-associated virus 2
GRSPaV	Grapevine rupestris stem pitting associated virus
HMA	Heteroduplex Mobility Assay
HSP70	Heat-shock protein 70
ML	Maximum likelihood
MP	Maximum parsimony
p60	~60 kDa protein
UPGMA	Unweighted Pair Group Method with Arithmetic mean

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Introduction

At least nine serologically-distinct viruses belonging to the *Closteroviridae* family are classified as grapevine leafroll-associated viruses. Most of them are members of the newly-created *Ampelovirus* genus, while only *Grapevine leafroll-associated virus 2* (GLRaV-2) belongs to the *Closterovirus* genus (Martelli et al. 2002). The symptoms associated with the presence of GLRaV-2 in grapevine are not clear. Indeed, this virus was initially correlated with grapevine leafroll disease, since it was identified for the first time in grapevines with leafroll symptoms (Gugerli et al. 1984). Later on, a close association was found between GLRaV-2 and the Kober 5BB graft-incompatibility condition in French and Italian grapevines (Greif et al. 1995). More recently, union-incompatibility on sensitive rootstocks and decline in young vines have been reported from GLRaV-2 infected vineyards in California and New Zealand (Uyemoto et al. 2001; Bonfiglioli et al. 2003).

Sometimes, it has been possible to associate different symptoms with the presence of genetically-different virus variants. Indeed, many GLRaV-2 isolates have been identified worldwide. The viral genome of six isolates was completely or nearly completely sequenced: PN, Sem and OR1 from the USA (Zhu et al. 1998; Abou Ghanem et al. 1998; Liu et al. 2009), RG from California (Rowhani et al. 2000), 93/955 from South Africa (Meng et al. 2005) and BD from Italy (Bertazzon et al. 2006). A high genetic distance has been detected between some of these isolates: the RG variant, in particular, differed at nucleotide level by 26 % from PN, while the BD variant showed 25 % nucleotide differences with both PN and RG sequences. Partial sequences were obtained from other GLRaV-2 isolates (Abou Ghanem-Sabanadzovic et al. 2000; Nakaune and Nakano 2006; Xu et al. 2006; Prosser et al. 2007; GenBank accession numbers Y15890, DQ911147, EU053125, EU053126, EU204909 to EU204912, EF118013 to EF118033, EF122992 to EF123037, FJ195742 to FJ195744, EU760836 to EU760853 and FJ786017 to FJ786020), but only three of them were new atypical variants (PV20, PV124 and PG11) (Beuve et al. 2007).

Studies on the pathological properties of GLRaV-2 isolates are available mainly on *Nicotiana benthamiana;* it was ascertained that 93/955 had more virulent effects than the PN variant, whereas no differences were evident in the type and severity of symptoms between the PN and RG isolates (Goszczynski et al. 1996; Meng et al. 2005). On grapevine, the PN variant seemed to be associated with leafroll and graft incompatibility symptoms, while the RG isolate induced only graft incompatibility reactions (Rowhani et al. 2000; Pirolo et al. 2006). The Alfie variant of GLRaV-2 is very similar to BD in the 2,000 bp sequenced and is responsible for decline in young plants associated with a graft incompatibility syndrome (Bonfiglioli et al. 2003).

In this work, GLRaV-2 variants were characterized at the molecular level and pathological properties of some isolates were determined on grapevine. First of all, the genetic variability of the coat protein (CP) gene was investigated by sequencing and a phylogenetic analysis of the isolates was performed. Afterwards, the study was extended to other GLRaV-2 infected grapevine accessions and to the putative heat-shock protein (HSP70) and ~60 kDa protein (p60) genes. Finally, biological trials for leafroll and graft incompatibility were performed in order to investigate the pathological properties of phylogenetically-different virus variants.

Materials and methods

Virus sources

A total of 78 grapevine accessions which had previously been found to be infected by GLRaV-2 through immunosorbent tests were used in this study (Table 1). Thirty-one samples were from vineyards located in different geographic regions in Northern, Central and Southern Italy. Fifteen accessions were from the grapevine international variety collection of the "Centro di Ricerca per la Viticoltura" (CRA-VIT) in Conegliano (Italy). Thirty-two samples came from Greece, France, Switzerland, Spain, Belgium, the Czech Republic, the USA and Brazil; six of them (AL224, Ch8386, H4, MuS, PN and Sem) were kindly provided by different research institutions.

RNA extraction, cDNA synthesis, PCR amplification and cloning

Samples consisted of leaves which showed leafroll symptoms or mature canes. Leaf veins or cortical

Table 1 Characteristics of infected grapevine accessions, GLRaV-2 genetic variants identified and their clustering according to Fig. 1, different Heteroduplex Mobility Assay profiles obtained in three genomic regions and accession numbers of the coat protein gene nucleotide sequences obtained in this work

