EVALUATION OF DIFFERENT PCR PRIMERS FOR IDENTIFICATION OF TUMORIGENIC BACTERIA ASSOCIATED WITH GRAPEVINE CROWN GALL

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SUMMARY

Grapevine crown gall caused by *Allorhizobium vitis*, or less frequently by *Agrobacterium tumefaciens* complex and *Rhizobium rhizogenes*, may seriously impact production in nurseries and vineyards worldwide. Although rapid and efficient detection and identification of tumorigenic bacteria is facilitated by PCR-based methods, high genetic diversity of these pathogens may hinder use of these methods in the disease diagnosis. Therefore, reliability of 11 primer pairs targeting fragments located on Ti plasmid or chromosomal DNA was tested on extensive collection of *All. vitis*, *A. tumefaciens* complex and *R. rhizogenes* strains isolated from grapevine throughout the world. Only primers VCF3/VCR3 targeting *virC* gene located on Ti plasmid clearly and accurately identified all tested tumorigenic strains associated with grapevine crown gall. Moreover, this primer pair coupled with primers specific for chromosomal *pehA* gene (PGF/PGR) in duplex PCR, may be recommended as the method of choice for routine preliminary identification of tumorigenic strains and differentiation of *All. vitis*.

Keywords: Allorhizobium vitis, *Agrobacterium tumefaciens* complex, *Rhizobium rhizogenes*, tumor-inducing plasmid, diagnosis.

INTRODUCTION

Crown gall is one of the most significant and widespread bacterial diseases of grapevine (*Vitis vinifera* L.) that may severely impact production in nurseries and vineyards throughout the world (Burr *et al*., 1998; Burr and Otten, 1999). Although *Allorhizobium vitis* is recognized as the predominant species causing this disease, tumorigenic strains belonging to *Agrobacterium tumefaciens* species complex (i.e. *A. tumefaciens*/biovar 1) and *Rhizobium rhizogenes* (i.e. *Agrobacterium rhizogenes*/biovar 2) were occasionally isolated from galled grapevine worldwide.

Pathogenicity of the bacteria causing crown gall primarily depends on the presence of tumor-inducing (Ti) plasmid in their genome (Zhu *et al*., 2000). The Ti plasmids are highly diverse and are classified by the type of opines produced in tumors as a consequence of genetic transformation of infected plants (Dessaux *et al*., 1998). Accordingly, *All. vitis* Ti plasmids have been classified into three major groups: octopine/cucumopine (O/C), nopaline (N) and vitopine (V) (Szegedi *et al*., 1988; Paulus *et al*., 1989). *A. tumefaciens* complex strains isolated from galled grapevine so far possessed agropine (A), N, octopine (O) and *All. vitis* O/C-type Ti plasmids, while a few *R. rhizogenes* strains associated with grapevine crown gall harbored N-type Ti plasmid (Ridé *et al*., 2000; Szegedi *et al*., 2005; Palacio-Bielsa *et al*., 2009b).

All. vitis is able to survive systemically in grapevine, which represents a serious risk for pathogen dissemination via latently infected propagation material (Burr *et al*., 1998; Burr and Otten, 1999). Once introduced in a vineyard, the pathogen may survive in grapevine debris in soil for more than 2 years after the infected plants are removed (Burr *et al*., 1995). However, *All. vitis* and other species causing grapevine crown gall are not considered quarantine pathogens in many countries. They are commonly regarded as harmful, widespread pathogens that can reduce the value of propagation material (quality pathogens). Therefore, in international trade, grapevine material exchanges are not a subject of strict phytosanitary control for the presence of these pathogens. In addition, phytosanitary diagnostics of tumorigenic bacteria in grapevine is hampered by a lack of standardized protocols for pathogen detection and identification. Consequently, incidence of grapevine crown gall that has been recorded in many European countries in the last 15 years was likely associated with pathogen introduction and spread by infected propagation material (Kuzmanović *et al*., 2015a).

Crown gall diagnosis was generally based on isolation of bacteria on semiselective media, biochemical tests and pathogenicity assay (Moore *et al*., 2001). Although time consuming and laborious, pathogenicity assay is the only reliable method for determining tumorigenicity of bacteria associated with grapevine crown gall. On the other hand, application of polymerase chain reaction (PCR) provides

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Table 1. Strains of *Allorhizobium vitis*, *Agrobacterium tumefaciens* complex and *Rhizobium rhizogenes* isolated from grapevine that were used in this study and results of PCR analysis using different primer sets.

