Vitis 30, 97-105 (1991)

Identification of the agent of grapevine fleck disease

by

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Identifizierung des Grapevine-Fleck-Erregers

Zusammenfassung: Ein Antiserum gegen ein italienisches Isolat von GPLIV (grapevine phloem-limited isometric virus) wurde dazu verwendet, um mittels ELISA die natürliche Verbreitung des Virus und seine Verbindung mit der Fleck-Erkrankung festzustellen. Insgesamt wurden 591 Pflanzen von *Vitis rupestris* auf das Vorkommen von GPLIV geprüft. Von 150 Reben mit Fleck-Symptomen waren 138 (92 %) ELISA-positiv und 12 (8 %) negativ. Von 441 symptomfreien *V.-rupestris*-Reben waren 435 (98,6 %) ELISA-negativ und 6 (1,4 %) positiv bezüglich GPLIV. Das Virus wurde bei ungefähr 30 % von 694 Reben verschiedener Herkunft, die in Apulien ausgepflanzt waren, entdeckt. Den stärksten Verseuchungsgrad (53 %) wies eine kommerziell genutzte Rebanlage mit der Sorte Italia auf, am geringsten infiziert (8 %) war eine Parzelle mit zertifizierten und visuell selektierten Unterlagsreben. Fleck-infizierte, nicht jedoch Fleck-freie *V.-rupestris* Reben enthielten im Phloemgewebe Viruspartikeln und vesikuläre Einschlußkörper. Pflänzchen der Sorte LN 33 aus *in-vitro*-Kulturen von Triebspitzen waren GPLIV-frei, wie mittels ELISA und Transmissionselektronenmikroskopie nachgewiesen werden konnte. Durch Pfropfung dieser Reben auf *V. rupestris* konnten keine Fleck-Symptome ausgelöst werden. Es wird gefolgert, daß GPLIV der Erreger der Fleck-Erkrankung ist, weshalb er in Grapevine-Fleck-Virus (GFKV) umbenannt werden sollte.

Key words: Vitis, varietiy of vine, rootstock, virosis, fleck, analysis, serology, ultrastructure, sanitation, Italy

Introduction

A non mechanically transmissible virus with isometric particles (GPLIV) was first detected in 1983 in the phloem of leafroll-affected vines (Castellano *et al.* 1983). Peculiar vesiculated inclusion bodies derived from heavily modified mitochondria (Castellano and Martelli 1984), were also observed in cells containing GPLIV and proven to be strictly associated with it (Castellano *et al.* 1985). GPLIV was successfully purified from naturally infected vines. It was shown to contain RNA, two types of particles (empty shells and nucleoproteins) about 30 nm in diameter, and to possess properties unlike those of any of the plant viruses assigned to current taxonomic groups (Boulila *et al.* 1990).

From the very beginning, the association of GPLIV with leafroll disease appeared to be rather loose. The virus was originally found in half of a number of leafroll-infected samples examined in Italy (Castellano *et al.* 1983) and was shortly afterwards detected also in symptomless vines in Italy (Belli *et al.* 1985; Castellano *et al.* 1985) and Switzerland (Gugerli *et al.* 1984). More convincing evidence of the lack of a cause-effect relationship between GPLIV and leafroll disease, was recently provided by the persistence of leafroll symptoms in vines from which GPLIV had been eliminated by heat treatment (Boulila *et al.* 1990).

A critical re-examination of our indexing records relative to the last 5 years showed that among graft-transmissible virus-like diseases, fleck was the one with the highest degree of association with the presence of GPLIV. The availability of an anti-

serum to GPLIV that could be used reliably in ELISA allowed to carry out studies aimed at investigating in detail the nature of the GPLIV-fleck association. The results of these studies, a preliminary account of which has already been given (BOSCIA *et al.* 1990), are reported in the present paper.