Grapevine cultivar	Geographic origin	GLRaV-2 isolate	ORF3 (HSP70)[a]	ORF4 (p60)[a]	ORF6 (CP)[a]	ORF6 Acc. No.	GLRaV-2 clade
17.37	CRA-VIT Collection[b]	17.37	–[d]	–[d]	–[d]		BD[f]
101.14	CRA-VIT Collection[b]	101.14	–[d]	–[d]	–[d]		BD[f]
110 Richter	Greece	110R	a	b	a	DQ314587	PN1[f]
157.11 Couderc	CRA-VIT Collection[b]	157.11C	a	m	g		PN2[f]
Alphonse Lavallée	France	AL224	a	w	h		PN2
Antinello	CRA-VIT Collection[b]	Ant	b	n	g		PN2
Arvino	Tuscany, Italy	Arv1	–[d]	y	m	DQ214588	PN3[f]
Arvino	CRA-VIT Collection[b]	Arv2	a	c	d	DQ314590	PN1[f]
Bianca	Tuscany, Italy	Bia1	a+e	g	mixed[e]		
Big Perlon	USA	BigP	c	mixed[e]	a		PN1
Black Magic	USA	BlaM	a	u	g		PN2
Bonarda	Sardinia, Italy	Bon	a	b	a		PN1[f]
Cannonao	Sardinia, Italy	Can	e	α	o	DQ314589	H4[f]
Castiglione	CRA-VIT Collection[b]	Cast1	a+e	t	mixed[e]		
Chasselas	Switzerland	Ch8386	a	mixed[e]	c		PN1
Ciliegiolo	Tuscany, Italy	Cil	a	b	f		PN1
Coloretto	Tuscany, Italy	Col	b	mixed[e]	g		PN2
Counoise	INRA Collection[c]	Z127	a	a	a		PN1+ BD[f]
Crimson	Apulia, Italy	Cris	a	b	a		PN1[f]
Cabernet Sauvignon	France	Cs1	a	h	a		PN1[f]
Cabernet Sauvignon	France	Cs2	a	j	a		PN1
Cabernet Sauvignon	France	Cs3	a	f	a		PN1
Cabernet Sauvignon	France	Cs4	a	e	a		PN1
Cabernet Sauvignon	Brazil	Cs5	a	f	a		PN1[f]
Cabernet Sauvignon	France	Cs6	a	–[d]	a		PN1
Don Mariano	CRA-VIT Collection[b]	BD	–[d]	–[d]	–[d]	DQ286725	BD[f]
Garnacha	Spain	Gar	a	mixed[e]	mixed[e]		
V. rupestris St. George	USA	H4	d	z	n		H4
Isabella	USA	Isa	a	–[d]	g		PN2
Isabella nera precoce	USA	IsP	a	r	g	DQ314591	PN2[f]
Italia	Apulia, Italy	Ita	–[d]	–[d]	mixed[e]		PN1+PN2+RG[f]
Leopoldo III	Belgium	Leo	–[d]	–[d]	–[d]	DQ314592	RG[f]
Mantonico bianco	CRA-VIT Collection[b]	MantB	a	b	mixed[e]		
Mayolet	Aosta Valley, Italy	My	–[d]	–[d]	–[d]		BD[f]
Melissa	Apulia, Italy	Mel	a	c	mixed[e]		
Merlot	France	Mer	–[d]	b	a		PN1[f]
Montepulciano	Umbria, Italy	Mon	a	l	b	DQ314593	PN1
Moscato di Terracina	CRA-VIT Collection[b]	MoT	a	t	g		PN2
Muskat moravsky	Czech Rep.	MuM	a	–[d]	–[d]	DQ314594	PN1
Muscat de Samos	Greece	MuS	b	s	g		PN2
Negro amaro	Apulia, Italy	NA1	a	t	g	DQ314595	PN2
Negro amaro	Apulia, Italy	NA2	b	p	e	DQ314596	PN1[f]

Table 1 (continued)

Grapevine cultivar	Geographic origin	GLRaV-2 isolate	ORF3 (HSP70)[a]	ORF4 (p60)[a]	ORF6 (CP)[a]	ORF6 Acc. No.	GLRaV-2 clade
Nebbiolo chiavennasca	Lombardy, Italy	NebC	a	b	a		PN1[f]
Nera	Tuscany, Italy	Ner	–[d]	–[d]	m	DQ314598	PN3[f]
Nero d'avola	Sicily, Italy	NerAv1	a	t	a		PN1
Nero d'avola	Sicily, Italy	NerAv2	a	v	g		PN2
Nerello calabrese	CRA-VIT Collection[b]	NerC	e	–[d]	p	DQ314597	H4[f]
Perle de Csaba	INRA Collection[c]	Z225	a	f	a		PN1+BD[f]
Pinot noir	France	PN	a	a	a		PN1
Pollera nera	Tuscany, Italy	Pol1	–[d]	y	m	DQ314599	PN3+H4[f]
		Pol2	e	–[d]	o	DQ314600	
Primitivo	Apulia, Italy	Prim	a	k	i	DQ314601	PN2
Primitivo	Apulia, Italy	Prim2	a	b	a		PN1
Primitivo	Apulia, Italy	Prim3	a	o	mixed[e]		
Ravanese	Tuscany, Italy	Rav	–[d]	d	a		PN1
Red globe	Apulia, Italy	Red1	–[d]	–[d]	a	DQ314602	PN1
Red globe	Apulia, Italy	Red2	a	m	g	DQ314603	PN2
Red globe	Apulia, Italy	Red3	a	–[d]	g		PN2
Red globe	Apulia, Italy	Red4	a	b	a		PN1
Red globe	Apulia, Italy	RG1	–[d]	–[d]	–[d]	DQ314605	RG[f]
Red globe	CRA-VIT Collection[b]	RG2	–[d]	–[d]	–[d]	DQ314606	RG[f]
Regina	CRA-VIT Collection[b]	Rei	a	i	a	DQ314604	PN1[f]
Ruby cabernet	INRA Collection[c]	Z170	a	s	g		PN2+BD[f]
Sauvignon	France	S317	c	mixed[e]	a		PN1
Semillon	USA	Sem	a	c	a		PN1
Sangiovese	Tuscany, Italy	Sg	a	b	f	DQ314607	PN1
Saint Pierre doré	INRA Collection[c]	Z241	a	m	g		PN2+BD[f]
Susipaniello	CRA-VIT Collection[b]	Sus	c	q	g		PN2
Tempranillo	Spain	Tempr	a	t	l	DQ314608	PN2
Tempranillo	Spain	Tempr2	e	α	o		H4
Tuccanese	CRA-VIT Collection[b]	Tuc	a	–[d]	–[d]	DQ314609	PN2
Unknown	INRA Collection[c]	T76	c	mixed[e]	mixed[e]		PN2+ PN3+ BD[f]
Unknown	INRA Collection[c]	T94	a	mixed[e]	mixed[e]		PN2+ PN3+ BD[f]
Unknown	INRA Collection[c]	T113	c	mixed[e]	mixed[e]		PN2+ PN3+ BD[f]
Unknown	INRA Collection[c]	T130	a	b	a		PN1+BD[f]
Verduschia	Tuscany, Italy	Vd	a	c	a		PN1[f]
Verdello di Bracciano	CRA-VIT Collection[b]	VerB	b	mixed[e]	mixed[e]		
Vermentino	Tuscany, Italy	Verm	a	x	g		PN2
Victoria	Apulia, Italy	Vic	a	mixed[e]	a		PN1

