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Genetic diversity of tumorigenic bacteria associated with crown gall disease of raspberry in Serbia

Nemanja Kuzmanović · Anđelka Prokić · Milan Ivanović · Nevena Zlatković · Katarina Gašić · Aleksa Obradović

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Abstract During the last 3 years, crown gall disease was observed in some young raspberry plantations throughout Serbia, causing considerable economic losses. Based on biochemical and physiological tests, PCR targeting the 23S rRNA gene, and 16S rRNA and *recA* gene sequence analysis, at least two different species were identified as causal agents of disease. Out of 14 strains isolated from raspberry tumors, 12 were identified as tumorigenic *Rhizobium rhizogenes*, one belonged to *Agrobacterium tumefaciens* genomic species G8, while the remaining strain formed a separate phylogenetic lineage within *A. tumefaciens* species complex, different from all known genomic species. All strains investigated harbored nopaline-type of Ti plasmid and showed identical pathogenic properties by inoculating several test plants. However, they were divided into two genetic groups based on PCR-RFLP analysis of Ti plasmid *virA-virB2* region. Furthermore, total of nine unique ERIC-PCR profiles were identified among the strains studied. Although strains of *R. rhizogenes* exhibited similar ERIC-PCR profiles, they were differentiated into six distinct genetic groups. Based on the fact that some genetic groups were composed of strains originating from different geographic areas, it can be assumed that they have a common origin and were probably disseminated by movement of infected plant material.

Keywords Crown gall · Raspberry · *Agrobacterium* · *Rhizobium* · Ti plasmid

The DDBJ/EMBL/GenBank accession numbers for the partial 16S rRNA gene sequences of strains KFB 323, KFB 330 and KFB 337 are: KP172481-KP172483, respectively. Accession numbers for the partial *recA* gene sequences of strains KFB 323, KFB 330, KFB 337, MAL 1.1.2, MAL 1.1.4, 39/7^T and NRCPB10^T are: KP172484-KP172489 and KP284164, respectively.

N. Kuzmanović (✉) · A. Prokić · M. Ivanović · N. Zlatković · A. Obradović
University of Belgrade-Faculty of Agriculture, Nemanjina 6, 11080 Belgrade, Serbia
e-mail: kuzmanovic1306@gmail.com

K. Gašić
Department of Plant Pathology, Institute for Plant Protection and Environment, Teodora Drajzera 9, 11040 Belgrade, Serbia

Introduction

Tumorigenic agrobacteria affect various fruit species and are responsible for crown gall disease that may cause significant economic losses in orchards and nurseries (Puławska 2010). Tumor formation on crown and roots of the host plant is a typical symptom of the disease. Pathogenicity of the strains is mainly determined by the presence of conjugative tumor-inducing (Ti) plasmid in their genome (Van Larebeke et al. 1974; Kerr et al. 1977).

Taxonomy of the genus *Agrobacterium* is debatable and still not fully resolved. Although Young et al. (2001) proposed inclusion of all *Agrobacterium* species into the genus *Rhizobium*, this taxonomy revision was disputed and not widely accepted (Farrand et al. 2003). The transfer of *Agrobacterium rhizogenes* (biovar 2) to genus *Rhizobium* was subsequently supported, while the rest

of the members remained within *Agrobacterium* genus (Lindström and Young 2011). However, a formal proposal with a definition or the new borders of genus *Agrobacterium* has not yet been published. Since *Agrobacterium tumefaciens* (biovar 1) is not a homogenous species but composed of at least 11 genomic species (G1 to G9, G13 and G14), it was proposed that they should be collectively called the *A. tumefaciens* species complex until being formally named (Costechareyre et al. 2010; Lindström and Young 2011). So far, only genomic species G2, G4 and G14 were formally named as *Rhizobium pusense* (Panday et al. 2011), *Agrobacterium radiobacter* (Conn 1942) and *Rhizobium nepotum* (Puławska et al. 2012a), respectively. Despite the proposal of name "*Agrobacterium fabrum*" for genomic species G8 (Lassalle et al. 2011), its standing in nomenclature still pending (http://www.bacterio.net/).

A. tumefaciens genomic species can be clearly delineated by genotypic-based methods: DNA-DNA hybridizations (De Ley 1974; De Ley et al. 1973; Popoff et al. 1984), amplified fragment length polymorphism (AFLP) (Portier et al. 2006; Mougel et al. 2002), sequence analysis of housekeeping *recA* gene (Costechareyre et al. 2010) and *recA*-based PCR approach (Shams et al. 2013). Although 16S rRNA gene sequencing may be suitable for identification of *Agrobacterium* species, it lacks the resolution power to discriminate among genomic species of *A. tumefaciens* complex (Mougel et al. 2002).

Raspberry is a natural host of tumorigenic *Agrobacterium* and *Rhizobium* strains. The disease may be particularly serious on certain cultivars (Burr et al. 1993). While the *Agrobacterium rubi* is recognized as causal agent of cane gall disease of *Rubus* spp. (Hildebrand 1940), tumorigenic strains of *A. tumefaciens* species complex (Alippi et al. 2012; Milijašević et al. 2007) and *Rhizobium rhizogenes* (Weller et al. 2004; Burr et al. 1993; Hobolth 1973; Peluso et al. 2003; Süle 1978) were predominantly isolated from raspberry showing crown gall symptoms. Moreover, nonpathogenic strain belonging to recently described species *R. nepotum* (Puławska et al. 2012a) was also recovered from galled raspberry.

Serbia is one of the world's leading raspberry producers (96,078 t in 2012 [http://faostat3.fao.org/]) and exporters. Raspberry production and export is of a strategic importance for agriculture in this country. During the last 3 years, high incidence of crown gall disease was recorded in some young raspberry plantations throughout Serbia. Infected plants showed stunted growth and significantly decreased yield. Newly introduced cultivars Polka and Tulameen were associated with disease.

