CHARACTERIZATION OF IRANIAN GRAPEVINE ISOLATES OF *RHIZOBIUM* (*AGROBACTERIUM*) spp.

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SUMMARY

To assess the diversity of *Rhizobium* (*Agrobacterium*) species and strains, associated with crown gall disease of grapevine (*Vitis vinifera*) in the major grapevine growing areas of northern Iran, 105 strains of the suspected bacterium were isolated from tumors of the crown and vines collected from vineyards in the East and West Azarbayjan and Ardabil provinces. Bacterial strains were characterized by their phenotypic features, presence of *virD2* and polygalacturonase genes and opine markers. Seventy seven strains were identified as *Rhizobium vitis* and the remaining 28 strains as *R. radiobacter*. Among the 99 strains of *Rhizobium*-carried *vir* gene sequences, 49, 35 and 8 strains appeared to have opine synthase genes of octopine, vitopine and nopaline types, respectively. The opine-type of the Ti plasmids of the remaining seven strains could not unequivocally be determined. Based on these findings the predominant species inducing tumor on grapevines in northern Iran is *R. vitis* and vitopine producers are the prevalent opine-type in *R. vitis* populations.

Key words: crown gall, opine markers, polygalacturonase gene, *virD2* gene, *Vitis vinifera.*

INTRODUCTION

Crown gall of grapevine (*Vitis* spp.) is a widespread and serious disease of this plant worldwide. The pathogen spreads systematically through latently infected tissues of symptomless *Vitis* spp., and persists in them and in crop debris that remains in the vineyards (Burr and Katz, 1983, 1984).

This disease can adversely affect vine vigour and growth, thus reducing crop yield. *Agrobacterium vitis* (Ophel and Kerr, 1990), formerly referred to as *Agrobacterium tumefaciens* biovar 3 (Kerr and Panagopoulos, 1977) and more recently reclassified as *Rhizobium vitis* (Young *et al*., 2001), is the predominant species causing crown gall on grapevines (Burr *et al.*, 1998). Only in *ca.* 10% of the cases, the disease is caused by *Agrobacterium tumefaciens* (Smith and Townsend, 1907) currently called *Rhizobium radiobacter* (Young *et al*., 2001). Both bacterial species can be distinguished from others on the basis of opine markers encoded by differently arranged T-DNA genes. *R. vitis* strains have mainly been found to harbour octopine, nopaline or vitopine type tumor inducing plasmids, pTis (Szegedi *et al*., 1988). Opines are specific compounds synthesized by crown gall tumors elicited by *Agrobacterium* strains (Dessaux *et al*., 1998). Not all opines are present in any given tumor. The classes of opines synthesized depend on the particular Ti plasmid possessed by the inciting *Agrobacterium* strains. There is a strong correlation between the opine-phenotypes of crown gall tumors and the *Agrobacterium* strains, because every opine detected in a tumor is a growth substrate for the inciting strain (Dessaux *et al*., 1998).

In recent years the use of the non-pathogenic *R. vitis* strain F2/5 for the biological control of crown gall disease of grapevine is gaining more popularity (Burr and Reid, 1993; Burr e*t al*., 1998). The crown gall bacterium was first isolated in Iran from Qazvin province by Amani (1966) who, based on biochemical characteristics, identified *R. radiobacter* (*A. tumefaciens*) as the causal agent of grapevine crown gall disease in the country. In the past 25 years, grape crown gall has been reported from different regions of Iran including Fars, Kohgiluyeh and Boyer-Ahmad, Tehran and Qazvin provinces (Salahi-Ardakani *et al*., 2000; Salehi *et al*., 2006).

Based on available reports, the dispersion and diversity of the causal agents of grapevine crown gall in Iran is not clear and contradictions occur. For example *R. vitis* and *R. radiobacter* were identified as incitants of grapevine stem and crown gall in Karaj or Fars provinces. However, in one study almost all strains belonged to *R. vitis* and only a few were identified as *R. radiobacter*, while in an earlier report, only *R. radiobacter* was identified as the incitant of the disease in Qazvin province (Amani, 1966; Salahi-Ardakani *et al*., 2000).

Different primer pairs based on plasmid and chro-

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mosomal DNA sequences have been designed for the molecular detection and identification of tumorigenic *Rhizobia* spp. and PCR analysis has successfully been employed for *R. vitis* detection, prediction of strain tumorigenicity and T-DNA type (Eastwell *et al*., 1995; Haas *et al*., 1995; Louws *et al*., 1999; Szegedi and Bottka, 2002; Kawaguchi *et al*., 2005; Genov *et al*., 2006).