[a] HMA profile obtained in the three genomic regions. Each letter represents a different HMA profile in that particular ORF, [b] Accessions maintained in the grapevine international variety collection of CRA-VIT in Conegliano (Italy), [c] Accessions maintained in the grapevine virus collection of INRA in Colmar (France), [d] The amplicon was not obtained in that ORF, [e] More than one GLRaV-2 predominant variant was present in that ORF, but their HMA profiles were not classified, [f] Accessions used in the biological trials

scrapings were homogenized in liquid nitrogen and RNA was extracted according to MacKenzie et al. (1997). Isolated RNA was converted into cDNA with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). The reaction was carried out by denaturation at 95°C for 5 min and reverse transcription at 42°C for 50 min with DNA random primers (Roche Diagnostic).

PCR was carried out with GLR2CP1/CP2 primer pair, which amplifies the entire GLRaV-2 major CP cistron (597 bp, ORF6) (Abou Ghanem-Sabanadzovic et al. 2000), and LR2-U2/L2 and LRaV-2(1)/(2) primer pairs, located in the HSP70 gene (332 bp, ORF3) and p60 gene (821 bp, ORF4), respectively (N. Abou Ghanem, unpublished results; Abou Ghanem et al. 1998). Infected samples, which had tested negative with all the primer pairs, were tested with V2dCPf2/CPr1 primers, which have a wider detection spectrum, in order to amplify a 534 bp fragment on the minor and major CP cistrons (Bertazzon and Angelini 2004) (Table 2).

The DNA amplifications were carried out in 50 µl total reaction volume. Five µl of cDNA were mixed with 45 µl of the amplification mixture, consisting of 50 µM each dNTP, 120 nM each primer, 1 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Polymed) and 10x *Taq* buffer. The PCR cycling was as follows: 40 cycles of denaturation for 20 s at 94°C, annealing for 30 s at 54°C for V2dCPf2/CPr1 primer pair and 52°C for all the other primer pairs, extension for 45 s at 72°C, final extension for 5 min at 72°C. The PCR products were analyzed by electrophoresis on 1 % agarose gel, stained with ethidium bromide and visualized using a UV transilluminator.

The GLR2CP1/CP2 amplicon obtained from cv Pollera nera, an autochthonous Italian variety infected by two predominant variants, was cloned. The RT-PCR product was cut with *Eco*RV (Mbi Fermentas), then ligated into the pBluescript SK(+) vector (Stratagene), before been cloned in *Escherichia coli* Top 10 F^I using standard protocols.

DNA sequence analyses

Some infected vine accessions from different cultivars and from several separate geographic sites were selected for the study of the variability of the viral CP gene. Nucleotide sequencing was carried out on amplicons from 24 GLRaV-2 isolates obtained with GLR2CP1/CP2 or V2dCPf2/CPr1 primer pairs. The amplicons were double-strand sequenced using automated equipment (BMR Service, Padova, Italy) and sequences were deposited in GenBank (Table 1).

Multiple alignments and a preliminary dendrogram were performed between the 24 newly-obtained sequences and all the available GLRaV-2 CP sequences (Table 3). On the basis of the obtained clustering, 11 representative GLRaV-2 isolates out of the 61 available in GenBank were selected for further phylogenetic analysis (93/955, CF, H4, PN, RG, Sem, SL10, Pg11, Pv20, Pv44, Pv124). The most informative blocks of nucleotides (388 bp) and amino acids (121 aa) of the 24 new sequences and the sequences of the 11 representative isolates were selected for molecular phylogenetic analysis from the multiple alignment of sequences using Gblock software (Castresana 2000). The phylogenetic studies were performed according to the maximum

Table 2 Characteristics of the primers used in this study: name, target gene, sequence, lenght of the amplicon, references. The last column points out in which experiment they were used

