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Detection of *Agrobacterium vitis* by PCR using novel *virD2* gene-specific primers that discriminate two subgroups

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Abstract Tumour tissue samples were collected from vines grown in various regions of Italy and other parts of Europe and extracted for detection of *Agrobacterium vitis*. Fifty strains were isolated on agar plates and screened by PCR with consensus primers from the *virD2* gene. They were confirmed as *A. vitis* with a species-specific monoclonal antibody. The isolates were further analyzed by PCR for their opine synthase genes and ordered into octopine, nopaline and vitopine strains. Primers designed on the octopine synthase gene did not detect octopine strains of *Agrobacterium tumefaciens*. For quantitative PCR, *virD2* fragments were sequenced: two classes of *virD2* genes were found and two primer sets designed, which detected octopine and nopaline strains or only vitopine strains. For simultaneous identification of all opine-type strains, multiplex real-time PCR with either primer pair and SYBR Green was performed: the combined sets of primers gave signals with DNA from any *A. vitis* strain. Specificity of the new primers for real-time PCR was evaluated using several unidentified bacterial isolates from grapevines and other plant species. An elevated level of non-specific background was observed when the combined primer sets were used in multiplex PCR assays. The real-time PCR protocol was also used to detect *A. vitis* cells directly from grapevine tumours; avoiding direct isolation procedures a sensitivity in the range of one to ten cells per assay was found. Inhibition of the PCR reaction by plant material was overcome by treating tumour extracts with a DNA purification kit as a step for the isolation of nucleic acids.

Keywords *Agrobacterium vitis* · Opine-type · Real-time PCR · *virD2* gene

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Introduction

Agrobacterium vitis is the predominant species causing crown gall tumours on grapevines (Burr et al. 1998), and only in approx. 10% of cases, infections are incited by *Agrobacterium tumefaciens* (Süle 2006, pers. comm.). Both bacterial species are grouped on the basis of opine markers encoded by differently arranged T-DNA genes, and *A. vitis* strains have mainly been classified into octopine, nopaline and vitopine-types (Szegedi et al. 1988). Opines are low-molecular weight molecules that act as crucial

molecular signals in plant-agrobacteria communications (Dessaux et al. 1992).

Recurrent and severe attacks of the disease in several viticultural areas at different latitudes are responsible for serious economic losses in nurseries and vineyards. The systemic nature of the disease is conducive to an endophytic phase of *A. vitis* living unevenly distributed within vascular tissue of vines where it can latently persist for a long period without causing any visible symptoms. Evidence that the bacterium also persists in the cortex was provided by Jäger (1990). A saprotrophic phase of the life cycle is also known, indicating the ability of the bacterium to survive in grapevine debris in the soil (Burr et al. 1998). Therefore, important measures for controlling the spread of crown gall should rely on suitable and reliable detection methods of the pathogen in both symptomatic and asymptomatic plant materials and in the soil. Various PCR protocols have been developed for the detection of virulent *Agrobacterium* spp. (Nesme et al. 1989; Sawada et al. 1995; Haas et al. 1995; Weller and Stead 2002; Benjama et al. 2004; Pulawska and Sobiczewski 2005) and *A. vitis* strains (Schultz et al. 1993; Szegedi and Bottka 2002) and the targets used to design primers were located on Ti/Ri plasmids and/or chromosomal gene sequences. In our standard laboratory assays, we used the protocol of Haas et al. (1995) with primers designed from the *virD2* genes of *Agrobacterium rhizogenes* (strain A4) and *A. tumefaciens* (strains C58 and A6), but not all the assayed *A. vitis* strains gave clearly detectable signals. On the basis of such observations, we decided to sequence the amplicons obtained with the primers of Haas et al. (1995) to design new *A. vitis*-specific primers for fluorescent-based real-time PCR.

Further information concerning the biodiversity of *A. vitis* strains might be useful to characterise their populations from different ecological niches. A significant part of such information comes from knowledge of the opine synthesis encoding T-DNA: for example, Szegedi and Bottka (2002) designed *A. vitis* pTiS4 vitopine synthase gene-specific primers, and used them for bacterial identification in PCR assays. In the present work, a PCR protocol was set up for the identification of octopine and nopaline-types of *A. vitis* strains of different origin isolated in our laboratory. Vitopine-type strains were also identified using the specific primers of Szegedi and Bottka (2002).