							Primer sets[e]							
Strain	**P**[a]	**pTi**[b]	**Geographical origin**	**Y**[c]	**Source**[d]	**Literature**	A/C'	CYT/ CYT'	PGF/ PGR	tms2F1/ tms2R2	VCF3/ VCR3	VirD2S4F$_{716}$/ VirD2S4R$_{1036}$	VirFF$_1$/ VirFR$_2$	UF/B1R/B2R/ AvR/ArR
Allorhizobium vitis														
AB3	T	OS	Hungary	1982	S. Süle	(Szegedi *et al.*, 1988)	(+)	–	+[j]	–	+	–	+	Av[j]
AB4	T	N	Hungary	1982	IPV-BO	(Szegedi *et al.*, 1988)	+	+	+[j]	+[j]	+	–[j]	+[j]	Av[j]
Av2	T	O/C	Croatia	2006	IPV-BO	(Kuzmanović *et al.*, 2015a)	+	+	+	+	+	–	+	Av
BPIC 956	T	O/C	Greece	1987	BPIC	UN	+	+	+	+	+	–	+	Av
CG47	T	N	USA	1979	CU	(Otten *et al.*, 1996)	+	+	+	+	+	–	+	Av
CG49	T	N	USA	1979	IPV-BO	(Burr *et al.*, 1987)	+	+	+[j]	+[j]	+	–[j]	+[j]	Av[j]
CG56	T	N	USA	1979	CU	(Burr *et al.*, 1995)	+	+	+	+	+	–	+	Av
CG78	T	V	USA	1981	CU	(Otten *et al.*, 1996)	+	–	+	–	+	+	–	Av
CG81	T	V	USA	1983	CU	(Otten *et al.*, 1996)	+	–	+	–	+	+	–	Av
CG102	T	OL	USA	1984	IPV-BO	(Bazzi *et al.*, 1988)	+	+	+[j]	+	+	–[j]	+[j]	Av
CG108	T	OL	USA	1985	CU	(Otten *et al.*, 1996)	+	+	+	+	+	–	+	Av
CG228	T	V	USA	1981	CU	(Otten *et al.*, 1996)	+	–	+	–	+	+	–	Av
CG415	T	OS	USA	1984	CU	(Otten *et al.*, 1996)	(+)	–	+	–	+	–	+	Av
CG447	T	N	USA	UN[i]	CU	(Otten *et al.*, 1996)	+	+	+	+	+	–	+	Av
CG475	T	OS	USA	1986	CU	(Otten *et al.*, 1996)	(+)	–	+	–	+	–	+	Av
CG511	NP		USA	1992	CU	(Burr *et al.*, 1999)	–	–	+	–	–	–	–	Av
F2/5	NP		South Africa	UN	IPV-BO	(Staphorst *et al.*, 1985)	–	–	+[j]	–	–	–	–	Av
IPV-BO 1861-5	T	V	Italy	1984	IPV-BO	(Bini *et al.*, 2008b)	+	–	+[j]	–	+	+[j]	–[j]	Av
IPV-BO 2152	T	V	Italy	UN	IPV-BO	(Bini *et al.*, 2008b)	+	–	+[j]	–	+	+[j]	–[j]	Av
IPV-BO 5159	T	V	Italy	2003	IPV-BO	(Bini *et al.*, 2008b)	+	–	+[j]	–	+	+[j]	–[j]	Av
IPV-BO 5162	T	O/C	Italy	2003	IPV-BO	(Bini *et al.*, 2008b)	(+)	–	+[j]	–	+	–[j]	+[j]	Av
IPV-BO 5372	T	O/C, V[g]	Italy	2003	IPV-BO	(Bini *et al.*, 2008b)	+	–	+[j]	–	+	+[j]	+[j]	Av
IPV-BO 5761	T	O/C	Moldova	2004	IPV-BO	(Bini *et al.*, 2008a)	(+)	–	+	–	+	–	+	Av
IPV-BO 5881	T	O/C	Italy	2005	IPV-BO	(Bini *et al.*, 2008a)	(+)	–	+	–	+	–	+	Av
IPV-BO 6048A1	T	O/C	Montenegro	2005	IPV-BO	(Bini *et al.*, 2008a)	(+)	–	+	–	+	–	+	Av
IPV-BO 6186	T	O/C	Italy	2006	IPV-BO	(Bini *et al.*, 2008a)	(+)	–	+	–	+	–	+	Av
IPV-BO 6207	T	O/C	Serbia	2006	IPV-BO	(Bini *et al.*, 2008a)	+	+	+	+	+	–	+	Av
IPV-BO 6209	T	O/C	Serbia	2006	IPV-BO	(Bini *et al.*, 2008a)	(+)	–	+	–	+	–	+	Av
IPV-BO 6570	T	O/C	Bulgaria[h]	2006	IPV-BO	(Bini *et al.*, 2008b)	(+)	–	+[j]	–	+	–[j]	+[j]	Av
IPV-BO 6571	T	V	Bulgaria[h]	2006	IPV-BO	(Bini *et al.*, 2008b)	+	–	+[j]	–	+	+[j]	–[j]	Av
IPV-BO 7104	T	O/C	Italy	2007	IPV-BO	(Kuzmanović *et al.*, 2015a)	(+)	–	+	–	+	–	+	Av
IPV-BO 8463	T	O/C	Morocco	2011	IPV-BO	(Kuzmanović *et al.*, 2015a)	(+)	–	+	–	+	–	+	Av
IPV-BO 8816	T	V	Italy	2011	IPV-BO	(Kuzmanović *et al.*, 2015a)	+	–	+	–	+	+	–	Av
IVIA 339-26	T	O/C	Spain	1982	IVIA	(Palacio-Bielsa *et al.*, 2009b)	+	+	+	+	+	–	+	Av
IVIA 2680-2-a3	T	V	Spain	UN	IVIA	(Palacio-Bielsa *et al.*, 2009b)	+	–	+	–	+	+	–	Av
IVIA 2739-16	T	O/C	Spain	UN	IVIA	(Palacio-Bielsa *et al.*, 2009b)	+	+	+	+	+	–	+	Av
K309[T]	T	O/C	Australia	1977	IPV-BO	(Ophel and Kerr, 1990)	+	+	+	+	+	–	+	Av[j]
KFB 239	T	O/C	Serbia	2010	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 241	NP[f]	O/C	Serbia	2010	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 242	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 243	T	V	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	+	–	+	–	+	+	–	Av
KFB 245	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 247	NP[f]	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 249	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 250	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 253	T	O/C	Serbia	2011.	KFB	(Kuzmanović *et al.*, 2014)	+	+	+	+	+	–	+	Av
KFB 254	T	O/C	Serbia	2011.	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 255	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 262	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 264	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	+	+	+	+	+	–	+	Av
KFB 267	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 269	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 272	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 273	T	O/C	Serbia	2011	KFB	(Kuzmanović *et al.*, 2014)	(+)	–	+	–	+	–	+	Av
KFB 315	T	V	France	2013	KFB	This study	+	–	+	–	+	+	–	Av
KFB 317	NP		Serbia	2011	KFB	This study	–	–	+	–	–	–	–	Av