Primer pair	Target gene	Sequences 5'-3'	Amplicon length	References	Experiment
GLR2CP/CP2	ORF6 (CP)	ATGGAGTTGATGTCCGAC TACATAACTTCCCTTCTACC	597	Abou Ghanem-Sabanadzovic et al. 2000	Sequencing and HMA ORF6
LR2-U2/ L2	ORF3 (HSP70)	ATAATTCGGCGTACATCCCCACTT GCCCTCCGCGCAACTAATGACAG	332	Abou Ghanem, unpublished	HMA ORF3
LRaV-2(1)/2(2)	ORF4 (p60)	AGGCGGATCGACGAATAC ATCCTGTCCGGCGCTGTG	821	Abou Ghanem et al. 1998	HMA ORF4
V2dCPf2/CPr1	ORF5-6 (CP, CPm)	ACGGTGTGCTATAGTGCGTG GCAGCTAAGTACGAATCTTC	534	Bertazzon and Angelini 2004	Sequencing ORF6

Table 3 Nucleotide sequences of the coat protein gene from GLRaV-2 isolates obtained by other authors used in this work for sequence comparisons and phylogenetic analyses

GenBank Acc. No.	GLRaV-2 Isolate	Country	Author
Y14131	Sem	USA	Abou Ghanem et al.
AF039204	PN	USA	Zhu et al.
AF314061	RG	USA	Zhang et al.
AY456132	Alfie	New Zealand	Bonfiglioli et al.
AY697863	H4	USA	Abou Ghanem et al.
AY842932	Shandong	China	Xiang et al.
AY881628	93/955	South Africa	Meng et al.
AY881629	94/970	South Africa	Meng et al.
DQ286725	BD	Italy	Angelini et al.
DQ911147	SL10	China	Wang et al.
EF012717	Pv124	France	Beuve et al.
EF012718	Pv44	France	Beuve et al.
EF012720	Pg11	France	Beuve et al.
EF012721	Pv20	France	Beuve et al.
EF118013 to EF118033	Various	France	Marais et al.
EU053125, EU053126, EU204909 to EU204912	M/C, L/I, SE, IT, MH, RI	Brazil	Radaelli et al.
EU760836 to EU760853	Various	USA	Jarugula et al.
FJ195742 to FJ195744	PnTM, Ch, CF	France	Fuchs et al.
FJ436234	OR1	USA	Liu et al.
FJ786017	LN	China	Wang et al.

likelihood (ML) and maximum parsimony (MP) methods (Felsenstein 2003). The ML analysis was performed with the PHYML 2.4 program (Guindon and Gascuel 2003). The Dayhoff and JC69 substitution matrices (Dayhoff et al. 1978) were used in the tree reconstruction, while site heterogeneity was modelled with a four category gamma distribution. The MP analysis was carried out using the PHYLIP 3.6 program (Felsenstein 2002). Non-parametric bootstrap resampling (BT) (Felsenstein 1985) was performed to test the robustness of the tree topologies obtained from the MP and ML analyses (1,000 replicates).

Heteroduplex mobility assay analyses

Heteroduplex Mobility Assay (HMA) experiments, carried out according to Wang and Hiruki (1999), were performed separately on LR2-U2/L2, LRaV-2 (1)/(2) and GLR2CP1/CP2 amplification products in order to study the variability of the HSP70, p60 and CP genes, respectively. A distance and a similarity matrix were generated from the data obtained in every genomic region from all the combinations of heteroduplex pairings according to Delwart et al. (1995). The UPGMA approach was used to perform cluster analyses and to construct a dendrogram for each DNA fragment. The statistical analyses were carried out using the Student's *t*-test.

Biological trials

Dormant cuttings of 30 grape cultivars and 4 rootstocks were selected from the grapevine accessions infected with different GLRaV-2 isolates as determined by CP gene sequence and HMA analyses (Table 1, last column). They included 8 plants from the INRA virus collection in Colmar (France), which were chosen because of their graft incompatibility condition, while the others had never shown symptoms of decline in the field. Most of the samples contained a single GLRaV-2 variant, whereas 10 accessions had mixed infections of different GLRaV-2 isolates. The sanitary status of the samples had previously been checked serologically and molecularly for the presence of other viruses associated with major grapevine diseases (fanleaf, leafroll and rugose wood). Only GLRaV-2 infected accessions

which were negative for the other grapevine leafroll-associated viruses were selected for leafroll indexing. Another six accessions which belonged to the same cultivars as the selected GLRaV-2 infected samples were taken as a negative control, while three accessions infected with GLRaV-1, GLRaV-3 or GLRaV-6 were chosen as a positive control for leafroll symptoms.

Two types of indexing trials, initiated in 2004, were carried out: 1) healthy accessions from cv Cabernet franc were used as an indicator of leafroll; 2) healthy rootstocks from *V. berlandieri* x *V. riparia* cv Kober 5BB (K5BB), SO4, 5C and *V. berlandieri* x *V. rupestris* cv 1103P were used in order to examine graft incompatibility. According to the quantity and quality of the woody materials, between 5 and 20 grafted plants per scion-rootstock combination were produced in nursery with omega grafting, using only dormant canes suitable for grafting. In total, 144 and 1044 grafted plantlets were obtained in the leafroll and the graft incompatibility trials, respectively (Tables 4 and 5).

Grafted plants were inspected for leafroll symptoms two to three years after graft-inoculation. The visual observations on graft incompatibility were carried out in three phases: 1) on unrooted plants before planting in the nursery; 2) on rooted grafts after the first year in nursery; 3) at the end of the second year in vineyard. Non-saleable plants were discarded at phase 2. The development of canes and roots was measured at phases 2 and 3 in all the grafted plants used in the incompatibility trial.