Materials and methods

Source material

Grapevine sources of various kinds were investigated for the presence of GPLIV. These were: (a) plants of *Vitis rupestris* indicators with or without symptoms of fleck, coming from our indexing plots, other Italian regions and Malta; (b) vines of different European grapevine varieties either from commercial vineyards, or from collections of foreign accessions or selected clones from Italy; (c) certified or visually selected clones of American rootstocks; (d) vines that had undergone sanitation by heat treatment or meristem tip culture *in vitro*.

Serology

Serological investigations were done by standard DAS-ELISA (CLARK and ADAMS 1977) using an antiserum to an Italian isolate of GPLIV with a titer of 1 : 128. The antiserum was absorbed with concentrated clarified healthy grapevine leaf extracts. Immunoglobulins were recovered by fractionation through a Protein A-Sepharose column from which they were eluted with 0.1 M glycine-HCl buffer pH 3. Positive and negative controls, consisting of tissue extracts from symptomatic and symptomless *V. rupestris*, were used in each test.

Electron microscopy

Small fragments of vein tissues were excised from leaves of symptomless ELISA-negative *V. rupestris* and LN 33, and from symptomatic ELISA-positive *V. rupestris* plants. These samples were double fixed in phosphate-buffered glutaraldehyde and osmium, processed for thin sectioning and stained according to standard procedures (MARTELLI and RUSSO 1984).

Results

Association of GPLIV with fleck

Of 150 *V. rupestris* vines with mild to severe fleck symptoms, 138 were ELISA positive for GPLIV (Table 1). The association of this virus with the disease was very high (92 % on average) regardless of the geographical origin of the source material, and ranged between 85 % (Emilia-Romagna) and 100 % (Sardinia and Veneto). By contrast, symptomless *V. rupestris* were virtually GPLIV-free, for about 99 % of the samples (435 out of 441) gave a negative ELISA response.

Distribution of GPLIV in the field

GPLIV was detected in about 30 % of field-grown vines (Table 2). The highest incidence (53 %) of ELISA-positive samples was in a commercial vineyard and the lowest (8 %) in a collection of American rootstock types containing virus-tested certified clones and visually selected candidate clones. Average infection levels (36 %) were

observed in a collection of *V. vinifera* varieties from European, Mediterranean and Middle Eastern countries.

It is worth pointing out that data of field detection of GPLIV by ELISA, tally with average fleck infection levels previously recorded in table and wine grapes visually selected in Southern Italy (SAVINO *et al.* 1985), or reported from Soviet Moldavia for European grapevine cultivars and American rootstocks (VERDEREVSKAYA *et al.* 1983).

Table 1

Presence of GPLIV in *Vitis rupestris* with and without symptoms of fleck

Vorkommen von GPLIV in *Vitis rupestris* mit Fleck-Symptomen und ohne Fleck-Symptome

Source	Symptomatic vines			Symptomless vines		
	Vines tested	ELISA-positive	ELISA-negative	Vines tested	ELISA-positive	ELISA-negative
A. Italy						
Apulia	101	94	7	413	3	410
Emilia-Romagna	20	17	3	13	1	12
Sardinia	11	11	0	7	0	7
Tuscany	4	3	1		—	—
Veneto	5	5	0	3	0	3
B. Malta	9	8	1	5	2	3
Total	150	138 (92 %)	12 (8 %)	441	6 (1.4 %)	435 (98.6 %)

Table 2

Distribution of GPLIV in different grapevine sources grown in Apulia

Verbreitung von GPLIV in Reben verschiedener Herkunft, die in Apulien ausgepflanzt wurden

Source	Vines tested	ELISA-positive	Percentage of infection
Visually selected clones of different varieties from Abruzzo (Central Italy)	162	53	33 %
Commercial vineyard of cv. Italia	148	79	53 %
Collection from foreign countries	101	36	36 %
Volunteer *V. rupestris* from commercial vineyards	55	17	30 %
Certified and visually selected rootstocks	228	19	8 %
Total	694	204	29 %

Presence of GPLIV in grapevine indicators

ELISA testing of *Vitis* indicators graft-inoculated with 10 selections of European grape varieties and American rootstock types, all of which had induced typical fleck responses in *V. rupestris* showed that GPLIV was readily detectable in all indicators.