Table 1 (continued). Strains of *Allorhizobium vitis*, *Agrobacterium tumefaciens* complex and *Rhizobium rhizogenes* isolated from grapevine that were used in this study and results of PCR analysis using different primer sets.

							Primer sets[e]							
Strain	**P**[a]	**pTi**[b]	**Geographical origin**	**Y**[c]	**Source**[d]	**Literature**	A/C'	CYT/ CYT'	PGF/ PGR	tms2F1/ tms2R2	VCF3/ VCR3	VirD2S4F$_{716}$/ VirD2S4R$_{1036}$	VirFF$_1$/ VirFR$_2$	UF/B1R/B2R/ AvR/ArR
KFB 318	NP		Serbia	2011	KFB	This study	–	–	+	–	–	–	–	Av
KFB 319	T	O/C	Serbia	2012	KFB	This study	(+)	–	+	–	+	–	+	Av
KFB 320	T	O/C	Serbia	2012	KFB	This study	(+)	–	+	–	+	–	+	Av
KFB 322	T	O/C	Serbia	2012	KFB	This study	(+)	–	+	–	+	–	+	Av
MAFF 211676	T	V	Japan	2003	NIAS	(Kawaguchi *et al.*, 2005)	+	–	+	–	+	+	–	Av
MAFF 211908	T	O/C	Japan	1998	NIAS	(Misawa and Takeuchi, 2006)	(+)	–	+	–	+	–	+	Av
MAFF 211942	T	O/C	Japan	1988	NIAS	(Sawada and Ieki, 1992)	+	+	+	+	+	–	+	Av
MAFF 302147	T	V	Japan	1988	NIAS	UN	+	–	+	–	+	+	–	Av
MAFF 663001	T	V	Japan	1987	NIAS	(Sawada *et al.*, 1990)	+	–	+	–	+[j]	+	–	Av
S4	T	V	Hungary	1981	S. Süle	(Szegedi *et al.*, 1988)	+	–	+[j]	–	+	+	–	Av
Tm4	T	O/C	Hungary	1980	IPV-BO	(Szegedi *et al.*, 1988)	+	+	+[j]	+[j]	+	–[j]	+[j]	Av[j]
WIN 4.2.3	T	O/C	Poland	2010	InHort	(Kuzmanović *et al.*, 2015a)	(+)	–	+	–	+	–	+	Av
WIN 4.2.4	T	O/C	Poland	2010	InHort	(Kuzmanović *et al.*, 2015a)	(+)	–	+	–	+	–	+	Av
Agrobacterium tumefaciens complex														
II-2/2	T	O/C	France	2002	E. Szegedi	(Szegedi *et al.*, 2005)	+	+	–	+	+	–	+	At
II-5/1	T	O/C	France	2002	E. Szegedi	(Szegedi *et al.*, 2005)	(+)	–	–	–	+	–	+	At
15/6	T	N	Hungary	1976	E. Szegedi	(Szegedi *et al.*, 2005)	+	+	–	+	+	–	–	At
16/6	T	N	Hungary	1976	E. Szegedi	(Szegedi *et al.*, 2005)	+	+	–	+	+	–	–	At
CG 628	T	A	USA	1983	CU	(Burr *et al.*, 1999)	+	+	–	+	+	–	–	At
IVIA 2709-2b-1-2	T	O/C	Spain	UN	IVIA	(Palacio-Bielsa *et al.*, 2009b)	(+)	–	–	–	+	–	+	At
Sh-1	T	O	Georgia	UN	E. Szegedi	(Dandurishvili *et al.*, 2011)	+	+	–	+	+	–	+	At
Rhizobium rhizogenes														
IVIA 1698-2b-2	T	N	Spain	UN	IVIA	(Palacio-Bielsa *et al.*, 2009b)	+	+	–	+	+	–	–	Rr

[a] Pathogenicity: T, tumorigenic; NP, nonpathogenic.

[b] Type of Ti plasmid: A, agropine; N, nopaline; O, octopine; O/C, octopine/cucumopine; OL, O/C with large TA-DNA; OS, O/C with small TA-DNA; V, vitopine.

[c] Year of isolation.

[d] S. Süle, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary; IPV-BO, Plant Pathology Department, University of Bologna, Bologna, Italy; BPIC, Benaki Phytopathological Institute Collection, Kifissia, Greece (provided by M.K. Holeva); CU, Cornell University, Geneva, USA (provided by T.J. Burr and D. Zheng); IVIA, Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain (provided by M.M. López); KFB, Collection of Phytopathogenic Bacteria, University of Belgrade-Faculty of Agriculture, Belgrade, Serbia; NIAS, National Institute of Agrobiological Sciences (NIAS) Genebank, Tsukuba, Japan; InHort, Research Institute of Horticulture, Skierniewice, Poland (provided by J. Puławska).