Table 4 Number of plants infected with single or mixed GLRaV-2 variants which showed leafroll symptoms on leafroll indicator

Sanitary status	GLRaV-2 isolates	No. symptomatic/ total grafts
GLRaV-2	PN1	6/46
	PN3	4/5
	RG	0/18
	BD	6/28
	PN3+H4	3/3
	PN1+PN2+RG	0/4
GLRaV-1		9/9
GLRaV-3		6/6
GLRaV-6		6/6
Negative		0/19

Statistical tests were performed with the SPSS program, using the analysis of variance test (ANOVA) after normalization of the data with angular transformation and the chi-square test (χ^2 test) with a contingency table.

Results

Genetic variability and phylogenetic analysis of the CP gene

The sequence analyses of the CP gene in the 24 selected GLRaV-2 variants showed high values of genetic variability. The comparison with the 61 sequences from GeneBank showed that the 24 isolates sequenced in this work represented almost all the GLRaV-2 genetic variability detected up to now (data not shown). ML and MP phylogenetic cladograms were constructed using the 24 newly-obtained sequences and the published sequences of another 11 isolates representative of all the GLRaV-2 CP sequences deposited in GenBank. The consensus cladograms obtained by applying the two methods were largely congruent in the resolution of the main clades (Fig. 1). However, the relationships were not always the same within the clades, particularly in the PN clade, where two minor nodes received very poor BT support. The proposed topology highlighted five well-resolved clades, named Pv20, BD, RG, H4 and PN. The Pv20, RG-BD and H4-PN clades were resolved as monophyletic groups and these findings were strongly supported by high BT values (100/100, 93/89 and 100/100, respectively).

The PN and H4 clades proved to be sister groups. The PN clade showed three well-resolved subclades (PN1, PN2, PN3), supported by high BT values (89, 99/99 and 99/99, respectively). Two nodes in the subclades PN1 and PN2 were not well resolved in both the ML and MP analyses, but these points were supported by the sequence alignment observation, which highlighted the elevated nucleotide and amino-acid identity between the various isolates (97 % and 99 %, respectively). A larger sequence portion is required to resolve this clade completely. The H4 clade showed two subclusters: the NerC, Can and Pol2 isolates on the one hand and the H4 variant on the other, with 7.5 % and 5 % divergence between them at the nucleotide and amino acid level, respectively.

Table 5 Number of surviving grapevines compared to the initial grafted plants, according to the rootstock, at the three phases: 1, before plantation in nursery; 2, after the 1st year in nursery; 3, end of the 2nd year in vineyard

GLRaV-2 isolate	K5BB			5C			1103P			SO4		
	1	2	3	1	2	3	1	2	3	1	2	3
Neg.	99/100	66/100	63/100	55/56	44/56	34/56	31/31	27/31	22/31	57/58	41/58	39/58
PN1	88/90	25/90*	23/90*	40/40	31/40	30/40	35/35	22/35	22/35	43/44	35/44	34/44
PN2	14/20	9/20	9/20	10/10	5/10	5/10	7/10	0/10*	0/10*	10/10	10/10	10/10
PN3	8/8	3/8	3/8							4/4	4/4	4/4
H4	20/20	3/20*	2/20*	10/10	8/10	8/10	10/10	8/10	8/10	9/10	6/10	4/10
RG	40/40	15/40	11/40	50/50	5/50*	5/50*	20/20	12/20	12/20	19/20	18/20	18/20
BD	91/100	46/100	45/100	98/100	70/100	60/100	85/86	60/86	50/86	90/100	55/100	54/100

*Significant difference in yield compared to the negative control ($p<0.05$)

The BD clade branched off as a sister of the RG clade with strong BT support (93/89). The BD and RG clades were very divergent among them; however, the Pv20 clade included the most divergent GLRaV-2 isolates (Table 6). The cladograms obtained with amino acid data were very similar to those obtained with nucleotide data.

Genetic variability of additional isolates in three genomic regions

The HMA method was used to investigate the genetic variability of the HSP70, p60 and the CP genes of 67 GLRaV-2 infected vine samples, including the samples previously sequenced in the CP gene. The primers used for the amplification of the three regions were not able to detect all the GLRaV-2 variants. Indeed, an RT-PCR product was obtained from 88.5 % (69 out of 78) of the accessions using GLR2CP1/CP2 primers, 82 % using LR2-U2/L2 primers and 78 % using LRaV-2(1)/(2)primers.

Most of the infected samples showed only a single predominant GLRaV-2 variant, while 17 accessions were infected with at least two distinct dominant variants, which were readily identified from mixed HMA patterns (Table 1).

A good degree of sequence heterogeneity was found in the genes investigated. In all the genomic fragments tested the majority of GLRaV-2-infected vine accessions showed a similar HMA profile, while very divergent patterns were less frequently detected. A total of 5, 27 and 14 different HMA patterns were found in the HSP70, p60 and CP genes, respectively (Table 1).