Therefore, in this study we isolated and identified tumorigenic bacteria from diseased plants originating from six localities throughout Serbia and investigated their pathogenic properties. Furthermore, we evaluated genetic diversity of isolated strains and their Ti plasmids by using PCR, PCR-RFLP and ERIC-PCR methods. Phylogenetic position of representative strains was elucidated by sequence analysis of 16S rDNA and *recA* housekeeping gene. Although Milijašević et al. (2007) identified *A. tumefaciens* as causal agent of the disease in Western Serbia, assessment of genotypic variation among tumorigenic strains occurring on raspberry in Serbia has not been performed so far.

Materials and methods

Bacterial strains

A total of 16 *Agrobacterium* strains isolated from raspberry, including 14 from Serbia and two from Poland, were used in this study (Table 1). Strains from Serbia were isolated from tumor tissue of diseased plants collected in six localities during 2011–2013. Nonselective yeast mannitol agar (YMA; 10 g l^{-1} mannitol, 1 g l^{-1} yeast extract, 1 g l^{-1} $CaCO_3$, 0.1 g l^{-1} NaCl, 0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 18 g l^{-1} agar; pH 7.2) and/or selective MG agar medium amended with tellurite (K_2TeO_3; 70 μg ml^{-1}) (Mougel et al. 2001) were used for isolations. Plates were incubated at 27 °C for 3–5 (isolation on YMA) or 4–7 days (isolation on MG agar medium amended with tellurite).

Bacterial colonies resembling *Agrobacterium* spp. phenotype were purified and maintained on YMA medium for further testing. Two strains from Poland were previously identified as tumorigenic *A. tumefaciens* and *R. rhizogenes* (J. Puławska, unpublished data) (Table 1). In addition to *Agrobacterium* strains isolated from raspberry, ten reference strains of *Agrobacterium* spp. and *Rhizobium* spp. were also used in this study (Table 1). For DNA extraction, bacteria were grown on King's medium B (King et al. 1954) at 27 °C for 24–48 h, to reduce production of polysaccharides.

Table 1 Strains of *Agrobacterium* and *Rhizobium* spp. used in this study

Strain	Species	Pathogenicity and opine-type of Ti/Ri plasmid[a]	Biological source[b]	Geographical origin	Year of isolation	Source[c]	PCR-RFLP profile of *virA-virB2*	ERIC-PCR pattern
Strains isolated from raspberry								
KFB 323	*R. rhizogenes*	Ti, N	Polka	Šabac, SRB[d]	2011	KFB	N1	I
KFB 324	*R. rhizogenes*	Ti, N	Polka	Šabac, SRB	2011	KFB	N2	II
KFB 325	*R. rhizogenes*	Ti, N	Polka	Valjevo, SRB	2011	KFB	N1	III
KFB 328	*R. rhizogenes*	Ti, N	Tulameen	Arilje, SRB	2012	KFB	N1	III
KFB 326	*R. rhizogenes*	Ti, N	Tulameen	Vrbas, SRB	2011	KFB	N2	IV
KFB 327	*R. rhizogenes*	Ti, N	Tulameen	Vrbas, SRB	2011	KFB	N2	IV
KFB 329	*R. rhizogenes*	Ti, N	Tulameen	Arilje, SRB	2012	KFB	N1	V
KFB 332	*R. rhizogenes*	Ti, N	Polka	Pirot, SRB	2012	KFB	N1	V
KFB 333	*R. rhizogenes*	Ti, N	Polka	Pirot, SRB	2012	KFB	N1	V
KFB 338	*R. rhizogenes*	Ti, N	Tulameen	Mladenovac, SRB	2013	KFB	N1	V
KFB 339	*R. rhizogenes*	Ti, N	Tulameen	Mladenovac, SRB	2013	KFB	N1	V
KFB 334	*R. rhizogenes*	Ti, N	Tulameen	Arilje, SRB	2013	KFB	N2	VI
MAL 1.1.2	*R. rhizogenes*	Ti, N	Polka	Poland	2010	J. Puławska	N2	VI
KFB 330	*A. tumefaciens* species complex	Ti, N	Tulameen	Arilje, SRB	2012	KFB	N1	VII
KFB 337	*A. tumefaciens* genomic species G8	Ti, N	Tulameen	Arilje, SRB	2013	KFB	N1	VIII
MAL 1.1.4	*A. tumefaciens* genomic species G1	Ti, N	Polka	Poland	2010	J. Puławska	N1	IX
Control strains								
CFBP 5522^T	*A. radiobacter*	NP	soil	The Netherlands	UD	CFBP	ND[f]	ND
B6	*A. radiobacter*	Ti, O	*Malus* sp.	UD[e]	1935	IPV-BO	ND	ND
C58	*A. tumefaciens* genomic species G8	Ti, N	*Prunus cerasus*	USA	1958	S. Süle	N2	ND
NRCPB10^T	*R. pusense*	NP	Rhizosphere soil of *Cicer arietinum*	India	UD	S.K. Das	ND	ND
39/7^T	*R. nepotum*	NP	*Prunus cerasifera*	Hungary	1989	J. Puławska	ND	ND
K309^T	*A. vitis*	Ti, O/C	*Vitis vinifera*	Australia	1977	IPV-BO	ND	ND
ATCC 13335^T	*A. rubi*	Ti	*Rubus ursinus* var. *loganobaccus*	USA	1942	UF	ND	ND
AF3.10^T	*A. larrymoorei*	Ti, C/N	*Ficus benjamina*	Florida, USA	1991	UF	ND	ND

Table 1 (continued)