[e] Results obtained by using different primer pairs: +, corresponding DNA fragment was amplified; (+), weak positive amplification signal was recoded; –, corresponding DNA fragment was not amplified; Av, DNA fragment specific for *All. vitis* was amplified; At, DNA fragment specific for *A. tumefaciens* complex was amplified; Rr, DNA fragment specific for *R. rhizogenes* was amplified.

[f] These strains did not induce tumors in pathogenicity assay on grapevine (Kuzmanović *et al.*, 2014), although PCR analysis suggested presence of Ti plasmid in their genome.

[g] Strain IPV-BO 5372 possessed both octopine/cucumopine and vitopine synthase genes (Bini *et al.*, 2008b).

[h] In the paper of Bini *et al.* (2008a) Italy is erroneously listed as a geographic origin of this strain.

[i] UN, unknown.

[j] Our results are congruent with previous work when testing these primers on the same strains.

fast detection of pathogenicity-associated genes that are mainly located on Ti plasmid, and pathogen identification to the species level by amplification of chromosomal genes. For these purposes, a number of PCR primers and protocols have been developed (Palacio-Bielsa *et al.*, 2009a). However, the presence of remarkable genetic diversity in these pathogens and their Ti plasmids can make diagnostics difficult.

The aim of this study was to evaluate reliability and accuracy of different PCR primers previously designed for the identification of bacteria causing crown gall, by using *All. vitis*, *A. tumefaciens* complex and *R. rhizogenes* strains isolated from grapevine throughout the world.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The collection of *All. vitis* (n = 69), *A. tumefaciens* complex (n = 7), and *R. rhizogenes* (n = 1) strains isolated from grapevine in

Table 2. Reference strains used in PCR analysis.

Strain	Species	P[a]	pTi[b]	Biological source	Geographical origin	Year of isolation	Source[c]
KFB 330^T	*Agrobacterium arsenijevicii*	T	N	*Rubus idaeus*	Serbia	2011	KFB
AF3.10^T	*Agrobacterium larrymoorei*	T	C/N	*Ficus benjamina*	USA	1991	UF
39/7^T	*Agrobacterium nepotum*	NP		*Prunus cerasifera*	Hungary	1989	InHort
C 3.4.1	*Agrobacterium nepotum*	NP		"Colt" rootstock	Poland	2008	InHort
NRCPB 10^T	*Agrobacterium pusense*	NP		Rhizosphere soil of *Cicer arietinum*	India	UN	S.K. Das
CFBP 5522^T	*Agrobacterium radiobacter*	NP		Soil	Netherlands	UN	CFBP
B6^T	*Agrobacterium radiobacter*	T	O	*Malus* sp.	USA	1935	IPV-BO
TR3^T	*Agrobacterium rubi*	T	N	*Rubus ursinus var. loganobaccus*	USA	1942	UF
Ch11^T	*Agrobacterium skierniewicense*	T	UN[d]	*Chrysanthemum* sp.	Poland	UN	InHort
MAFF 106578	*Agrobacterium tumefaciens* complex	R	UN	*Cucumis melo*	Japan	1985	NIAS
Ach5	*Agrobacterium tumefaciens* genomic species G1	T	O	UN	UN	UN	IPV-BO
C58	*Agrobacterium tumefaciens* genomic species G8	T	N	*Prunus cerasus*	USA	1958	S. Süle
MAFF 211950	*Allorhizobium vitis*	T	V	*Actinidia* sp.	Japan	1987	NIAS
ATCC 11325^T	*Rhizobium rhizogenes*	R	UN	*Malus domestica*	UN	UN	UF
K84	*Rhizobium rhizogenes*	NP		Orchard soil	Australia	UN	KFB
MAFF 211707	*Rhizobium rhizogenes*	T	UN	*Prunus persica*	Japan	1988	NIAS
MAFF 211729	*Rhizobium rhizogenes*	T	UN	*Prunus salicina*	Japan	1988	NIAS
MAFF 211731	*Rhizobium rhizogenes*	T	UN	*Prunus avium*	Japan	1988	NIAS

[a] Pathogenicity: T, tumorigenic; R, rhizogenic; NP, nonpathogenic.
[b] Type of Ti plasmid: N, nopaline; C/N, chrysopine/nopaline; O, octopine; V, vitopine.
[c] KFB, Collection of Phytopathogenic Bacteria, University of Belgrade-Faculty of Agriculture, Belgrade, Serbia; UF, University of Florida, Gainesville, USA (J.B. Jones, G.V. Minsavage); InHort, Research Institute of Horticulture, Skierniewice, Poland (provided by J. Puławska); S.K. Das, Institute of Life Sciences, Bhubaneswar, India; CFBP, Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; IPV-BO, Plant Pathology Department, University of Bologna, Bologna, Italy; NIAS, National Institute of Agrobiological Sciences (NIAS) Genebank, Tsukuba, Japan; S. Süle, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary.
[d] UN, unknown.

Table 3. Primer pairs used in this study.