Materials and methods

Bacterial strains and DNA extraction

The *A. vitis* strains used in this study are listed in Table 1; *A. tumefaciens* strains C58, T37 (nopaline-type) and Ach5 and B6 (octopine-type) were also included for analysis. The majority of the *A. vitis* strains (50 out of 53) were isolated in our laboratory on semi-selective RS medium (Roy and Sasser 1983) from grapevine tumours collected in various Italian and other European viticultural areas, identified with a species-specific monoclonal antibody (Bishop et al. 1989) and screened for tumourigenic properties by classical PCR using consensus primers designed from

Table 1 *A. vitis* strains used in this study

Origin of grapevine samples	Strain
Emilia-Romagna	IPV-BO 1861-5, IPV-BO FC_2/14
Friuli-Venezia Giulia	IPV-BO 5159 to 5172, IPV-BO 5231, IPV-BO 5232, IPV-BO 5237, IPV-BO 5238, IPV-BO 5278, IPV-BO 5279, IPV-BO 5389, IPV-BO 5398, IPV-BO 5807, IPV-BO 5808, IPV-BO 5810, IPV-BO 5811, IPV-BO 5820, IPV-BO 6068, IPV-BO 6082, IPV-BO 6086, IPV-BO 6088, IPV-BO 6172
Tuscany	IPV-BO 6186, IPV-BO 6187R
Apulia	IPV-BO 5881, IPV-BO 5898
Moldavia	IPV-BO 5761, IPV-BO 5763, IPV-BO 5766
Montenegro	IPV-BO 6048
Serbia	IPV-BO 6207 to 6214
Hungary	Tm4, AB4
USA	CG49

IPV-BO Istituto Patologia Vegetale—Bologna

the *virD2* gene (Haas et al. 1995). Additional reference strains were: Tm4, octopine-type (Szegedi et al. 1988), AB4 and CG49, nopaline-type (Szegedi et al. 1988; Otten et al. 1996), and IPV-BO FC_2/14, vitopine-type (Bazzi et al. 1987; Schultz et al. 1993). All strains were grown on YMA (Miller et al. 1990) for 48 h at 27°C; at this time, bacterial suspensions ($A_{600\ nm}$=0.1, approx. 1×10^8 cfu ml^{-1}) were prepared for the PCR assays in 0.1% Tween 20 and lysed at 95°C for 15 min.

Primers and standard PCR conditions for detection of octopine, nopaline and vitopine-type strains of *A. vitis*

Specific primers designed by Bini et al. (2008) on sequences of octopine and nopaline synthase genes of *A. vitis* strains Tm4 and AB4, respectively (GenBank, accession numbers: U83987 and X77327) were used; for vitopine synthase gene detection, we applied primers published by Szegedi and Bottka (2002) (Table 2). PCR assays with primers designed on octopine and nopaline synthase genes were performed in the Mastercycler (Eppendorf) in 50 µl reaction volume with the following mixture: 1× buffer [20 mM Tris–HCl, pH 8.7, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$], 0.2 mM dNTPs, 0.2 µM each primer, 0.5 U DNA polymerase (recombinant, Fermentas) and 10 µl bacterial lysates. The applied thermal profile was: 96°C for 1 min followed by 34 cycles at 94°C for 20 s, 52°C for 20 s and 72°C for 40 s; final extension at 72°C for 5 min. Samples were run on a 1% agarose gel and stained with ethidium bromide. The PCR conditions and the reaction mixture for identification of vitopine type strains were described by Szegedi and Bottka (2002).

Sequencing and design of primers on *virD2* gene

Fragments of *virD2* PCR obtained with primers VIRD2A–VIRD2C (Haas et al. 1995) from seven *A. vitis* strains, IPV-BO 5159 and IPV-BO 5160 (vitopine-type), IPV-BO 5162, IPV-BO 5163 and Tm4 (octopine-type), CG49 and AB4 (nopaline-type) were commercially sequenced (SeqLab, Göttingen, Germany). Nucleotide sequences were aligned with the programme Align Plus 4 (Scientific & Educational Software). Novel primers for real-time PCR assays were designed on the basis of *virD2* sequences (Table 2).

Real-time PCR assay with novel primers on *virD2* gene

Real-time PCR amplification was carried out in a 50 µl reaction volume: 1× buffer (20 mM Tris–HCl, pH 8.7, 20 mM KCl, 10 mM $(NH_4)SO_4$, 2.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.2 µM each primer, SYBR Green I diluted 1.5×10^5-fold from a commercial stock solution (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 U TEMPase Hot Start polymerase (Ampliqon, Biomol GmbH, Germany) and 10 µl bacterial suspension. Real-time PCR experiments were performed in an i-CycleriQ Real-time PCR

Table 2 Primer sequences applied for PCR assays

Primers	Sequence (5′–3′)	Amplicon size (bp)
For strain differentiation		
OCTF[a]	GAA TAT GAG AAATCC GTC TCG	
OCTR[a]	ACT CAG AGC TCG TGG CCT TG	475
NOPF[a]	GCA AAC GTA AGT GTT GGA TC	
NOPR[a]	CAA GCG AAT ACT CGA GAC G	394
VisF[b]	CCG GCC ACT TCT GCT ATC TGA	
VisR[b]	CCA TTC ACC CGT TGC TGT TAT T	561
From *virD2* gene for real-time PCR		
VIRD59F26	ATT GGA ATA TCT GTC CCG	
VIRD59R122	GGC GAG ATC GCG GAT ATT	96
VIRD62F23	AAC CAT TCA GCA GGT TAT	
VIRD62R135	TGG TAA TAT GAT CAG GCG	102
From *virD2* gene[c]		
VIRD2A	ATG CCC GAT CGA GCT CAA GT	
VIRD2C	TCG TCT GGC TGA CTT TCG TCA TAA	224

[a] Bini et al. (2008)
[b] Szegedi and Bottka (2002)
[c] Haas et al. (1995)

Detection System (Bio-Rad, Hercules, CA, USA) according to the following cycling conditions: 95°C 30 min; 41×94°C 20 s, 52°C 30 s, 72°C 30 s, 94°C 1 min, 4°C hold. Real-time PCR experiments were also performed by adding both primer sets in the same reaction mixture (multiplex PCR) with a concentration of 0.2 μM each.