The average distance values, calculated from band migration in the gel, were used to assess the genetic distance between GLRaV-2 isolates and to construct three UPGMA dendograms. Average genetic diversity in the three different genome regions was: 0.0311 ± 0.0335 for the HSP70 gene, 0.0567±0.0794 for the p60 gene and 0.0326±0.0405 for the CP gene. The difference between the average value from p60 gene versus the values from HSP70 and CP genes was statistically significant ($p<0.01$), confirming that the p60 gene was the most variable. The maximum divergence value between the two isolates was lower for the HSP70 gene (0.100), while the p60 gene showed the highest genetic distance (maximum 0.280). Data for the CP gene was intermediate (maximum 0.174). The topology of the UPGMA dendrograms was conserved in the three genomic regions analyzed, confirming the clustering previous-

Table 6 Percentage of nucleotide (above) and aminoacid (below) identities in the CP gene between the isolates representative of the five phylogenetic clusters of GLRaV-2

	PN	H4	RG	BD
H4	89,1			
	93,9			
RG	76,7	77,4		
	90,9	88,9		
BD	74,7	77,6	79,1	
	86,4	85,9	85,9	
Pv20	73,4	73,7	73,0	71,4
	80,8	79,3	79,8	77,8

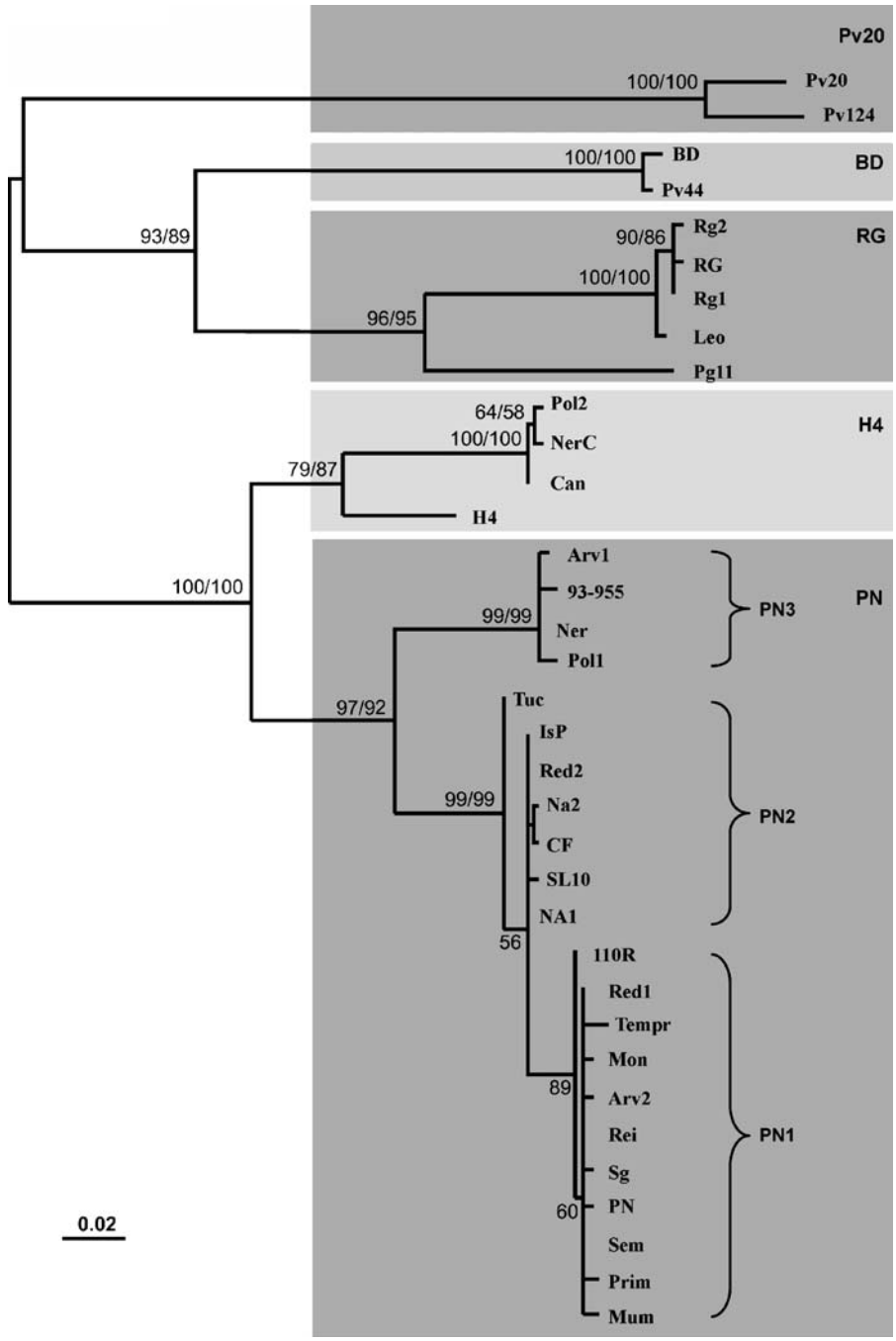

Fig. 1 Evolutionary relationships among the GLRaV-2 nucleotide sequences analysed in the coat protein gene (388 bp). The figure shows the best unrooted likelihood tree (−lnL=2476.029858) obtained using the PHYML program. Maximum parsimony tree topology was largely congruent. Bootstrap values obtained using both procedures are reported at the node (Maximun likelihood/Maximun parsimony), but only if they were higher than 50. The five squares in different colours, named Pv20, BD, RG, H4 and PN, identify the main clades. The length of the branches represents the number of reconstructed changes of state over all sites (the bar represents 0.02 substitution per site)

ly obtained in the CP gene for the 24 selected isolates (data not shown). No additional divergent variants were identified. Most of the viral variants fell into the PN group in all the dendrograms, even though the varietal and geographic origins of the vine samples were very different.