Strain	Species	Pathogenicity and opine-type of Ti/Ri plasmid[a]	Biological source[b]	Geographical origin	Year of isolation	Source[c]	PCR-RFLP profile of *virA-virB2*	ERIC-PCR pattern
Ch11[T]	*R. skierniewicense*	Ti	*Chrysanthemum* sp.	Poland	UD	J. Puławska	ND	ND
ATCC 11325[T]	*R. rhizogenes*	Ri	*Malus domestica*	UD	UD	UF	ND	ND

[a] Ti, tumor inducing; Ri, root-inducing; NP, nonpathogenic; N, nopaline-type; O, octopine-type; O/C, octopine/cucumopine-type; C/N, chrysopine/nopaline-type

[b] Respective cultivar is given for strains isolated from raspberry

[c] CFBP, Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; S. Süle, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary; J. Puławska, Research Institute of Horticulture, Skierniewice, Poland; S.K. Das, Institute of Life Sciences, Bhubaneswar, India; UF, University of Florida, Gainesville, USA (J.B. Jones, G.V. Minsavage); IPV-BO, Plant Pathology Department, University of Bologna, Bologna, Italy (E. Biondi)

[d] SRB, Serbia

[e] UD, undocumented

[f] ND, not determined

Pathogenicity tests

Pathogenicity of the strains was determined by inoculating carrot root discs, young tomato (*Lycopersicon esculentum* L.) plants and sunflower (*Helianthus annuum* L.) seedlings. One-year old raspberry plants (cv. Meeker), as a natural host, were inoculated with five representative strains. Four carrot root discs were inoculated with each strain by pipetting 100 μl of bacterial suspension (approx. 10^6 CFU/ml) on the abaxial side of the disc (Moore et al. 2001). Tomato and sunflower plants were inoculated as described before (Kuzmanović et al. 2014). Potted raspberry plants were inoculated in stem internodes following the same procedure as for tomato and sunflower. Three plants were inoculated per strain. Inoculated plants were maintained in a greenhouse at 24±3 °C. Tumor formation was scored during 3–4 weeks (carrot, tomato, and sunflower) or 2 months (raspberry) after inoculation.

Physiological and biochemical properties

The strains were characterized using following physiological and biochemical tests: oxidase reaction, growth in 2 % NaCl and at 35 °C, 3-ketolactose production, acid clearing on PDA amended with $CaCO_3$, ferric ammonium citrate test, motility at pH 7.0, citrate utilization, production of acid from meso-erythritol (erythritol) and D-(+)-melezitose monohydrate (melezitose), and alkali from L-(+)-tartaric acid disodium salt (tartrate) (Moore et al. 2001).

DNA extraction

Genomic DNA was isolated from bacterial suspensions (approx. 10^8 CFU/ml) using the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality was checked by agarose gel electrophoresis. The DNA samples were diluted to 10–20 ng/μl and stored at −20 °C for further analysis.

PCR analysis

Bacterial strains isolated from raspberry were analyzed by PCR using primers specific for plasmid *virC* (VCF3/VCR3) (Suzaki et al. 2004), *virD2* (A/C') and *ipt* (CYT/CYT') (Haas et al. 1995), and *tms2* (tms2F1/tms2R2) (Puławska and Sobiczewski 2005) genes, as well as chromosomal 23S rRNA gene (UF/B1R/B2R/

AvR/ArR) (Puławska et al. 2006). The primers specific for *virD2* and *ipt* genes, and 23S rRNA gene-specific primers were used in duplex (Haas et al. 1995) and multiplex PCR (Puławska et al. 2006) reactions, respectively.

PCR amplifications was also conducted with primer pair F749 (Mougel et al. 2001) and F14 (aka vir-14 or FGPvirG15') (Picard et al. 1992) designed to amplify the intergenic region between *virB11* and *virG* genes of Ti plasmid. This primer pair amplifies 432 bp product from nopaline-type (e.g. pTiC58 and pTi-SAKURA), or 384 bp product from octopine-type (e.g. pTiAch5 and pTi15955) and agropine/mannopine-type (e.g. pTiBo542) Ti plasmids, as determined by NCBI Primer-Blast tool (Ye et al. 2012). The opine-type of pathogenic plasmid was further examined with primers derived from octopine-type (ocsF/ocsR) and nopaline-type (RBF/RBR) Ti plasmids (Tan et al. 2003), and *virF* gene of octopine-type Ti plasmid (virFF1/virFR2) (Bini et al. 2008) used in separate PCR reactions.

PCR amplifications were carried out using a 2720 Thermal Cycler (Applied Biosystem, Foster City, CA, USA). The reactions were performed in 15 μl mixtures and contained 1× DreamTaq Green Buffer (Thermo Scientific, Vilnius, Lithuania), 200 μM of each dNTP, 0.5 μM of each primer, 0.3 U of DreamTaq DNA polymerase (Thermo Scientific, Vilnius, Lithuania) or Taq DNA polymerase (Invitrogen, Sao Paulo, Brazil), and 2 μl of template DNA. The thermal profiles were as described in the original publications, except for PCR amplifications with primers VCF3/VCR3 and F749/F14. The thermal profile used for PCR with primer pair VCF3/VCR3 was as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and elongation at 72 °C for 1 min, and final extension at 72 °C for 7 min. With primer pair F749/F14 thermal cycling conditions were programmed as described by Rhouma et al. (2006). The PCR products were resolved by electrophoresis in 1.5 % (w/v) agarose gel in 1× TAE buffer. The gels were stained in ethidium bromide solution (1 μg/ml) and the amplicons were visualized under UV light.