In the present study, PCR primer pairs designed on *virD2* gene sequences were used to differentiate the tumor-inducing *Rhizobium* strains from other Rhizobia (Haas *et al*., 1995). Identification of the opine types was attempted by PCR with opine-specific primers (Szegedi and Bottka, 2002; Bini *et al.*, 2008a).

MATERIALS AND METHODS

Bacterial strains. Samples from grapevine crowns and stems bearing whitish young tumors were transferred to the laboratory where the tumors were washed in running tap water and surface-sterilized for 2 min in 1% sodium hypochlorite. Tumors were then rinsed with several changes of sterile distilled water (SDW), crushed in an about equal volume of SDW and left at room temperature. After 30 min a loopful (15 µl) of the suspension was streaked onto plates of potato dextrose agar (PDA) and RS (Roy and Sasser, 1983) media and incubated at 28°C.

The isolates of *Rhizobium* used in this study, their year and place of isolation are listed in Table 1. *R. vitis* ICMP 10752 (octopine type), *R. vitis* ICMP 10753 (nopaline type), *R. radiobacter* ICMP 5856 (tumorigenic) and ICMP 5785 (saprophytic) were used as reference strains.

Tumorigenicity assays. Strains were grown on PDA for 48 h at 28°C. Bacterial cells were collected on the flat end of a sterile toothpick and placed onto stems of tobacco, tomato and sunflower plants in the greenhouse (22-29°C). A single wound was then made through the inoculum into the stem with a sterile insect-mounting pin (Burr *et al*., 1999). Two plants were inoculated for each strain and the test was repeated once. Plants were observed for the appearance of tumors at the inoculation sites for up to 1 month.

Physiological and biochemical tests. A standard set of physiological and biochemical tests were performed for differentiation of *Rhizobium* spp. (Moore *et al*., 2001), including evaluation of growth on D1M agar, 3-ketolactose production, growth in 2% NaCl, growth at 35°C, acid production from melezitose, clearing of PDA plus $CaCO_3$ medium, utilization of tartrate and malonate, motility at pH 7.0, citrate utilization, and oxidase reaction.

Preparation of template DNA. Bacteria were grown on glucose/yeast-extract agar (Szegedi *et al*., 2005) or nutrient agar at 27°C for 48 h. Cells were scraped off the medium, suspended in sterile distilled water (A_{600} nm = 0.1, *ca.* 10^8 CFU/ml) and were lysed by the addition of 1:10 volume of 3% KOH and heating the suspension at 95°C for 2 min with subsequent cooling on ice. Lysates were centrifuged at 8000 *g* for 2 min and the supernatants were used directly for PCR or stored at -20°C until used.

Primers and PCR conditions for determining tumorigenicity and the opine-type of strains. Strains were screened for tumorigenicity by PCR using the primer pairs designed for amplification of a fragment of the *virD2* gene (Haas *et al*., 1995). Primers designed by Bini *et al*. (2008b) for amplification of sequences of octopine and nopaline synthase genes and the ones designed for the vitopine synthase gene of *R. vitis* were employed for the determination of the opine-type of

Table 1. Name, location and year of isolation of *Rhizobium* strains used.

Strains	Locality of collection	Year of isolation
F1 to F5	Jolfa, East Azarbayjan	2009
AH1 to AH7	Ahar, East Azarbayjan	2009
N1 to N7	Miandoab, West Azarbayjan	2010
MR1,MR2,MR3	Marand, East Azarbayjan	2009
SA1 to SA6	Sarab, East Azarbayjan	2009
Ta1 to Ta4	Tabriz, East Azarbayjan	2009
S1 to S6, D1, D2	Osku, East Azarbayjan	2009
K1 to K4	Salmas, West Azarbayjan	2010
AZ1 to AZ10	Azarshahr, East Azarbayjan	2009
R1 to R7	Ardabil, Ardabil	2010
Bd1 to Bd13	Bonab, East Azarbayjan	2009
Marq1 to Marq9, G1 to G7	Maragheh, East Azarbayjan	2009
H1 to H7, E1 to E4	Mianeh, East Azarbayjan	2009
AJ1 to Aj4	Ajabshir, East Azarbayjan	2009

strains (Szegedi and Bottka, 2002).

A partial sequence of the polygalacturonase gene (*pehA*) was amplified using the primer pair PGF/PGR (Szegedi and Bottka, 2002) for verification of the strains assigned to *R. vitis*. PCR was done in a final volume of 25 µl containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 2.5µl of 10X buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.4), 1.25 U Taq DNA polymerase (CinnaGen, Iran) and 3 µl of template DNA. Temperature and time profiles of the amplification reactions were an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50-54°C for different primers (VirD2A/VirD2C and NOPF/NOPR, 50°C; OCTF/OCTR, 52°C; PGF/PGR and VisF/VisR, 54°C) for 1 min, and extension at 72°C for 1 min. Final elongation was for 10 min at 72°C.