Biological trials for leafroll symptoms

Typical leaf symptoms of grapevine leafroll disease were induced in the indicators by the presence of GLRaV-1 and GLRaV-3, which are the most widespread grapevine leafroll viruses. The identification of

symptoms was very easy, given the down-rolling and the reddening of almost all the leaves from the early stage of veraison. On the other hand, GLRaV-2 infections showed weaker symptoms than the other two leafroll viruses. In particular, the symptoms only appeared in the late vegetative season and the leaves were affected unevenly. Only at the very end of the season did symptoms become evident and allow a clear distinction between healthy and infected plants. The leaves remained flat or slightly rolled and the colouration was less intense, with red garnet tones accompanied by yellow-orange nuances in the older leaves. The symptoms appeared mainly on the edges of the basal leaves on two year old canes (Supplementary Fig. 2).

After three years of visual observation all the plants infected with GLRaV-1, GLRaV-3 and GLRaV-6 showed evident leafroll symptoms, while only a small part of the GLRaV-2 infected accessions (19/105) were symptomatic (Table 4). The appearance of the symptoms was consistently associated with the presence of different GLRaV-2 isolates. Indeed, accessions with the RG variant never showed leafroll symptoms. On the other hand, accessions with the PN3 group in single or mixed infection were almost always symptomatic. In several cases different plants infected with the same isolate did not show the same biological behaviour: leafroll symptoms were observed only in 6 out of 46 plants infected with the PN1 variants and in 6 out of 28 accessions infected with the BD variant. Plants with mixed infection of isolates belonging to the PN1, PN2 and RG groups did not show leafroll, confirming previous results on PN1 and RG single infections and giving an idea of the biological properties of the PN2 group variants, which could not be tested as a single infection.

The viral sanitary status of accessions with leafroll symptoms was compared with that of asymptomatic plants, in order to discover if another virus could interfere with the manifestation of leafroll symptoms. Once the association with major grapevine viruses had been excluded, the correlation with the *Grapevine rupestris stem pitting associated virus* (GRSPaV) was studied. Despite the presence of this virus in most of the samples, no correlation was found with the appearance of leafroll symptoms.

Biological trials for graft incompatibility

Many young vines showed symptoms associated with graft incompatibility, such as cracking and strong swelling of the graft union, chromatic changes of leaves and weak shoot growth. Virus-free accessions did not show any symptoms, while visible symptoms associated with the graft incompatibility syndrome were present in a large number of the plants discarded at phase 2, in particular those infected with RG and PN1 isolates (Supplementary Fig. 3).

The average yield of healthy unrooted and rooted plants was in line with normal values obtained in nursery: about 85 % at phase 1 and about 70 % at phase 2. The number of surviving grafted grapevines was mostly invariant at phase 3; only some virus-free accessions grafted on 5C died. The total average yield of unrooted plants (phase 1) was very high even for GLRaV-2-infected plants. The number of surviving grafted infected grapevines strongly decreased at phase 2; some other plants died at phase 3.

The influence of rootstock was analysed first. Grapevines grafted on K5BB showed the most serious damage with a drop in yield of 50 % in infected plants. On the other hand, plants grafted on SO4 did not show any effects associated with the presence of GLRaV-2 infection. The influence of each virus isolate was subsequently investigated. The presence of each variant induced yield decreases, ranging from 18 % for BD isolates to 50 % for RG isolates. Other variants which were genetically similar to each other (PN1, PN2, PN3 and H4) showed intermediate effects (28 % yield decrease on average). Overall, the number of surviving grapevines was affected both by the cultivar of the rootstock and by the kind of viral variant, with a statistically significant interaction ($p<0.01$) (Table 5). Grapevine grafted on K5BB, 5C and 1103P showed a reduction in yield caused by all the virus variants; in particular, the most serious problems were associated with the presence of PN1 and H4 variants in K5BB, the presence of the RG isolate in 5C and the presence of the PN2 variant in 1103P, all of which caused the death of almost all the grafted grapevines. Generally, mixed infections with different GLRaV-2 isolates produced the same effect as single infections, though in some cases the mortality increased.

The development of canes and roots on saleable plants was evaluated at phase 2 after the first year in nursery (data not shown). The ANOVA test showed that there was a significant growth decrease in the infected plants compared to the healthy ones, and this was affected by both the viral isolate and the rootstock cultivar. All the GLRaV-2 infected plants grafted on K5BB showed a reduction in growth both in the canes ($p<0.01$) and in the roots ($p<0.05$). The development of grapevines grafted on 1103P was modified only by the RG and BD isolates ($p<0.05$), while plants grafted on 5C showed reduced vigour in the presence of BD ($p<0.01$) and PN2 ($p<0.05$) isolates. The development of grapevines grafted on SO4 was not affected by the presence of GLRaV-2 infections ($p>0.05$). It is worth noting that all surviving grapevines infected with the BD variant and grafted on K5BB, 1103P and 5C showed a reduction in vigour in comparison to the healthy plants.

Visible differences in plant growth disappeared after two years in vineyard and no other symptoms of graft incompatibility, such as weak shoot and solid red colouration, appeared.

Discussion

The study of the genetic variability of the CP gene by nucleotide sequencing revealed clustering of the GLRaV-2 known isolates into five main groups. The number of clades did not tally with that proposed by Meng et al. (2005), which identified four type variants: PN, 93/955, H4 and RG. In the present paper two very divergent groups were added—BD and Pv20—while the 93/955 variant fitted into the PN3 subcluster of the PN group.