Primer pair	Target organism	Location of the target sequence[a]	Target gene	Size of amplicon (bp)	Reference
A/C'	Tumorigenic/rhizogenic bacteria	pTi/pRi	*virD2*	224	(Haas *et al.*, 1995)
CYT/CYT'	Tumorigenic bacteria	pTi	*ipt* (*tmr*)	427	(Haas *et al.*, 1995)
PGF/PGR	*All. vitis*	C	*pehA*	466	(Szegedi and Bottka, 2002)
tms2F1/tms2R2	Tumorigenic bacteria	pTi	*iaaH* (*tms2*)	617	(Puławska and Sobiczewski, 2005)
UF/B1R	*A. tumefaciens* complex	C	23S rRNA	184	(Puławska *et al.*, 2006)
UF/B2R	*R. rhizogenes*	C	23S rRNA	1066	(Puławska *et al.*, 2006)
UF/AvR	*All. vitis*	C	23S rRNA	478	(Puławska *et al.*, 2006)
UF/ArR	*A. rubi*	C	23S rRNA	1006	(Puławska *et al.*, 2006)
VCF3/VCR3	Tumorigenic/rhizogenic bacteria	pTi/pRi	*virC*	414	(Suzaki *et al.*, 2004)
VirD2S4F$_{716}$/VirD2S4R$_{1036}$	Tumorigenic *All. vitis*[b]	pTi	*virD2*	320	(Bini *et al.*, 2008b)
VirFF$_1$/VirFR$_2$	Tumorigenic *All. vitis*[c]	pTi	*virF*	382	(Bini *et al.*, 2008b)

[a] pTi, Ti plasmid; pRi, Ri plasmid; C, Chromosomes.
[b] Primer pair VirD2S4F$_{716}$/VirD2S4R$_{1036}$ amplifies the corresponding DNA fragments for *All. vitis* vitopine-type pTi.
[c] Primer pair VirFF$_1$/ VirFR$_2$ amplifies the corresponding DNA fragments for *All. vitis* octopine/cucumopine-type and nopaline-type pTi, but also for octopine-type pTi of *A. tumefaciens* complex strains.

various geographic areas was tested in this study (Table 1). Out of the total *All. vitis* strains, 63 were tumorigenic, while the remaining six were nonpathogenic. Eighteen reference *Agrobacterium* and *Rhizobium* strains were also included in the PCR analysis (Table 2).

All the strains were cultured on yeast mannitol agar (YMA; 10 g/l mannitol, 1 g/l yeast extract, 1 g/l $CaCO_3$, 0.1 g/l NaCl, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \times 7H_2O$, 18 g/l agar; pH 7.2) medium at 27°C. Cultures were stored on YMA medium at 4°C for short term maintenance or in nutrient broth amended with 30% (w/v) glycerol at –80°C for long term preservation.

DNA preparation. Pure culture genomic DNA templates were prepared from bacteria grown on King's medium B (King *et al.*, 1954) at 27°C for 24-48 h. The bacterial suspensions (approx. 10^8 CFU/ml) were heated at 95°C for 10 min. Obtained lysates were incubated on ice for 5 min and centrifuged for 5 min at 8,000 rpm. The supernatants were used directly for PCR amplifications.

Table 4. PCR protocols used in this study.

	PCR protocols				
PCR mix	**Duplex PCR: A/C' CYT/CYT'**	**Duplex PCR: PGF/PGR VCF3/VCR3**	**Multiplex PCR: PGF/PGR VirD2S4F$_{716}$/VirD2S4R$_{1036}$ VirFF$_1$/VirFR$_2$**	**Simplex PCR: tms2F1/tms2R2**	**Multiplex PCR: UF/B1R/B2R/AvR/ArR**
Buffer[a]	1×	1×	1×	1×	1×
$MgCl_2$ (mM)	/	1.5	3	/	/
dNTPs (mM)	0.2	0.2	0.2	0.2	0.2
Primers (µM)	0.4	0.5	0.4/0.5[c]	0.5	0.5
DMSO (%)	/	/	5	/	/
DNA Polymerase (U)[b]	0.5	0.5	0.5	0.3	0.3
Template DNA (µl)	2.5	2	5	1.5	1.5
Final volume (µl)	25	25	25	15	15
Thermal cycling conditions					
Initial denaturation	94°C-1 min	94°C-5 min	94°C-1 min	94°C-1 min	94°C-1 min
Cycles number	40	35	40	35	35
Denaturation	[94°C-1 min	[94°C-1 min	[94°C-1 min	[94°C-1 min	[94°C-1 min
Annealing	50°C-1 min	56°C-1 min	60°C-1 min	63°C-1 min	67°C-1 min
Extension	72°C-1 min]	72°C-1 min]	72°C-1 min]	72°C-1.5 min]	72°C-1.5 min]
Final extension	72°C-5 min	72°C-5 min	72°C-5 min	72°C-10 min	72°C-10 min

[a]DreamTaq Green Buffer (10×; contains 20 mM $MgCl_2$; Thermo Scientific, Vilnius, Lithuania) was used for duplex PCR assay with A/C' and CYT/CYT' primers, simplex PCR assay with tms2F1/tms2R2 primer pair and multiplex PCR assay with UF/B1R/B2R/AvR/ArR primer set. For duplex PCR assay with PGF/PGR and VCF3/VCR3 primer pairs *Taq* Buffer with KCl (10×; Thermo Scientific, Vilnius, Lithuania) was used. For multiplex PCR assay with PGF/PGR, VirD2S4F$_{716}$/VirD2S4R$_{1036}$ and VirFF$_1$/VirFR$_2$ primers Colorless GoTaq Flexi buffer (no $MgCl_2$ in buffer; Promega, Madison, WI) or DreamTaq Green Buffer (10×; contains 20 mM $MgCl_2$; Thermo Scientific, Vilnius, Lithuania) were used.