Virus concentration was highest in symptomatic *V. rupestris* and in the symptomless hybrid *V. rupestris* × *V. berlandieri* 110 R (Table 3).

Distribution of GPLIV within grapevine plants

In earlier serological testing, it was noticed that inconsistent ELISA responses were sometimes obtained, which seemed to depend on the relative position on the vine of tissue samples analyzed. The distribution of GPLIV in different grapevine organs was therefore investigated, as follows:

(a) A shoot each from ELISA-positive vines of LN 33 and Primitivo di Gioia was chosen and the presence of GPLIV was checked in the petiole of each leaf and in each tendril from base to top. In both varieties (Table 4) ELISA readings were highest in the basal leaves. Antigen concentration decreased towards the top of the shoots, but only in the uppermost leaves of LN 33 it dropped below detection level. Tendrils of both cultivars proved to be good antigen sources for ELISA, especially for LN 33, in which virus concentration seemed to be higher in tendrils than leaves.

(b) Shoots from 20 ELISA-positive *V. rupestris* were collected and the presence of GPLIV was verified in the basal symptomless leaves, in the middle leaves with symptoms and in the apical leaves. ELISA readings showed that there was a gradient of antigen concentration, with highest values in the basal leaves and low unreliable readings in the apical leaves.

(c) Cuttings from cold-stored mature canes of 26 fleck-infected selections of different *V. vinifera* cultivars were rooted and tested for GPLIV. The virus was readily detected in cortical tissues (bark scrapings) and roots of all selections.

Effect of sanitation treatments on GPLIV

Of 14 plantlets obtained from a fleck-infected clone of Primitivo di Gioia, 5 (over 35 %) were found to be free from GPLIV after not less than 60 d of heat treatment at 38 °C (SAVINO *et al.* 1985).

Table 3

Presence of GPLIV in grapevine indicators inoculated with fleck-infected ELISA-positive donors

Vorkommen von GPLIV in Indikatorreben nach Inokulation mit Fleck-infizierten ELISA-positiven Donatoren

Donor varieties	ELISA readings (A_{405})				
	Vitis rupestris	Kober 5 BB	Mission	*Vitis riparia*	110 R
Bolgar	1.348	0.746	1.393	0.660	0.225
Italia 1	1.028	0.746	0.109	1.051	0.705
Italia 2	0.392	0.248	1.195	0.646	1.075
Italia 3	1.322	0.705	nt	1.189	1.355
Primitivo 1	1.246	0.540	0.256	1.170	0.520
Primitivo 2	0.076	0.795	**0.010**	1.350	1.346
Rcatzitelli	0.996	0.219	1.132	1.240	0.480
Verdeca	1.260	0.406	nt	**0.020**	1.115
157/11	0.792	0.157	nt	0.050	1.341
41 B	0.897	0.231	1.242	0.660	0.225

nt = not tested. Figures in bold are ELISA negative samples. Positive samples are considered those giving an A_{405} absorbance value of 0.050 or higher.