The GLRaV-2 population observed in this work showed the presence of many slightly differing variants in most of the vine samples tested. The majority of the accessions under study were infected by a single, predominant GLRaV-2 variant; however, in some cases, HMA patterns composed of several bands revealed the presence of mixed infections. Analysis of GLRaV-2 variability in three ORFs showed high genomic conservation, with values similar to those reported for other closteroviruses (Kong et al. 2000) and ampeloviruses (Little et al. 2001; Turturo et al. 2005).

PN group variants, which were the most common, were identified both in autochthonous cultivars and in commercial varieties from different geographical areas, without any relationship with the original growth site. GLRaV-2 variants of the other groups were mostly identified in varieties of little economic importance or in autochthonous cultivars, such as Arvino, Cannonao, Leopoldo III, Nera, Nerello calabrese and Pollera nera. These data suggested that human activities played a major role in the dissemination of the virus through the world trade of GLRaV-2-infected grapevine propagative material.

Phylogenetic analyses of GLRaV-2 variants served as a basis for determining the pathogenic properties of the virus. Genetic variants can, indeed, cause different types of symptoms in the host, as in the case of CTV (Lee and Bar-Joseph 2000). In this work, after demonstrating that the GLRaV-2 population includes extremely divergent variants, their different pathological properties were studied.

The ability of GLRaV-2 isolates to produce leafroll symptoms was investigated first. A small percentage of infected plants showed mild leafroll symptoms, which appeared at the very end of the season. It was proved that only a few GLRaV-2 clusters had the ability to induce leafroll symptoms in all the infected plants, while in most cases symptoms appeared only in some accessions. Vines infected with the RG isolate were always asymptomatic, confirming previous results (Rowhani et al. 2000). On the whole, while these data proved that the manifestation of leafroll symptoms is related to the kind of GLRaV-2 cluster that infects the plant, they also suggest that other factors may contribute to the disease. Possible differences in susceptibility due to the different original varieties were removed with the use of the same leafroll indicators for all the accessions. The involvement of major grapevine viruses seems to be excluded. Also GRSPaV, whose involvement had been suggested by Meng et al. (2005), was not correlated with the presence of leafroll symptoms. Differences in symptoms caused by environmental conditions can be excluded, since all the plants were located in the same vineyard for the indexing trial and inspections were made over a period of two to three years. A reasonable hypothesis which might explain why the same GLRaV-2 cluster caused leafroll symptoms in only a few accessions could be the

unknown interaction of GLRaV-2 with other graft-transmissible agents, originating from the original accessions used as a rootstock, or the different virus concentrations in the plants; further studies on the correlation between the virus titre and the leafroll symptoms development should be performed. Moreover, it is possible that specific and still unknown viral molecular determinants, not always associated with the phylogenetic grouping, are responsible for the manifestation of leafroll symptoms in grapevine.

Importation, certification and registration programs for grapevines depend largely on biological indexing for leafroll disease, with the use of sensitive cultivars as an indicator for leafroll agents. The results reported in this work call the use of biological assays for GLRaV-2 diagnosis into question. Indeed, more than 80 % of the accessions which tested positive for GLRaV-2 with molecular assay did not show any leafroll symptoms, and it is worth noting that most of these variants are internationally widespread. For these reasons, serological and molecular procedures should definitely be used together with the biological assays for the detection of GLRaV-2 in certification and registration programs.

GLRaV-2 is able to induce not only leafroll symptoms but also graft incompatibility. In this work it was proved that damage caused by the different GLRaV-2 variants was strongly linked to the rootstock. Mortality, in particular, was higher when K5BB was used in grafting, followed by 5C and 1103P, in agreement with literature data (Uyemoto et al. 2001; Pirolo et al. 2006). The kind of virus isolate present also caused different reactions. It was shown that graft incompatibility is associated mainly with variants of the RG and PN groups. Decrease in yield or symptoms related to the graft incompatibility syndrome were not observed in vines infected with the BD isolate. Moreover, the reduction in vegetative growth detected after the first year in the nursery disappeared after two years in the field. The symptoms of early reddening and decline, observed by Bonfiglioli et al. (2003) on vines infected with the Alfie variant of GLRaV-2, which is very similar to the BD isolate, were not observed in this trial. However, the symptoms induced by the Alfie isolate appeared in three to four year-old vines; a further two years of visual observations on the accessions used in the present work would therefore be needed.

Given the symptoms induced in sensitive rootstocks, the RG variant was initially considered a different virus, named *Grapevine Rootstock Stem Lesion associated Virus* (GRSLaV) because of the distinct lesions found in the woody cylinders of specific rootstocks, which required more than one year to develop (Uyemoto et al. 2001). Further research is in progress to investigate these wood symptoms in GLRaV-2-infected grapevines. The removal of bark tissue, examination of the scion-rootstock junction and cross-sections of some surviving plants will be performed.

In this study the presence of symptoms associated with graft incompatibility did not represent the total damage. This was expressed much better by the nursery yield of rooted grafts. The disease caused strong initial damage, due mainly to the unsuccessful graft between scion and rootstock (phase 2); the overcoming of this critical phase did not lead to any significant further damage to the quality and surviving of grafted plants. Careful selection of rooted grafts by the nursery men before the sale does, therefore, help to minimize the damage caused by graft incompatibility syndrome on trading grapevine materials.

In conclusion, this work showed that GLRaV-2 has high genetic variability, congruent in the three genomic regions studied, with very divergent variants present only in a few grapevine samples. Biological trials revealed that extremely divergent variants have different pathological properties, mostly related to the phylogenetic grouping. This highlighted a clear correlation between genetic features and the biological behaviour of GLRaV-2 variants.

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