All strains listed in Table 1 were used in PCR tests and each reaction was repeated at least twice. PCR products were electrophoresed in 2% agarose gels in TEB buffer (90 mM Tris base, 2 mM EDTA, 90 mM boric acid, pH 8.3) at 85 V and stained with ethidium bromide (0.5 µg/ml). Gels were photographed under UV light.

RESULTS

Identification of strains. One hundred and five bacterial strains with white, mucoid and convex colonies on PDA+$CaCO_3$ were isolated from symptomatic crown and stem tissue, all of which were catalase and oxidase positive, motile, aerobic, grew on 2% NaCl and hydrolyzed esculin and Tween 80. None of the strains produced H_2S or indole. All isolates were identified as putative *Rhizobium* strains. Of these, 77 that produced colonies with dark red centers and white edge on RS medium, utilized L-tartrate and malonate, did not produce ketolactose from lactose and pellicle in ferric ammonium citrate broth were presumed to be *R. vitis* strains whereas the remaining 28 isolates were identified as *R. radiobacter*, as they produced ketolactose from lactose and pellicle in ferric ammonium citrate broth and utilized of D-tartrate (Table 2).

Tumorigenicity. All except for six strains produced galls on tomato, tobacco and sunflower within four weeks after puncture inoculation. Six strains identified as *R. vitis* (strains E1 to E4, D1 and D2) were apparently non-tumorgenic and did not produce galls on these plants.

Table 2. Phenotypic characteristics and possession of partial sequences of genes for opine synthesis, polygalacturonase and *virD2* genes in *Rhizobium* (*Agrobacterium*) strains isolated from grapevine.

Strain	Opine*	PG	*virD2*
Rhizobium vitis ICMP 10752	OCT	+	+
R. vitis ICMP 10753	NOP	+	+
Tumorigenic *R. vitis* SA1 to SA6, Ta1 to Ta4 S1 to S6, AZ1 to AZ10, Marq1 to Marq9	VIT	+	+
Bd1 to Bd13, H1 to H7 , F1 to F5	OCT	+	+
R1 to R7	ND	+	+
AJ1 to Aj4	NOP	+	+
R. radiobacter ICMP 5856	ND	-	+
R. radiobacter N1 to N7, MR1,MR2,MR3, AH1 to AH7, K1 to K4 G1 to G7	OCT NOP OCT	- - -	+ + -
Non-tumorigenic *R.vitis* E1 to E4, D1 and D2		+	-

* OCT: octopine, NOP: nopaline, VIT: vitopine, ND: not determined

PCR analysis of tumorigenicity. Ninety two of the 105 strains examined, yielded a 224 bp amplicon from the *virD2* gene in PCR (not shown). No amplification products were obtained from *R. vitis* strains E1 to E4 and D1 and D2 following PCR with *virD2* primers, suggesting that they may not harbour Ti plasmids. These isolates were non-pathogenic in the *in situ* tumorigenicity tests on the three indicator plants mentioned before.

Seventy seven strains produced a 466 bp fragment with the polygalacturonase gene-specific primers (PGF/PGR) (Fig. 1). These were all the strains that produced colonies with red centers on RS medium, typical for *R. vitis.*

Opine types of the strains. The opine type of each isolate was determined in parallel using primers designed for the amplification of a partial sequence of the octopine, nopaline and vitopine synthase genes (Bini *et al*., 2008a; Szegedi and Bottka, 2002).

Among the 99 strains of *Rhizobium* carried *vir* gene sequences, 49, 35 and 8 strains appeared to have opine synthase genes of octopine, vitopine and nopaline type, respectively (Table 2). Out of the 77 preliminarily characterized *R. vitis* strains assayed, 35 were identified as vitopine-type, 25 as octopine-type, 4 as nopaline-type and 7 strains not determined (Fig. 2 and 3).

Of the 28 *R. radiobacter* strains, 24 produced a 475 bp amplification product with the octopine primers OCTF/OCTR, and four were identified as nopaline type with the NOPF/NOPR primers (Fig. 2 and 3). One of the nonpathogenic strains (D1) was used as a negative control in some PCR trials, yielding no amplification product, as expected.

Thus the majority of *R. vitis* strains inducing tumors on grapevine in northern Iran appear to be the vitopine type.