PCR-RFLP analysis of Ti plasmid *virA-virB2* region

The *vir* region (*virA-virB2* genes) of Ti plasmids was further analyzed by PCR-RFLP method. PCR was carried out by using primer pair FGPvirA2275/FGPvirB$_2$164 amplifying 1673 bp fragment from nopaline-type Ti plasmids (Ponsonnet and Nesme 1994). The reaction mixtures (25 μl) contained 1× DreamTaq Buffer (Thermo Scientific, Vilnius, Lithuania), 200 μM of each dNTP, 0.4 μM of each primer, 10 % of dimethyl sulfoxide (DMSO), 0.6 U of DreamTaq DNA polymerase (Thermo Scientific, Vilnius, Lithuania) and 2 μl of template DNA. The thermal profile was as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, and elongation at 72 °C for 1.5 min, and final extension at 72 °C for 5 min.

PCR products were digested with restriction endonuclease *Hha*I (*Cfo*I) (Thermo Scientific, Vilnius, Lithuania), as recommended by the manufacturer. Restriction fragments obtained after 4 h of digestion were separated in 2.5 % agarose gel at 80 V for 5 h, stained with ethidium bromide and visualized under UV light.

ERIC-PCR

ERIC-PCR fingerprinting (Versalovic et al. 1991) was used to asses genetic diversity among strains studied. The PCR mixtures (25 μl) contained 1× PCR buffer (Invitrogen, Sao Paulo, Brazil), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 2 μM of each primer (ERIC1R and ERIC2), 0.16 mg/ml of bovine serum albumin, 10 % of DMSO, 2 U of *Taq* DNA polymerase (Invitrogen, Sao Paulo, Brazil) and 2 μl of template DNA. The thermal profile was as described previously by Rademaker and De Bruijn (Rademaker and De Bruijn 1997). Electrophoresis was performed in a 1.5 % (w/v) agarose gel in 1× TAE buffer at 60 V for 30 min and 75 V for 5 h. After the run, gels were stained in ethidium bromide solution and photographed under UV light.

Sequence analysis 16S rRNA and *recA* genes

Representative strains were selected for phylogenetic analysis of the 16S rRNA and *recA* housekeeping gene sequences. Region of 16S rRNA gene was amplified with primers fD1 and rP2 (Weisburg et al. 1991). The PCR reactions (50 μl) contained 1× DreamTaq Buffer (Thermo Scientific, Vilnius, Lithuania), 200 μM of each dNTP, 0.2 μM of each primer, 1.5 U of DreamTaq DNA polymerase (Thermo Scientific, Vilnius, Lithuania) and 3 μl of template DNA. The thermal profile was as described above for amplifying *virA-virB2* region, except that an annealing temperature of 55 °C was used. The

recA gene sequences were amplified by using primers F2898 and F2899 (Costechareyre et al. 2010; Shams et al. 2013). The reaction mixtures (50 μl) contained 1× DreamTaq Buffer (Thermo Scientific, Vilnius, Lithuania), 200 μM of each dNTP, 0.5 μM of each primer, 5 % of DMSO, 1 U of DreamTaq DNA polymerase (Thermo Scientific, Vilnius, Lithuania) and 3 μl of template DNA. The thermal profile was as described by Costechareyre et al. (2010).

The sequencing of PCR-amplified partial 16S rRNA gene fragments was performed using the same primers as for amplifications, while the *recA* PCR products were sequenced with standard T7 and T3 primers by Macrogen Europe (The Netherlands). The obtained sequences were compared with those in the NCBI database (October 2014) by using the BLASTn algorithm (Altschul et al. 1997). The sequence alignment was conducted using ClustalW algorithm implemented in MEGA version 6 software package (Tamura et al. 2013). Reference sequences of related *Agrobacterium* and *Rhizobium* species were also included. Phylogenetic analysis was carried out by maximum likelihood (ML) method using the raxmlGUI 1.3 (Silvestro and Michalak 2012) and the GTR+G model (general time-reversible model with gamma-distributed rates) of evolution with 100 thorough bootstraps.

Results

Identification of the isolated strains

Bacterial colonies phenotypically resembling *Agrobacterium* spp. were isolated on YMA and MG agar medium amended with tellurite. In order to differentiate pathogenic ones, isolated strains were subsequently subjected to PCR analysis using VCF3/VCR3 primers specific for Ti/Ri plasmid *virC* gene. Out of 46 tested strains, 36 were determined to be phytopathogenic. A total of 14 strains were selected for further study (Table 1). In duplex PCR using *virD2* and *ipt* gene-specific primers, two specific DNA fragments were amplified in all tested strains from raspberry. Likewise, strains were also positive in PCR with primers specific for *tms2* gene and intergenic region between *virB11* and *virG* genes of Ti plasmid. Overall, based on PCR analysis, Ti plasmid was detected in 14 tested strains originating from raspberry.

In order to differentiate strains to the species level, we performed multiplex PCR assay targeting 23S rRNA gene sequences. Twelve strains isolated in Serbia were identified as *R. rhizogenes*, one strain as *A. tumefaciens*, whereas the remaining one gave two amplification products specific for both these species. The strains identified by PCR as *A. tumefaciens* and *R. rhizogenes* exhibited bacteriological properties typical for these species, while the atypical strain KFB 330 showed features of *A. tumefaciens*, except for positive reaction in citrate utilization test (Table 2).

Pathogenic properties of the strains

All strains studied induced typical tumors on carrot root discs, young tomato plants and sunflower seedlings. Five representative strains KFB 323, KFB 330, KFB 337, MAL 1.1.2 and MAL 1.1.4 that were tested for tumorigenicity by inoculation of raspberry plants caused characteristic symptoms at the inoculation sites. Control strains of *A. radiobacter* (B6) and *A. vitis* (K309^T) were tumorigenic on carrot root discs, young tomato plants and sunflower seedlings, while the strains of *A. tumefaciens* genomic species G8 (C58) and *A. rubi* (ATCC 13335^T) induced crown gall symptoms at stem internodes of raspberry. The plants inoculated with nonpathogenic strain of *R. nepotum* (39/7^T) and SDW developed no symptoms.