Table 4

Distribution of GPLIV in leaves and tendrils of two grapevine varieties

Verbreitung von GPLIV in Blättern und Ranken zweier Rebsorten

Position of the leaf or tendril, starting from the base of the shoot	ELISA readings (A_{405})			
	LN 33		Primitivo	
	Leaf	Tendril	Leaf	Tendril
1	0.433	0.068	0.448	0.214
2	0.101	0.578	0.451	0.220
3	0.362	0.105	0.296	0.103
4	0.050	0.051	0.242	0.089
5	**0.025**	0.181	0.079	0.229
6	0.050	**0.017**	0.256	0.216
7	0.062	0.171	0.251	0.060
8	0.054	0.879	0.195	0.240
9	0.103	0.371	0.161	0.264
10	0.304	0.654	0.198	0.050
11	0.219	0.406	0.211	0.091
12	0.264	0.701	0.305	0.260
13	0.177	—	0.161	—
14	0.149	—	0.212	—
15	0.070	—	0.125	—
16	0.121	—	0.079	—
17	0.079	—	0.233	—
18	0.113	—	—	—
19	**0.033**	—	—	—
20	**0.023**	—	—	—
21	**0.003**	—	—	—
22	**0.005**	—	—	—
23	**0.015**	—	—	—
24	**0.003**	—	—	—
Number of observations	24	12	17	12
Average of readings (A_{405})	0.117	0.349	0.230	0.168

Figures in bold are ELISA-negative samples. Positive samples are those giving an A_{405} absorbance value of 0.050 or above.

The serological screening of 309 rooted explants from a number of different heat-treated cultivars from Southern Italy showed that GPLIV persisted in 8.7 % of the explants (27 plantlets). Although the initial level of fleck contamination of these heat-treated vines was unknown, there are no reasons to believe that it differed from the average 30—40 % recorded in the area.

Absence of GPLIV was ascertained in 63 rooted explants propagated from meristem tips excised from a fleck-infected LN 33 vine and cultivated *in vitro*.

Graft transmission trials

Shoot tips from 4 sanitized rooted explants of LN 33 and from the fleck-infected LN 33 mother plant were green-grafted on a total of 30 pot-grown healthy *V. rupestris*

indicators (6 vines for each source of inoculum). Before grafting, all vines used in the experiment were re-checked by ELISA and found to be GPLIV-free except for the LN 33 mother plant. Serological tests made about 1 month after grafting showed that the 6 *V. rupestris* indicators inoculated with infected LN 33 shoots were ELISA-positive for GPLIV and exhibited incipient fleck symptoms. All indicators grafted with shoots from sanitized explants were ELISA-negative and remained symptomless. After a 3-month dormancy in the cold (4 °C), all grafted *V. rupestris* were moved to a glasshouse at 25 °C. Only the 6 vines inoculated with infected LN 33 explants showed clear-cut fleck symptoms on the new flush of vegetation and high ELISA readings.

Electron microscopy

Profiles of isometric virus particles (Fig., A) and/or vesiculated inclusion bodies (Fig., B) were found in thin-sectioned phloem tissues of 13 different ELISA-positive *V. rupestris* accessions from Italy and Malta but in none of 3 ELISA-negative vines from the same origins (Table 1). The same cytological features were observed in leaves of the fleck-infected LN 33 mother plant and in *V. rupestris* indicators that had been graft-inoculated with its shoots and were showing fleck symptoms. Virus particles and inclusion bodies were not seen in any of the ELISA-negative sanitized plantlets of LN 33, nor in *V. rupestris* indicators graft-inoculated with them.

Discussion

The present study has shown that GPLIV is readily transmitted by grafting to *Vitis* indicators. In grapevines, the virus is thoroughly systemic. It spreads quickly in the plants invading phloem tissues of roots, canes, leaves and tendrils, where it induces formation of highly characteristic vesiculated inclusion bodies. These cytopathological structures are sufficiently common and easy to detect to be regarded as useful GPLIV infection markers.

Additional and more significant results were the demonstration that : (a) GPLIV has a remarkably high and consistent association with fleck disease, as shown by its presence in *V. rupestris* indicators only when they show symptoms; (b) plantlets obtained by culturing *in vitro* meristem tips from fleck-infected vines no longer contain GPLIV, as ascertained serologically and by thin-sectioning and are unable to transmit fleck disease to *V. rupestris*.

