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Isolation and characterization of Agrobacterium strains from grapevines in Bulgarian vineyards and wild grapes, *V. vinifera* ssp. *silvestris*

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Summary

Twenty strains of *Agrobacterium* spp. were isolated from grapevines derived from 9 different grape-growing regions in Bulgaria and from symptomless wild grapes, *Vitis vinifera* ssp. *silvestris*, naturally growing in forests of South-East Bulgaria. The isolated strains were examined by biochemical and pathogenicity tests and PCR analysis of chromosomal and plasmid-located genes. Two of the studied strains resemble non-pathogenic *A. tumefaciens*, but the others were determined as tumorigenic *A. vitis*. Only one out of 18 *A. vitis* strains, isolated from grapevines, was found to harbor vitopine Ti plasmid type. Surprisingly, all 4 *A. vitis* strains isolated from symptomless *V. vinifera* ssp. *silvestris* plants, proved to be tumorigenic. Thus, for the first time we report the isolation of pathogenic *A. vitis* strains from wild grapes. The strict correlation between the results from biochemical and virulence tests and PCR analysis suggests that the chosen PCR procedures can be used efficiently for direct PCR testing for the presence and tumorigenicity of *A. vitis* in grapevines.

K e y w o r d s : *Agrobacterium*, grapevine, wild grapes, *V. vinifera* ssp. *silvestris,* pathogen strain, polymerase chain reaction.

Introduction

Crown gall is one of the most important and widespread bacterial diseases of grapevines. It is caused predominantly by *Agrobacterium vitis,* although *A. tumefaciens* was also found occasionally in infected vines (Burr *et al*. 1998). *Agrobacterium* survives systemically in infected grapes (Lehoczky 1968). There is no effective chemical control and the preventive measures including early detection of the pathogen and propagation of disease-free planting material are the only way out (Burr *et al*. 1998, Burr and Otten 1999). In recent years the use of a non-pathogenic *A. vitis* strain F2/5 for biological control was proposed (Burr and Reid 1993, Burr *et al*. 1998). A number of *A. vitis* strains were isolated from various grape-growing areas in the world; they were further characterized for their tumorigenic capacity, T-DNA structure, physiological and biochemical properties. Representative strain collections were established and well characterized reference strains were selected (Burr *et al*. 1998, Burr and Otten 1999). Ti plasmid-located genes are responsible for the tumor formation in infected plants (Burr and Otten 1999 and references therein). Restriction maps, cloned fragments, and sequences are now available for the major types of *A. vitis* Ti plasmids. With regard to the Ti plasmid, three basic structures have been defined: the octopine/cucomopine type, the nopaline type and the vitopine type (Szegedi *et al*. 1988, Burr and Otten 1999, Szegedi 2003). Different primer pairs based on plasmid and chromosome DNA sequences are designed and PCR analysis was successfully applied for detection of *A. vitis*, prediction of strain tumorigenicity and T-DNA type (Winans *et al*. 1987, Eastwell *et al*. 1995, Haas *et al*. 1995, Louws *et al*. 1999, Szegedi and Bottka 2002).

The presence of wild grapes, *V. vinifera* ssp. *silvestris*, naturally growing as single plants in the forests of South-East Bulgaria was reported by Negrul (1968) and Katerov (1968). The occurrence of Crown Gall disease in Bulgarian vineyards was described for the first time by Valachev (1902). Malenin (1980) reported Crown Gall to occur in all grape-growing regions of Bulgaria. Besides these studies no systematic collection, differentiation and characterization of *Agrobacterium* strains isolated from Bulgarian vineyards and naturally growing wild grapes was performed so far.

Here we describe the isolation and characterization of *Agrobacterium* strains from different grape-growing regions of Bulgaria. Moreover, the isolation of pathogenic *A. vitis* strains from wild grapes (*V. vinifera* ssp. *silvestris)* is reported. The application of biochemical and PCR tests for strain differentiation and the determination of strain tumorigenicity are discussed.