Characterization of Ti plasmid

The Ti plasmid of the strains originating from raspberry was characterized by PCR analysis targeting sequences specific for nopaline-type, octopine-type and agropine/mannopine-type Ti plasmids. In PCR using primer pair F749/F14 the product of 432 bp was amplified in all tested strains from raspberry, suggesting they carry nopaline-type of Ti plasmid. Furthermore, out of 16 strains, specific PCR products were amplified from 15 strains when primers specific for nopaline-type Ti plasmid (RBF/RBR) were used. The remaining strain KFB 324 gave a weak positive PCR signal with coamplification of multiple nonspecific fragments. Specific amplification products were not obtained when primers specific for octopine-type Ti plasmid (ocsF/ocsR) were used. Overall, Ti plasmid of all strains studied from raspberry was classified as nopaline-type (Table 1).

Based on PCR-RFLP analysis of Ti plasmid *virA-virB2* region two different restriction profiles (N1 and N2) were found (Table 1). The genetic group

Table 2 Bacteriological characteristics of strains isolated from raspberry compared to reference strains of *Agrobacterium* and *Rhizobium* spp

Test	Results[a]											
	Investigated strains			Control strains[b]								
	KFB 323–329, KFB 332–334, KFB 338, KFB 339, MAL 1.1.2	KFB 337, MAL 1.1.4	KFB 330	1	2	3	4	5	6	7	8	9
Oxydase reaction	−	+	+	+	+	+	+	+	+/−	+	+	−
Growth at 35 °C	−	+	+	+	+	+	+	+	+	+	−	−
Growth in 2 % NaCl	−	+	+	+	+	+	+	+	+	+	+	−
3-ketolactose production	−	+	+	+	+	+	+	−	−	−	−	−
Acid-clear on PDA amended with CaCO3	+	−	−	−	−	−	−	−	−	−	−	+
Motility at pH 7.0	+	+	+	+	+	+	+	−	−	+	−	+
Ferric ammonium citrate	−	+	+	+	+	+	−	−	−	−	–[c]	−
Citrate utilization	+	−	+	−	−	−	−	+	−	−	−	+
Acid from erythritol[d]	+	+	+	+	+	+	−	−	−	−	−	+
Acid from melezitose	−	+	+	+	+	+	−	−	−	−	−	−
Alkali from tartrate	+	−	−	−	−	−	−	+	−	−	−	+

[a] +, positive reaction; −, negative reaction; +/−, both weak positive and negative results were obtained in three replicate analyses

[b] Control strains: 1 – *R. radiobacter* (CFBP 5525^T), 2 - *A. tumefaciens* genomic species G8 – "*A. fabrum*" (C58), 3 - *R. pusense* (NRCPB10^T), 4 - *R. nepotum* (39/7^T), 5 - *A. vitis* (K309^T), 6 – *A. rubi* (ATCC 13335^T), 7 – *A. larrymoorei* (AF3.10), 8 - *R. skierniewicense* (Ch11^T), 9 – *R. rhizogenes* (ATCC 11325^T)

[c] Although it was reported that *R. skierniewicense* grows with pigmentation in ferric ammonium citrate broth (Puławska et al. 2012b), we got negative reaction in this test

[d] According to the literature, acid production from erythritol as a sole carbon source is restricted to *R. rhizogenes* (Moore et al. 2001)

N1 included 11 strains belonging to *R. rhizogenes* and *A. tumefaciens* species complex. The remaining five *R. rhizogenes* strains were characterized by N2 PCR-RFLP profile (Table 1). The PCR-RFLP groups were not associated with geographical origin of the strains or raspberry cultivars from which they were isolated.

ERIC-PCR genotyping

The genetic profiles generated by ERIC-PCR allowed differentiation of the strains and provided insight into their genetic diversity (Fig. 1). A total of nine unique ERIC-PCR patterns were identified among the strains studied (Table 1, Fig. 1). Although strains of *R. rhizogenes* exhibited similar genetic profiles, they were differentiated into six distinct genetic groups (Table 1, Fig. 1). On the other hand, *A. tumefaciens* strains KFB 337 and MAL 1.1.4, and atypical strain KFB 330 exhibited distinguishably different genetic profiles (Fig. 1). Associations between the strains' ERIC-PCR profile and geographic origin or raspberry cultivars from which they were isolated were not found. Strains belonging to genetic groups III, V and VI were isolated from different geographic areas (Table 1).

16S rRNA and *recA* phylogeny

The representative strains KFB 323, KFB 330, KFB 337, MAL 1.1.2 and MAL 1.1.4 were further characterized by partial sequencing of their 16S rDNA and *recA* housekeeping gene. Phylogenetic analysis of the 16S rRNA gene sequences (1304 bp for *R. rhizogenes* and 1308 bp for *A. tumefaciens* species complex) confirmed identity of strains KFB 323 and KFB 337 assigned as *R. rhizogenes* and *A. tumefaciens*, respectively (Fig. 2). Atypical strain KFB 330 was closely related to species belonging to *A. tumefaciens* complex (Fig. 2). Its closest relatives were type strains of *R. nepotum* (99.8 % sequence identity, two SNPs) and *A. radiobacter* (99.3 %, nine SNPs).