Under circumstances preventing the re-inoculation of *V. rupestris* with pure GPLIV cultures because the virus is not mechanically transmissible, the findings of the present investigation, taken together, seem to fulfill to the largest possible extent Koch's postulates. On these grounds, it seems plausible to suggest that GPLIV is the etiological agent of fleck and to propose it to be renamed, accordingly, grapevine fleck virus (GFKV).

The hypothesis that fleck was caused by non mechanically transmissible isometric virus was put forward by VERDEREVSKAYA *et al.* (1983) on the basis of ultrastructural observations whereby spherical virus-like particles were seen in phloem cells of fleck-

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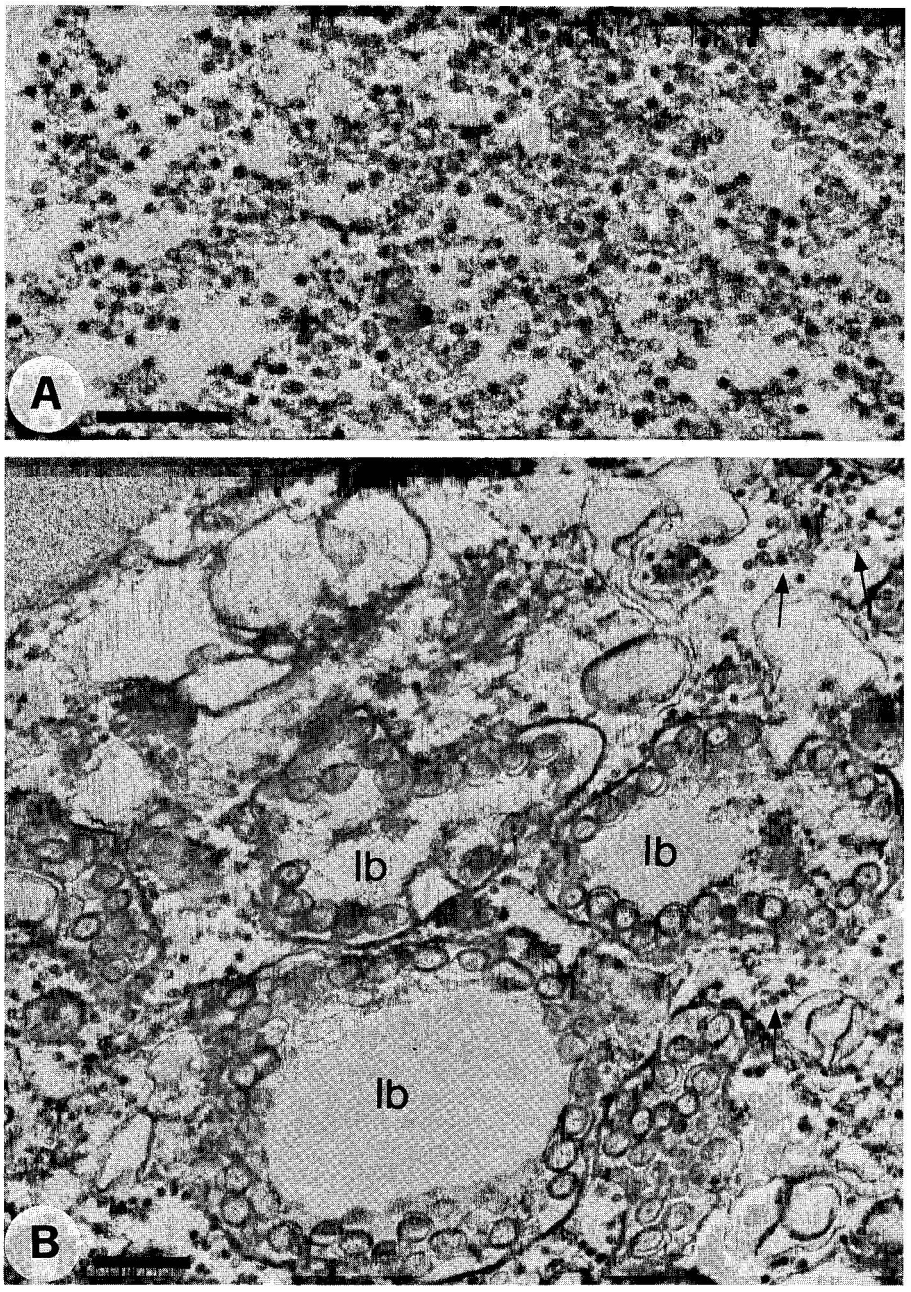
A) Gruppe isometrischer Partikeln in einer Phloemzelle von *V. rupestris* mit Fleck-Symptomen. Zahlreiche Partikeln liegen als leere Hüllen vor; wahrscheinlich handelt es sich hierbei um die T-Komponente von GPLIV. — B) Vesikuläre Einschlußkörper (Ib) , typisch bei GPLIV-Infektionen, in einer Phloemparenchymzelle von *V. rupestris* mit Fleck-Symptomen. Dieselbe Zelle enthält verstreute Gruppen isometrischer Viruspartikeln (Pfeile). — Balken = 200 nm.