DISCUSSION

Crown gall of grapevine, commonly caused by *R. vitis,* occurs throughout the world where grapevines are grown. In Iran the grapevine is a crop of major importance in all areas where the crown gall disease was observed, especially in the northern part of the country. Since *R. vitis* was isolated from the majority of vineyards that were sampled, it appears to be a common incitant of *Vitis vinifera* galls in Iran, as is the case in other parts of the world.

As mentioned, a previous study identified *R. radiobacter* (*A. tumefaciens*) as the agent of grapevine stem and crown gall in an important viticultural area of Iran (Amani, 1966). Subsequently *R. vitis* was reported as the causal agent of crown gall in Fars and Kohgiluyeh and Boyer-Ahmad provinces (Salahi-Ardakani *et al*., 2000). However, the latter authors had shown that isolates of the causal agents of grapevine crown gall in Fars and Kohgilueh and Boyer-Ahmad provinces represented a heterogeneous population, based on biochemical and serological tests and cell proteins pattern, i.e. 51%

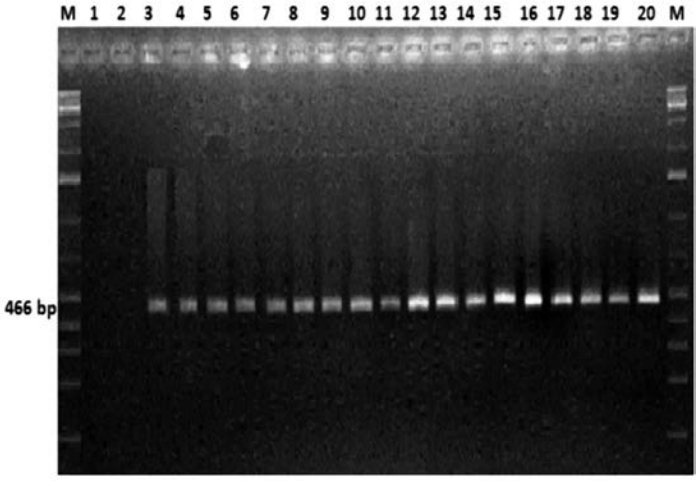

Fig. 1. PCR analysis of the strains with a polygalacturonase gene-specific primer pair (PGF/PGR) which amplifies a 466 bp fragment from all *Rhizobium vitis* strains. Lane 1, *R. radiobacter* ICMP 5856 (negative control); lane 2, *R.radiobacter* MR3; lane 3, *R. vitis* SA1; lane 4, Ta1; lane 5, S2; lane 6, S5; lane 7, E3; lane 8, AZ4; lane 9, AZ7; lane 10, Marq2; lane 11, Marq5; lane 12, H3; lane 13, F4; lane 14, F5; lane 15, Aj5; lane 16, R2; lane 17, D1; lane 18, Bd5; lane 19, Bd6; lane 20, *R.vitis* ICMP 10752 (positive control); lane M, 1Kb Plus DNA ladder (Fermentas, Lithuania).

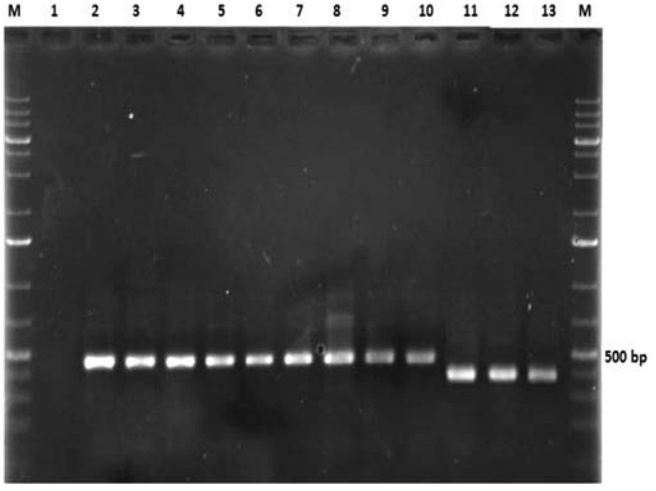

Fig. 2. PCR analysis of *Rhizobium radiobacter* and *R. vitis* strains with primers for octopine (OCTF-OCTR, 475 bp, lanes 2-10) and nopaline (NOPF-NOPR, 394 bp, lanes 11-13) synthase genes. Lane 1, H_2O (negative control); lane 2, *R.vitis* ICMP 10752 (positive control); lane 3, *R. vitis* Bd2; lane 4, Bd5; lane 5, H3; lane 6, F4; lane 7, F5; lane 8, *R. radiobacter* N2; lane 9, MR1; lane 10, AH3; lane 11, *R. vitis* ICMP 10753 (positive control); lane 12, *R.vitis* Aj1; lane 1, *R.radiobacter* K2; lane M, 1Kb plus DNA ladder (Fermentas, Lithuania).