[b]DreamTaq DNA polymerase (Thermo Scientific, Vilnius, Lithuania) was used for duplex PCR assay with A/C' and CYT/CYT' primers, simplex PCR assay with tms2F1/tms2R2 primer pair and multiplex PCR assay with UF/B1R/B2R/AvR/ArR primer set. For duplex PCR assay with PGF/PGR and VCF3/VCR3 primer pairs *Taq* DNA polymerase (recombinant; Thermo Scientific, Vilnius, Lithuania) was used. For multiplex PCR assay with PGF/PGR, VirD2S4F$_{716}$/VirD2S4R$_{1036}$ and VirFF$_1$/VirFR$_2$ primers GoTaq Flexi DNA polymerase (Promega, Madison, WI) or DreamTaq DNA polymerase (Thermo Scientific, Vilnius, Lithuania) were used.

[c]Concentration of 0.4 µM was used for PGF/PGR primer pair, while 0.5 µM was used for VirD2S4F$_{716}$/VirD2S4R$_{1036}$ and VirFF$_1$/VirFR$_2$ primer pairs.

PCR amplification. Total of 11 primer pairs targeting fragments located on Ti plasmid or chromosomal DNA were used in this study (Table 3). Primer pairs A/C' and CYT/CYT' were applied in duplex PCR, allowing identification of pathogenic *Agrobacterium* strains and differentiation between Ti and Ri plasmids (Haas *et al*., 1995). Primer pairs PGF/PGR and VCF3/VCR3 were also used in duplex PCR, providing differentiation between pathogenic *All. vitis* and *A. tumefaciens* (Kumagai and Fabritius, 2008). Primers PGF/PGR, VirD2S4F$_{716}$/VirD2S4R$_{1036}$ and VirFF$_1$/VirFR$_2$ were used in multiplex PCR allowing identification and differentiation between *All. vitis* strains carrying O/C and N-type Ti plasmids, *All. vitis* strains carrying V-type Ti plasmid and strains belonging to *A. tumefaciens* complex carrying O-type Ti plasmid (Bini *et al*., 2008b). Primer pair tms2F1/tms2R2 was used in simplex PCR, although they were originally designed for the first round of a semi-nested PCR method for detection of tumorigenic *Agrobacterium* in soil (Puławska and Sobiczewski, 2005). Primer set UF/B1R/B2R/AvR/ArR (1 universal forward and 4 species-specific reverse primers) was used in multiplex PCR enabling identification and differentiation of *A. tumefaciens* complex, *R. rhizogenes*, *All. vitis* and *Agrobacterium rubi* (Puławska *et al*., 2006).

PCR amplifications were conducted as described before (Bini *et al*., 2008b; Kuzmanović *et al*., 2014) or after slight modifications (Table 4). Negative controls (PCR-grade water) were included in all PCR assays. Thermal cycling conditions were as described previously in the respective publications (Table 4). PCRs were performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). The PCR products were separated by electrophoresis in a 1.5% agarose gel in 1× Tris-acetate-EDTA (TAE). In order to improve separation of fragments of similar size, 2% agarose gel was used for resolving products of duplex PCR with PGF/PGR and VCF3/VCR3 primers. The gels were stained in ethidium bromide solution (1 µg/ml) and visualized under UV light.

RESULTS

Primer pairs A/C' and VCF3/VCR3 amplified corresponding DNA fragments from all tumorigenic strains belonging to *All. vitis*, *A. tumefaciens* complex and *R. rhizogenes* used in this study (Table 1; Table 5). However, by using A/C' primers weak bands were obtained in 30 strains of *All. vitis* and two strains *A. tumefaciens* complex, all carrying O/C Ti plasmids (Table 1). Furthermore, CYT/CYT' primers used in duplex PCR with primers A/C' amplified corresponding DNA fragments only from 17 *All. vitis* strains, of which 12 carried O/C and 5 N-type of Ti

Table 5. Summarized results of PCR analysis of *Allorhizobium vitis*, *Agrobacterium tumefaciens* complex and *Rhizobium rhizogenes* strains isolated from grapevine using different primer sets[a].

Primer set	*Allorhizobium vitis* (n=69)[b]					*Agrobacterium tumefaciens* complex (n=7)	*Rhizobium rhizogenes* (n=1)
	O/C (n=42)	V (n=15)	N (n=5)	O/C,V (n=1)	NP (n=6)		
A/C'	42[c]	15	5	1	2	7[c]	1
CYT/CYT'	12	0	5	0	0	5	1
PGF/PGR	42	15	5	1	6	0	0
tms2F1/tms2R2	12	0	5	0	0	5	1
VCF3/VCR3	42	15	5	1	2	7	1
$VirD2S4F_{716}/VirD2S4R_{1036}$	0	15	0	1	0	0	0
$VirFF_1/VirFR_2$	42	0	5	1	2	4	0
UF/B1R/B2R/AvR/ArR	42[d]	15[d]	5[d]	1[d]	6[d]	7[e]	1[f]

[a] Number of strains that gave a positive amplification signal is indicated.

[b] Strains carrying octopine/cucumopine (O/C), vitopine (V) and nopaline (N) Ti plasmids, one possessing both octopine/cucumopine and vitopine synthase genes (O/C, V) and nonpathogenic (NP) strains were tested.

[c] Both weak and strong signals were recorded. Data for each strain are presented in Table 1.

[d] DNA fragments specific for *All. vitis* were amplified.