In the phylogenetic tree based on *recA* gene sequences (869 bp) the strains of *R. rhizogenes* (KFB

323 and MAL 1.1.2) were grouped with type strain of this species (Fig. 3). Furthermore, the *recA* sequence analysis revealed that strains MAL 1.1.4 and KFB 337 belong to *A. tumefaciens* genomic species G1 and G8, respectively. A total of 21 different *recA* alleles have been so far described among genomic species G1, whereas six unique alleles have been found within genomic species G8 (Costechareyre et al. 2010; Shams et al. 2013; Lamovšek et al. 2014). The strain MAL 1.1.4 possessed a *recA*-G1-15 allele, which was found among nonpathogenic strains isolated from agricultural soils in Slovenia (Lamovšek et al. 2014). On the other hand, novel allele named *recA*-G8-7 was identified in strain KFB 337. BLASTn searches on NCBI database failed to find sequences identical to one of strain KFB 337.

Atypical strain KFB 330 clustered within a clade of *A. tumefaciens* species complex (Fig. 3). However, this strain formed separate phylogenetic lineage, different from all known genomic species (Fig 3). This strain was closely related to genomic species G1 and had the highest level of identity (96.9 %) to the sequence of nonpathogenic strain CFBP 5622 isolated from rhizosphere of *Solanum nigrum* in France. Moreover, based on BLASTn searches, we were unable to find sequences more closely related than 96.9 %.

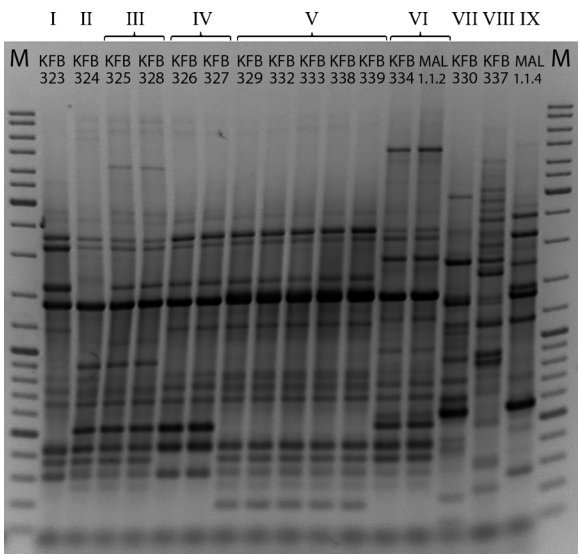

Fig. 1 ERIC-PCR patterns of the strains isolated from raspberry. The different ERIC-PCR patterns are indicated by Roman numerals above the lanes. Strain designations are indicated above the respective lanes. Lanes M, molecular size marker GeneRuler DNA Ladder Mix (#SM0333; Thermo Scientific, Vilnius, Lithuania)

Discussion

Insights into the genetic diversity of phytopathogenic *Agrobacterium* spp. and *Rhizobium* spp. may be particularly important for better understanding of the crown gall epidemiology and ecology of the disease causal agent. The aim of this study was to characterize and differentiate tumorigenic bacteria from raspberry in Serbia. The crown gall has been already recorded on raspberry in this country (Milijašević et al. 2007), but also on other *Rubus* sp. such as blackberry (Arsenijević 1989). However, during the last 3 years, crown gall disease was also observed in some young raspberry plantations suffering considerable losses.

Our results revealed extensive genetic diversity of tumorigenic bacteria associated with crown gall of raspberry in Serbia, although a limited number of strains were analyzed. At least two different species were identified as causal agents of the disease. Out of 14 strains isolated from the tumors, 12 were identified as *R. rhizogenes*, whereas the remaining two (KFB 330 and KFB 337) belonged to *A. tumefaciens* species complex (Table 1).

The high diversity of agrobacteria was observed within the one locality in western Serbia where strains of *R. rhizogenes* (KFB 328, KFB 329 and KFB 334), *A. tumefaciens* genomic species G8 (KFB 337) and atypical strain belonging to *A. tumefaciens* species complex (KFB 330) were isolated from the same raspberry plantation. Similarly, strains of *A. tumefaciens* and *R. rhizogenes* were also isolated from the same forest or stone fruit nursery, but also from a single tumor (Nesme et al. 1987; Nesme et al. 1992; Kuzmanović et al. 2013).

On the other hand, strains studied were generally homogenous with respect to opine-type of Ti plasmid, since they all harbored nopaline-type of Ti plasmid. Additionally, all strains showed identical pathogenic properties in pathogenicity test on several test plants. Nevertheless, they were differentiated into two genetic groups based on PCR-RFLP analysis of Ti plasmid *virA-virB2* region. The fact that strains belonging to different species or genomic species originating from same plantation (KFB 328, KFB 329, KFB 330 and KFB 337) share the same Ti plasmid genotype suggests that these Ti plasmids are transferable between distantly related *Agrobacterium* and *Rhizobium* strains in nature, confirming the previous findings (Nesme et al. 1992; Michel et al. 1990).

The strains belonging to different taxonomic entities of *Agrobacterium* sp. and *Rhizobium* sp. exhibited