Material and Methods

B a c t e r i a l s t r a i n s : The donor grapevine plants are located in different grapevine growing regions and represent old native *V. vinifera* cvs like Mavrud and Dimiat, widespread cvs like Cabernet Sauvignon, Merlot and Chardonnay and locally selected cv. Velika. *V. vinifera* ssp *silvestris* plants were found in the forests of the Arkutino area and the Stranja Mountain, South-East Bulgaria. *V. vinifera* trunks and canes with typical crown galls were collected from vineyards in the main grape-growing regions of Bulgaria. Stem samples from wild *V. vinifera*

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ssp. *silvestris* were collected in natural forests in South-Eastern Bulgaria. None of the sampled wild vines had any apparent galls on their roots and stems. To isolate *Agrobacterium* spp., the surface cell layers of the samples were removed with a scalpel; they were washed diligently with sterile distilled water. The samples were cut to small pieces, transferred into 1.5 ml centrifuge tubes and stored at -70 °C for one h. Following incubation at room temperature for 30 min the samples were centrifuged at 5678 g for 10 min. The suspension collected from the bottom of the centrifuge tubes was spread on semiselective Roy and Sasser medium (RS, Burr and Katz 1984) and incubated at 28 °C. Colonies typical to *A. vitis* were streaked on potato dextrose agar (PDA). All isolates were maintained on PDA for further characterization. The studied strains are listed in the Table. Several well-characterized strains of *Agrobacterium* spp. were used as controls. To perform the virulence test and DNA isolation, the bacterial strains were grown on liquid Luria Bertani medium (LB, Maniatis *et al*. 1982) overnight at 28 °C with 200 rpm shaking.

Physiological and biochemical tests: A standard set of physiological and biochemical tests to differentiate *Agrobacterium* spp. was applied (Moore *et al*. 2001). It included: evaluation of growth on D1M agar, 3-ketolactose production, growth in 2 % NaCl, growth at 35 °C, acid production from melezitose, acid clearing on PDA plus $CaCO_3$, motility at pH 7.0, citrate utilization, and oxidase reaction.

Virulence tests: *Vitis riparia* x *V. Berlandieri*, var. SO4 and *Lycopersicon esculentum*, cv. Triadica (tomato) plants were grown in the greenhouse. The stems of the plants were inoculated by a syringe with a sterile hypodermic needle. Bacterial suspensions (150 µl, optical density $(OD)_{600} = 0.2$) were applied to each plant by perforating stems. Tumor formation was scored 6 weeks after inoculation of *V. riparia* and 3 weeks after inoculation of tomato plants. Under laboratory conditions, well-developed carrots (*Daucus carota*) were surface-sterilized with 20 % bleach, cut into slices and placed onto a wet filter paper in sterile Petri dishes. The top of the slices was inoculated by spreading 150 µl bacterial suspensions. Tumor formation was scored 3 weeks after inoculation.

Molecular analyses of the strains: Bacterial DNAs were isolated from overnight cultures using the rapid Triton X-100 / sodium azide protocol (Abolmaaty *et al*. 2000, Szegedi and Bottka 2002). The PCR amplifications were carried out in the Gene Amp PCR System 9700 in 25 µl final volume. The reaction mixture consists of 1x *Taq* polymerase buffer, 1.8 mM $MgCl_2$, 0.4 µM of each primer, 160 µM of each dNTP, 1.25 unit Taq polymerase (Fermentas, USA) and 2 µl template DNA. The reaction conditions involved an initial denaturation step at 94 °C for 1 min, followed by 30-35 cycles at 94 °C, 50 °C for VirD2 and 54 °C for PG, VirE2 and VIS, 72 °C for 1 min at each temperature and final extension step at 72 °C for 5 min. Four PCR primer pairs were used. PGF (5'-GGGGCAGGATGCGTTTTTGAG) and PGR (5'-GACGGCACTGG-GGCTAAGGAT) specifically amplified a 466 bp fragment of the chromosome located polygalacturonidase (PG) gene of *A. vitis* (Herlache *et al*. 1997, Szegedi and Bottka 2002). VirD2A (5'-ATGCCCGATC-GAGCTCAAGT) and VirD2C (5'-TCGTCTGGCT-GACTTTCGTCATAA) amplified a 224 bp fragment of the plasmid *vir*D2 gene of *Agrobacterium* spp. (Haas *et al*. 1995). VirE2PF (5'-CGTGCTGCCGTCTCTACA) and VirE2PR (5'-ACTGAACGCGATCCCACA) amplified a 753 bp fragment of *vir* E2 gene from *Agrobacterium* sp., but not *A. vitis* harboring vitopine type Ti plasmid (Winans *et al*. 1987, Szegedi and Bottka 2002). VisF (5'-CCG-GCCACTTCTGCTATCTGA) and VisR (5'-CCATTCAC-CCGTTGCTGTTATT) specifically amplified a 561 bp fragment of *A. vitis* vitopine synthase (VIS) gene associated with vitopine type Ti-plasmid (Canaday *et al*. 1992, Szegedi and Bottka 2002).