A) A group of isometric particles in a phloem cell of a *V. rupestris* plant with fleck symptoms. Many of the particles appear as empty shells which are likely to represent the T component of GPLIV. — B) Vesiculated inclusion bodies (Ib) typically associated with GPLIV infections in a phloem parenchyma cell of *V. rupestris* with fleck symptoms. The same cell contains scattered groups of isometric virus particles (arrows). — Bars = 200 nm.

infected Moldavian vines. These vines were recently reported (DOLJA *et al.* 1990) to contain a dsRNA of a size compatible with that of the genomic RNA of GFKV as estimated by BOULILA *et al.* (1990). Although it is not known whether the Moldavian virus is the same as GPLIV and no ultimate evidence of its real involvement in the aetiology of fleck was provided by VERDEREVSKAYA *et al.* (1983), these authors had made a valuable observation, which lends support to the conclusion we now foster. This conclusion appears to be endorsed also by GUGERLI *et al.* (1990) and ENGELBRECHT and KASDORF (1990), who have reported that a virus serologically related to GPLIV is the possible agent of fleck in Switzerland and South Africa, respectively.

Summary

An antiserum against an Italian isolate of grapevine phloem-limited isometric virus (GPLIV) was used in an ELISA survey carried out for assessing the natural distribution of the virus and its association with fleck disease. A total of 591 vines of *Vitis rupestris* were checked for the presence of GPLIV. Of 150 plants with fleck symptoms, 138 (92 %) were ELISA-positive and 12 (8 %) negative. Of 441 symptomless *V. rupestris*, 435 (98,6 %) were ELISA-negative and 6 (1,4 %) positive for GPLIV. The virus was detected in about 30 % of 694 vines of different origin grown in Apulia (Southern Italy). The highest infection (53 %) was in a commercial vineyard of cv. Italia and the lowest (8 %) in a plot of certified and visually selected rootstocks. Fleck-infected, but not fleck-free *V. rupestris* contained virus particles and vesiculated inclusion bodies in phloem tissues. LN 33 plantlets derived from *in vitro* culture of meristem tips from ELISA-positive fleck-infected mother plants were found to be free from GPLIV, as ascertained by ELISA and thin-sectioning. These vines failed to induce fleck symptoms when grafted on *V. rupestris*. It is concluded that GPLIV is the agent of fleck and, therefore, it should be renamed grapevine fleck virus (GFKV).

Acknowledgements

Research supported by the National Research Council of Italy, Special Project RAISA, Subproject No. 2, Paper No. 46.

Some of the *V. rupestris* samples analyzed in the present work were kindly supplied by Drs. M. BORGO, Conegliano; R. CREDI, Bologna, E. TRIOLO, Pisa and U. PROTA, Sassari, whose help is gratefully acknowledged.

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Received, 28. 1. 1991

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