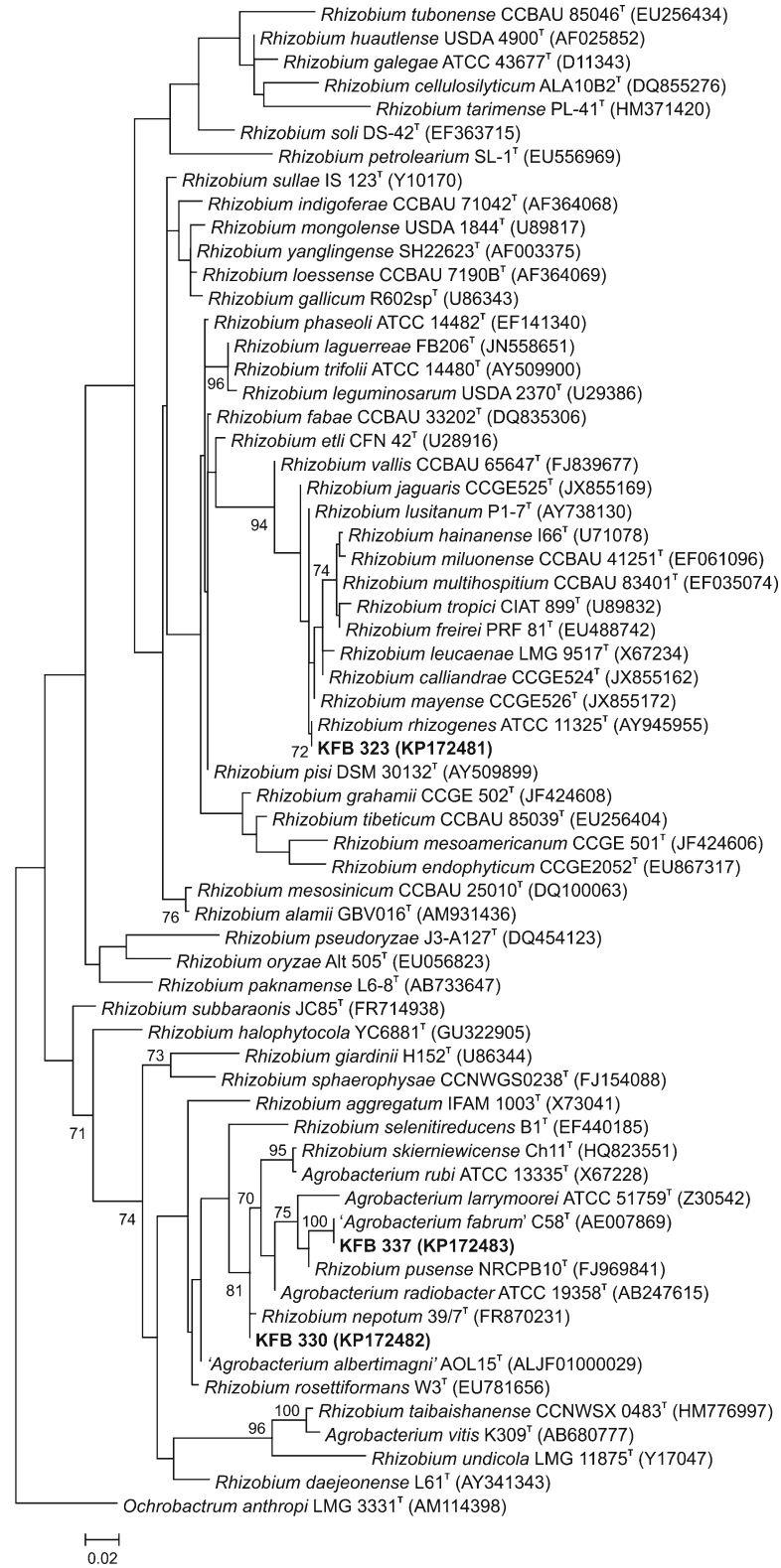

Fig. 2 Maximum likelihood tree based on 16S rRNA gene sequence indicating phylogenetic relationships of strains studied (marked in bold) and related members of the *Rhizobiaceae* family. Bootstrap values≥70 are shown at nodes. The scale bar represents the number of substitutions per site. DDBJ/EMBL/GenBank accession numbers are shown in parentheses. *Ochrobactrum anthropi* (strain LMG 3331^T) is used as the outgroup organism