[e] DNA fragments specific for *A. tumefaciens* complex were amplified.

[f] DNA fragments specific for *R. rhizogenes* were amplified.

plasmids. Total of five *A. tumefaciens* complex and one *R. rhizogenes* strain also gave positive results with this primer pair (Table 1; Table 5). Primer pair tms2F1/tms2R2 amplified corresponding DNA fragments from the same strains that gave a positive amplification signal with primers CYT/CYT' (Table 1).

Primers $VirD2S4F_{716}/VirD2S4R_{1036}$ exhibited specificity for tested *All. vitis* strains carrying V-type of Ti plasmid, while primer pair $VirFF_1/VirFR_2$ detected *All. vitis* strains harboring O/C and N Ti plasmids, as well as O/C and O strains of *A. tumefaciens* complex (Table 1; Table 5). In addition, atypical strain IPV-BO 5372 possessing both O/C and V synthase genes was positive both with $VirD2S4F_{716}/VirD2S4R_{1036}$ and $VirFF_1/VirFR_2$ primers, which was congruent with previous work of Bini *et al.* (2008b).

Out of the six nonpathogenic *All. vitis* strains tested, two produced positive amplification signal with primers A/C', VCF3/VCR3 and $VirFF_1/VirFR_2$. The remaining four nonpathogenic strains were negative in PCRs using primers specific for Ti plasmid genes (Table 1; Table 5).

All tested *All. vitis* strains gave a positive amplification signal with primer pair PGF/PGR, while strains of *A. tumefaciens* complex and *R. rhizogenes* were negative. Using UF/B1R/B2R/AvR/ArR primer set, corresponding DNA fragments were amplified for each of three species used in this study. Results of PCR analysis obtained with reference *Agrobacterium* and *Rhizobium* strains are presented in Table 6.

DISCUSSION

Although rapid and efficient detection and identification of tumorigenic bacteria responsible for crown gall is facilitated by PCR-based methods, high genetic diversity of these pathogens may hinder their use in disease diagnosis. In this study, different primers specific for the pathogenicity associated genes located on Ti plasmid and those amplifying chromosomal gene fragments were evaluated for identification of *All. vitis*, *A. tumefaciens* complex and *R. rhizogenes* strains isolated from grapevine throughout the world.

Two primer pairs (A/C' and VCF3/VCR3) were able to detect all 71 tumorigenic strains of *All. vitis*, *A. tumefaciens* complex and *R. rhizogenes*. Nevertheless, A/C' primers gave weak amplification signals with some *All. vitis* and *A. tumefaciens* complex strains carrying O/C-type Ti plasmid, most likely due to incomplete complementarity to the target sequence, which was in agreement with previous reports (Bini *et al.*, 2008b; Kumagai and Fabritius, 2008; Kuzmanović *et al.*, 2014).

The CYT/CYT' and tms2F1/tms2R2 primers amplified corresponding DNA fragments only from N and minority of O/C strains of *All. vitis*. In addition, corresponding DNA fragments were amplified from N, A, O and one O/C strain of *A. tumefaciens* complex and *R. rhizogenes* strain carrying N-type of Ti plasmid. Thus, two different PCR groups were evident in O/C strains. Indeed, T-DNA of O/C Ti plasmid, consisting of two independent T-DNA fragments (TA-DNA and TB-DNA), may differ in oncogene composition and arrangement (Paulus *et al.*, 1989; Burr *et al.*, 1998; Burr and Otten, 1999). In this respect, the O/C Ti plasmids are divided into two groups: OS and OL plasmids, having small and large TA-DNA region, respectively. In addition, another form of T-DNA, related to O TL-DNA and O/C TA-DNA, but lacking TB-DNA region, have also been detected (Otten *et al.*, 1996; Burr *et al.*, 1998; Burr and Otten, 1999). Interestingly, O/C strains that gave positive amplification signal with CYT/CYT' and tms2F1/tms2R2 primer pairs were the same as those giving strong amplification signal with primer pair A/C'.

Primer pairs $VirD2S4F_{716}/VirD2S4R_{1036}$ and $VirFF_1/VirFR_2$ provided reliable identification of all tumorigenic strains of *All. vitis*, allowing differentiation between

Table 6. Results of PCR analysis of reference strains.

Strain	Species	Primer sets							
		A/C'	CYT/ CYT'	PGF/ PGR	tms2F1/ tms2R2	VCF3/ VCR3	VirD2S4F$_{716}$/ VirD2S4R$_{1036}$	VirFF$_1$/ VirFR$_2$	UF/B1R/B2R/ AvR/ArR
KFB 330^T	*Agrobacterium arsenijevicii*	+	+	–	+	+	ND[a]	ND	At/Rr[b]
AF 3.10^T	*Agrobacterium larrymoorei*	–	–	–	–	+	ND	ND	ND
39/7^T	*Agrobacterium nepotum*	–	–	–	–	–	ND	ND	At/Rr
C 3.4.1	*Agrobacterium nepotum*	–	–	–	–	–	ND	ND	At/Rr
NRCPB 10^T	*Agrobacterium pusense*	–	–	–	–	–	–	–	At[c]
CFBP 5522^T	*Agrobacterium radiobacter*	–	–	–	–	–	ND	ND	At
B6^T	*Agrobacterium radiobacter*	+	+	–	+	+	–	+	At
TR3^T	*Agrobacterium rubi*	+	+	–	+	+	ND	ND	Ar[d]
Ch11^T	*Agrobacterium skierniewicense*	+	+	–	+	+	ND	ND	ND
MAFF 106578	*Agrobacterium tumefaciens* complex	+	–	–	–	+	–	–	At
Ach5	*Agrobacterium tumefaciens* genomic species G1	+	+	–	+	+	–	+	At
C58	*Agrobacterium tumefaciens* genomic species G8	+	+	–	+	+	–	–	At
MAFF 211950	*Allorhizobium vitis*	+	–	+	–	+	+	–	Av[e]
ATCC 11325^T	*Rhizobium rhizogenes*	+	–	–	–	+	ND	ND	Rr[f]
K84	*Rhizobium rhizogenes*	–	–	–	–	–	–	–	Rr
MAFF 211707	*Rhizobium rhizogenes*	+	+	–	+	+	–	–	Rr
MAFF 211729	*Rhizobium rhizogenes*	+	+	–	+	+	–	–	Rr
MAFF 211731	*Rhizobium rhizogenes*	+	+	–	+	+	–	–	Rr