Results and Discussion

Totally 20 isolates of *Agrobacterium* spp. were recovered after the isolation from the plant samples and subsequent screening on semi-selective RS medium. Sixteen of them were isolated from grapevine tumors and 4 from symptomless wild grapes. A previously isolated strain B-29 (Malenin 1973) was included in the study, as well. Although no direct comparison of different isolation protocols was performed, we readily isolated bacteria from all plant samples through a procedure including -70 °C freezing of the plant material. Introduction of the deep freezing step is assumed to facilitate the release of bacteria strongly attached to grape cell walls. The isolated strains, producing typical colonies on RS medium, were further subjected to a set of biochemical tests to differentiate *Agrobacterium* spp. (Moore *et al*. 2001). The results (Table) suggest that the strains IG-1 to IG-18 and B-29 resemble *A. vitis*, while the strains KT and TP show 3-ketolactose production and acid from melezitose and are *A. tumefaciens.* All studied strains were found to be motile at pH 7 and strains KT and TP utilize citrate, which were exceptions from the described typical characteristics of *A. tumefaciens* (Moore *et al*. 2001). The results demonstrated PCR amplification with the *A. vitis*-specific PGF/PGR primer pair for all 19 strains determined as *A. vitis* (Table, Figure, A), which confirms their correct differentiation by the biochemical tests. The 19 *A. vitis* strains were also positive by PCR with *vir*D2 primer pair (Table), which points out that they carry Ti plasmids. No PCR products were observed from the *A. tumefaciens* strains KT and TP after analysis with *vir*D2 and *vir*E2 genes, suggesting that they do not harbor Ti plasmids and are non-pathogenic (data not shown). The PCR analysis with Vis primers specific for the vitopine synthase gene revealed amplification for the *A. vitis* strain IG-18, suggesting that it harbours a vitopine type Ti plasmid (Canaday *et al*. 1992, Szegedi and Bottka 2002). All other studied *A. vitis* strains show amplification with *vir*E2 primers but were negative at PCR with vitopine synthase primers (Table, Figure, B, C), and could be considered to harbor a non-vitopine type of Ti plasmids. The virulence test confirms the results from PCR analysis as all 19 *A. vitis*

Table 1

Characterization of the *Agrobacterium* strains isolated from Bulgarian vineyards and wild grapes *V. vinifera* ssp. *silvestris*

Strains	Variety	Location	Species Pathogenic. (Ti plasmid type)	Physiological and biochemical tests										PCR analysis			Tumor induct. on		
				Growth	3-ketolact.	Growth	Growth	Acid from	Acid	Motility at	Citrate	Oxidase	PG	VirD2	VirE2	VIS	*V. riparia*	Tomato	Carrot
IG-1	Cab. Sauv.	Pleven / BG	*A. vitis*, P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	+
IG-2	Cab. Sauv.	Pleven / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-3	Cab. Sauv.	Pazardjik / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-4	Chardonay	Pravosl. / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	+
IG-5	Cab. Sauv.	Pomorie / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-6	Cab. Sauv.	Pomorie / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-7	Velika	Biala / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-8	Velika	Biala / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-9	Wild grape	Arkutino / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-10	Wild grape	Arkutino / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-11	Wild grape	Stranja / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-12	Wild grape	Stranja / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-13	Mavrud	Dragom. / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-14	Mavrud	Dragom. / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-15	Mavrud	Dragom. / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
IG-16	Cab. Sauv.	Sliven / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	NT
IG-17	Chardonay	Sliven / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	NT
IG-18	Merlot	Sliven / BG	*A. vitis,* P,vit^+	+	-	+	-	-	-	+	+	+	+	+	-	+	+	+	NT
B-29**	Cab. Sauv.	Pleven / BG	*A. vitis,* P^+	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-
KT	Dimiat	Korten / BG	*A.tum.,* NP^+	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-
TP	Cab. Sauv.	Topolovo / BG	*A.tum.,* NP^+	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-
S-4*	Szegedi *et al.* 1988		*A. vitis,* P, vit	+	-	+	-	-	-	+	+	+	+	+	-	+	+	+	NT
AB-3*	Szegedi *et al.* 1988		*A. vitis,* P, oct	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	NT
AT-1*	Szegedi *et al.* 1988		*A. vitis,* P, nop	+	-	+	-	-	-	+	+	+	+	+	+	-	+	+	NT
F2/5*	Burr and Reid 1993		*A. vitis,* NP	+	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-
K-84*	Kerr 1972		*A. rhiz.,* NP	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-
C-58*	Hooyakas *et al.* 1980		*A.tum.,* P, nop	+	+	+	-	+	-	+	-	+	-	+	+	-	+	+	+

*- control strains; **- provided by Malenin, (Malenin 1973); $^+$ determined in this study.