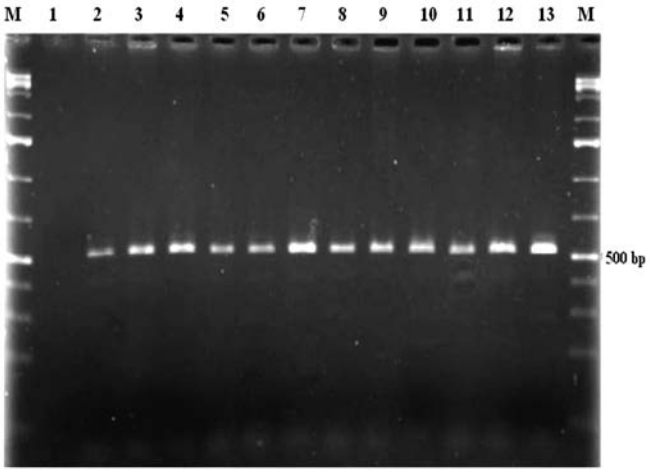

Fig. 3. PCR analysis of *Rhizobium vitis* strains with primers for the vitopine (VisF/VisR, 561 bp) synthase gene after electrophoresis on agarose gel. Lane 1, H_2O negative control; lane 2, *R.vitis* SA2; lane 3, SA3; lane 4, Ta2; lane 5, Ta4; lane 6, S1; lane 7, S5; lane 8, Az1; lane 9, Az2; lane 10, Az9; lane 11, Marq2; lane 12, Marq7; lane 13, Marq9; lane M, 1Kb plus DNA ladder (Fermentas, Lithuania).

of the *Rhizobium* strains belonged to *R. vitis* (tumorigenic), 3% belonged to *R. radiobacter* (tumorigenic) and 46% were saprophytic.

Javaheri *et al.* (2000) reported that most of the *Rhizobium* strains from the Iranian grape-growing provinces of Qazvin and Tehran contained octopine type Ti, and a few had nopaline type Ti plasmids, as determined by a rapid micro scale method (Otten and Schilperoort, 1978).

For identifying of *R. vitis* and *R. radiobacter* strains in northern Iran, we have now compared a set of primers designed on the chromosomal or Ti plasmid sequences, and tested several published primers for agrobacteria detection. So, the polygalacturonase gene-specific primers PGF/PGR amplified the expected products from all *R. vitis* strains, confirming their identification as determined by biochemical tests.

At 50°C annealing temperature VirD2A/VirD2C primers amplified the corresponding fragments from all the examined strains except for 13 of them (Table 2), indicating that the published primers are not fully complementary to the *virD2* genes of the tested strains, as already reported by Bini *et al.* (2008b).

PCR results with opine synthase-specific primers showed that 35 (49%) of the tumorigenic *R. vitis* isolates from Iran belong to the vitopine, 25 (35%) to the octopine group, and 4 (5%) to the nopaline group whereas the group of 7 strains was not determined. These results are not in accordance with previously published data for *Rizobium* strains (Burr *et al.*, 1998; Ride *et al.*, 2000) and field tumors (Szegedi, 2003). Formerly, octopine types were reported to be the most prevalent opine type in *R. vitis* populations (*ca.* 60%), followed by nopaline types (*ca.* 30%) and vitopine types (*ca.* 10%). Bini *et al.* (2008b) reported that 22 (38%) of the *R. vitis* isolates from Italy belonged to the octopine and 32 (56%) to the vitopine group, whereas nopaline type isolates were not found.

In this study, of the 28 *A. tumefaciens* strains, 24 produced a 475 bp amplification product with the octopine primers OCTF/OCTR and 4 strains were identified as nopaline type with the NOPF/NOPR primers, in accordance with previously data relative to *R. radiobacter* strains (Javaheri *et al.*, 2000).

In conclusion, our investigation has shown that tumor-bearing grapevines from northern Iran vineyards contain predominantly pathogenic vitopine type strains of *R. vitis.* The close correlation between the results of biochemical and virulence tests and PCR analysis of the isolated bacterial strains suggests that the PCR protocol employed can efficiently be used for direct PCR testing of propagative material for planting and for production of *Rhizobium*-free vines, similarly to the methods proposed by other researchers (Eastwell *et al.*, 1995; Kaufmann *et al.*, 1996; Louws *et al.*, 1999; Szegedi and Bottka, 2002).

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