[a] ND, not determined.
[b] DNA fragments specific for *A. tumefaciens* complex and *R. rhizogenes* were amplified.
[c] DNA fragments specific for *A. tumefaciens* complex were amplified.
[d] DNA fragments specific for *A. rubi* were amplified.
[e] DNA fragments specific for *All. vitis* were amplified.
[f] DNA fragments specific for *R. rhizogenes* were amplified.

strains carrying O/C and N, and those carrying V-type Ti plasmids. Although, *A. tumefaciens* complex strains harboring O and O/C-type Ti plasmids were amplified, strains of *A. tumefaciens* complex carrying A and N-type Ti plasmids, including *R. rhizogenes* strain carrying N-type Ti plasmid, were not amplified with these primers. Lack of specificity for *A. tumefaciens* complex strains carrying A and N-type Ti plasmids originating from different hosts, excluding grapevine, is reported for these primers (Bini *et al*., 2008b).

Although assigned as non-tumorigenic in pathogenicity assay on grapevine (Kuzmanović *et al*., 2014), strains KFB 241 and KFB 247 displayed PCR profiles as majority of tumorigenic O/C strains. These strains most likely harbor Ti plasmid since both *virC* and *virD2* gene fragments were detected by PCR, but for some reason they did not exhibit tumorigenic ability in inoculation experiments. However, as their potential role in crown gall disease remained unclear, elimination of samples containing such strains should be recommended.

From a practical point of view, PCR detection of sequences in Ti plasmid is sufficient for preliminary crown gall diagnosis. However, it is also important to identify tumorigenic strains to the species level. Based on results obtained in this study, primer pair PGF/PGR is reliable in identification of *All. vitis*, while application of UF/B1R/B2R/AvR/ArR primer set provided accurate identification and differentiation of *All. vitis*, *A. tumefaciens* complex and *R. rhizogenes*. It must be noted that *Agrobacterium nepotum*, which is member of *A. tumefaciens* complex (genomic species G14) may also occur on grapevine (Puławska *et al*., 2012). However, two amplification products specific for both *A. tumefaciens* complex and *R. rhizogenes* were amplified with UF/B1R/B2R/AvR/ArR primer set from strains belonging to this species (Puławska *et al*., 2006). This was also observed for recently described species *Agrobacterium arsenijevicii* that was isolated from raspberry and cherry plum (Kuzmanović *et al*., 2015b, 2015c).

Overall, only primers VCF3/VCR3 clearly and accurately identified all tumorigenic strains of *All. vitis*, *A. tumefaciens* complex and *R. rhizogenes* associated with crown gall of grapevine. In previous studies, these primers exhibited high level of specificity toward various tumorigenic and rhizogenic strains (Suzaki *et al*., 2004; Kawaguchi *et al*., 2005; Kumagai and Fabritius, 2008; Kawaguchi and Inoue, 2009; Süle *et al*., 2012). This primer pair coupled with primers PGF/PGR in duplex PCR (Kumagai and Fabritius, 2008), may be recommended as the method of choice for routine preliminary identification of tumorigenic strains and differentiation of *All. vitis* that is mostly responsible for grapevine crown gall.

Conventional PCR protocols used in this study are generally suitable for testing bacteria from pure culture. However, this approach most likely could not provide sufficient sensitivity for the pathogen detection in asymptomatic grapevine material which represents a serious risk for the disease introduction and spread. Real-time PCR approach which follows the efficient DNA extraction from grapevine tissue may overcome these shortcomings. So far, two real-time PCR protocols for detection of *All. vitis* were

published, both based on SYBR Green dye chemistry and targeting *virD2* gene sequences (Bini *et al.*, 2008a; Johnson *et al.*, 2013). Real-time PCR protocol developed by Bini *et al.* (2008a) is reported as suitable for detection of all opine-type strains of *All. vitis*; however, O and N strains of *A. tumefaciens* complex could not be identified. Johnson *et al.* (2013) developed assay incorporating several DNA extraction methods followed by real-time PCR assay for detection of diverse tumorigenic *All. vitis* strains in grapevine tissue. In order to further evaluate specificity of primers they designed, it would be interesting to test strains carrying OS Ti plasmid and those having atypical form of T-DNA mentioned above. Moreover, strains of *A. tumefaciens* complex and *R. rhizogenes* occurring on grapevine should be also included. In any case, further efforts should be directed towards the development of more specific and sensitive TaqMan-based real-time PCR assay.

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