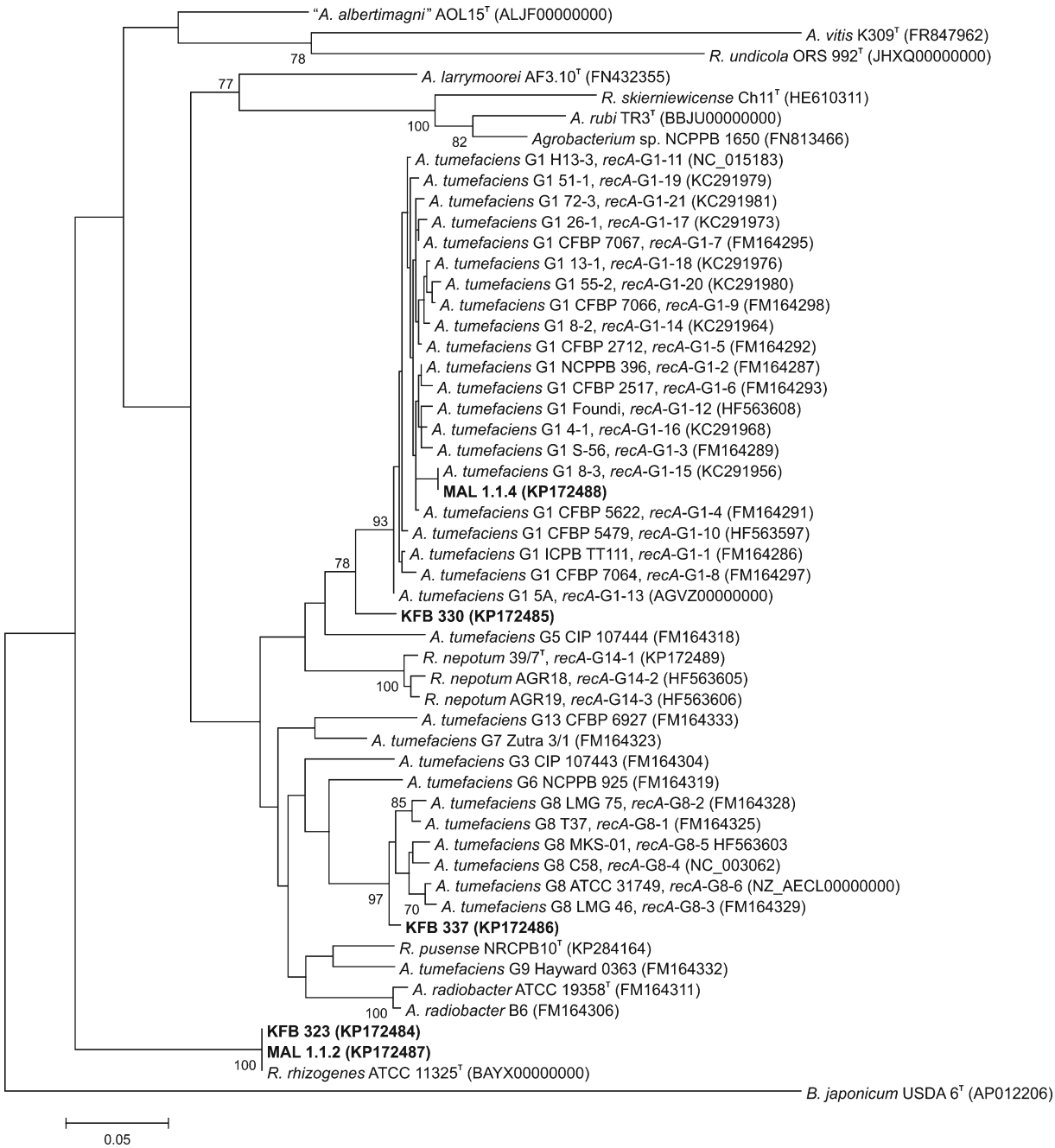

Fig. 3 Maximum likelihood tree based on *recA* gene sequence indicating phylogenetic relationships of strains studied (marked in bold) and related members of *Agrobacterium* sp. and *Rhizobium* sp. Bootstrap values≥70 are shown at nodes. The scale bar represents the number of substitutions per site. Allele codes for particular species/genomic species of *A. tumefaciens* complex are given. DDBJ/EMBL/GenBank accession numbers are shown in parentheses. *Bradyrhizobium japonicum* (strain USDA 6^T) is used as the outgroup organism

substantially different ERIC-PCR profiles (Fig. 1). However, when analyzing intraspecies diversity of ERIC-PCR patterns, strains of *R. rhizogenes* showed similar genetic profiles (Fig. 1). They were divided into six genetic groups (Table 1, Fig. 1). Since genetic groups III, V and VI were composed of strains originating from different geographic areas, it can be assumed that they have a common origin and were probably disseminated

by movement of infected plant material. It is likely that the pathogen systemically colonizes the raspberry plants and that may be latently present within propagation material. Systemic nature has been already proven for different agrobacteria in various plant species (Tarbah and Goodman 1987; Jones and Raju 1988; Cubero et al. 2006; Yakabe et al. 2012; Zoina et al. 2001).

By *recA* gene sequence analysis we confirmed identity of strains studied and differentiated those belonging to *A. tumefaciens* species complex (Table 1, Fig. 3). In addition, we also found novel allele (*recA*-G8-7) within genomic species G8, which increases the known diversity of this genomic species. Until now, total of 70 different alleles were found within *A. tumefaciens* species complex (Costechareyre et al. 2010; Lamovšek et al. 2014; Shams et al. 2013). Further assessment of allelic diversity among agrobacteria will contribute to the more efficient epidemiological surveillance and crown gall outbreak investigations.

Although strain KFB 330 was identified as a member of *A. tumefaciens* species complex, it had some atypical properties and therefore was not fully identified. In multiplex PCR targeting 23S rRNA gene sequences, it gave two amplification products specific for both *A. tumefaciens* and *R. rhizogenes*. This feature was also recorded for strains 39/7, 7/1 and 0 (Puławska et al. 2006), later described as novel species within *A. tumefaciens* species complex - *R. nepotum* (Puławska et al. 2012a). However, biochemical and physiological tests as well as sequence analysis of *recA* housekeeping gene showed that KFB 330 is not a member of *R. nepotum*, although this strain was clustered with type strain of this species by phylogenetic analysis of 16S rRNA gene sequences (Fig. 2). The phylogenetic position of atypical strain remained unclear since it formed separate phylogenetic lineage, different from all known genomic species of *A. tumefaciens* species complex (Fig 3).

Morphological, physiological and biochemical methods are the oldest tools for studying prokaryotes, but still essential for the characterization and classification of bacteria (Tindall et al. 2010). When we performed set of differential bacteriological tests, atypical strain KFB 330 generally exhibited characteristics of *A. tumefaciens*, apart from positive reaction in citrate utilization test (Table 2). Phenotypic variations among strains of *A. tumefaciens* have been already recorded in literature (Bouzar and Moore 1987; du Plessis et al. 1984; Holmes and Roberts 1981; Süle 1978). Presently, most of the genomic species of *A. tumefaciens* complex are not formally named since they still cannot be differentiated by clear and stable discriminative phenotypic traits. However, *R. pusense* (genomic species G2), *R. radiobacter* (G4) and *R. nepotum* (G14) are phenotypically distinguishable by physiological and biochemical tests (Puławska et al. 2012a; Panday et al. 2011). In addition, ability to degrade ferulic acid and caffeic acid is reported as specific characteristic feature of genomic species G8 ("*A. fabrum*") (Shams et al. 2012; Lassalle et al. 2011).

In summary, our results revealed the existence of a high degree of genetic variation among pathogenic *Agrobacterium* and *Rhizobium* strains isolated from tumor tissue. The data presented in this paper highlight the importance of crown gall bacteria on raspberry and contribute to taxonomic studies of agrobacteria.

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