P (NP) - (non) pathogenic; Ti plasmid type: vit- vitopine, oct- octopine, nop- nopaline; (NT) - not tested

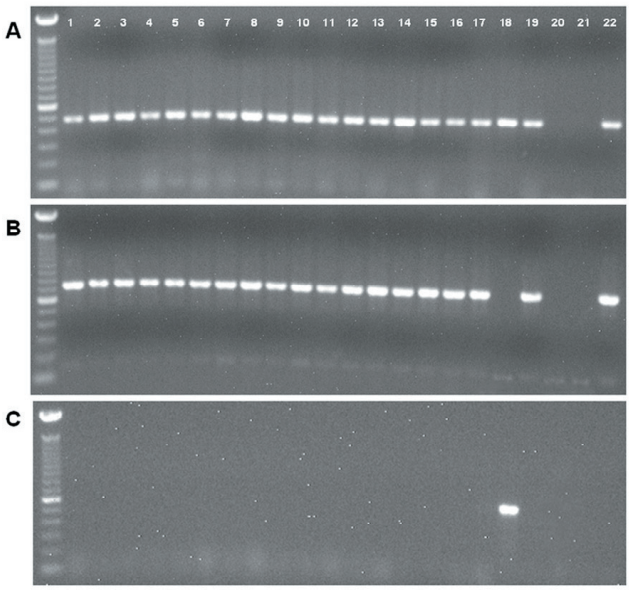

Figure: PCR analysis of the strains: **A,** PG primer pair amplifies a 466-bp product from all *A. vitis* strains. **B,** VirE primer pair amplifies 753-bp product from non-vitopine type strains. **C.** Vis primer pair amplifies a 561-bp product from vitopine type strains. Lane numbers correspond to the following strains: **1**, IG-1; **2**, IG-2; **3**, IG-3; **4** IG-4; **5**, IG-5; **6**, IG-6; **7**, IG-7; **8**, IG-8; **9**, IG-9; **10**, IG-10; **11**, IG-11; **12**, IG-12; **13**, IG-13; **14**, IG-14; **15**, IG-15; **16**, IG-16; **17**, IG-17; **18**, IG-18; **19**, B-29; **20**, KT; **21**, TP ; **22**, AB-3; Bands in size-marker lanes represent increments of 100 bp.

strains were found to induce tumors on greenhouse-grown *V. riparia* and tomato plants and some of them on carrot slices. At the same time no tumor formation was observed after inoculation with the *A. tumefaciens* strains KT and TP, determined as non-pathogenic (Table). The comparison of the isolated strains with the set of characterized control strains of *Agrobacterium* spp. in all performed tests demonstrates the accurate performing of the tests and the correct interpretation of data (Table).

The results from the present study demonstrate that sampled tumor bearing grapevine plants from vineyards in Bulgaria contain predominantly pathogenic non-vitopine type strains of *A. vitis.* Only the strain IG-18 is found to harbor vitopine type Ti-plasmid (Table, Figure, C). Similar results were obtained within other studies involving characterization of bacterial strains from grapevines in Germany, France, Australia, Hungary, Turkey and South Africa (Burr and Otten 1999, Argun *et al*. 2002, Szegedi *et al*. 2005). The close correlation between the results from biochemical and virulence tests and PCR analysis of the isolated strains suggests that the applied set of PCR analysis could be efficiently used for direct PCR testing of plant material for planting and production of *Agrobacterium*-free vines, similar to that proposed by Eastwell *et al*. (1995), Kaufmann *et al*. (1996), Louws *et al*. (1999), and Szegedi and Bottka (2002).

Two earlier studies on *A. vitis* strains colonizing wild *V. vinifera* ssp *silvestris* (C. Bazzi, unpubl. data, reviewed by Burr and Otten 1999) and *V. riparia* (Burr *et al*. 1998) demonstrated that all isolated strains are non-tumorigenic. Thus, the results of the present study are very interesting since they show that all 4 *A. vitis* strains isolated from wild *V. vinifera* ssp *silvestris* are tumorigenic (strains IG-9 to IG-12). The sampled wild grape plants are placed in the forest areas of south-eastern Bulgaria with no vineyards nearby. Thus, it is unlikely that the isolated pathogenic *A. vitis* strains are derived from contamination with commercial grapevines. The isolation of tumorigenic *A. vitis* strains from wild grapes lacking distinguished galls on their stems and roots raises a number of questions concerning their biodiversity and genetic relationship with other pathogenic strains isolated from vineyards in Bulgaria, as well as the extent of their host range. Presently we initiate a molecular marker study on the biodiversity of the isolated *A. vitis* strains and are in search of new locations with naturally growing wild grapes.

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