Bacterial extraction from grapevine tumours

Tumours were collected in an experimental vineyard from vines cv. Ancelotta/420A inoculated with the vitopine strain IPV-BO 5159 (Table 1). The samples were carefully washed under tap water, rinsed with sterile distilled water (SDW), and thin tissue slices (approx. 200 mg) were cut with a sterile scalpel and transferred in 1.5 ml of SDW in a Petri dish for 30 min. Supernatant (1 ml) was collected and filtered with a syringe and cottonwool and centrifuged (18,000×*g*, 4°C, 15 min). Pellet was resuspended in 300 μl of 0.1% Tween 20 and samples were heated at 95°C for 15 min. Bacterial DNA was purified for real-time PCR assays from plant debris with purification columns (NucleoSpin® Plant, Machery-Nagel GmbH & Co., Germany).

Sensitivity of the real-time PCR assay

Three samples of dried and necrotic tumours from naturally infected grapevines were used to evaluate the sensitivity threshold of real-time PCR. Extraction of *A. vitis* cells from tumours was performed following the protocol described in the previous paragraph. Ten-fold dilutions (10^{-1} up to 10^{-6}) of tumour extracts (1 ml) were prepared with the xylem extract obtained from healthy grapevine cuttings vacuum-washed with SDW (Bazzi et al. 1987), and 100 μl of each dilution were plated on RS medium (Roy and Sasser 1983) for colony counting. Plates were incubated at 27°C for 5–6 days. The remaining suspension of each dilution was concentrated and purified using DNA purification columns.

Results

Differentiation of vitopine, octopine and nopaline-type *A. vitis* strains

The primers designed on octopine and nopaline synthase genes and the primers designed by Szegedi and Bottka (2002) on the vitopine synthase gene were able to discriminate octopine, nopaline and vitopine-types of the *A. vitis* strains used in the present study. PCR assays with primers NOPF-NOPR (negative) and OCTF-OCTR (positive) are shown in Fig. 1. Out of seven *A. vitis* strains used for sequencing part of the *virD2* gene, two were classified into the vitopine-type, two into the octopine-type, and the control strains CG49, Tm4 and AB4 were confirmed to be nopaline, octopine and nopaline-type, respectively. In summary, out of the 50 preliminarily characterized *A. vitis* strains assayed, 12 were identified as vitopine-type, 38 as octopine-type and none as nopaline-type (Table 3). Among four *A. tumefaciens* strains, only the nopaline-type was amplified (Fig. 1).

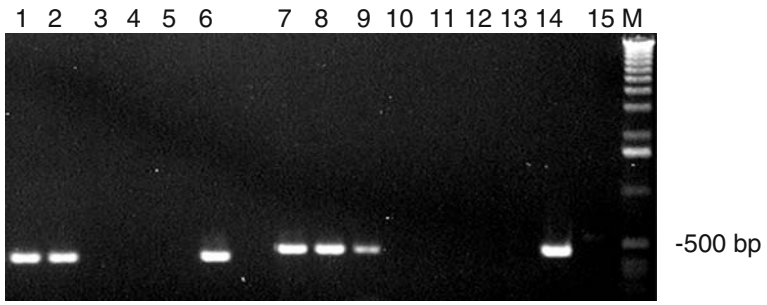

Fig. 1 Standard PCR assays of *A. vitis* and *A. tumefaciens* strains with primers from the nopaline (NOPF-NOPR, 394 bp, *lanes 1–6*) and octopine (OCTF-OCTR, 475 bp, *lanes 7–15*) genes. *Lanes 1*, *10*: *A. tumefaciens* C58 (nop +; oct −); *lanes 2*, *11*: *A. tumefaciens* T37 (nop +; oct −); *lanes 3*, *12*: *A. tumefaciens* Ach5 (nop −; oct −); *lanes 4*, *13*: *A. tumefaciens* B6 (nop −; oct −); *lanes 5*, *14*: *A. vitis* Tm4 (oct +, control); *lanes 6*, *15*: *A. vitis* AB4 (nop +, control); *lane 7*: *A. vitis* IPV-BO 5166 (oct +; nop−); *lane 8*: *A. vitis* IPV-BO 5167 (oct +; nop−); *lane 9*: *A. vitis* IPV-BO 5169 (oct +; nop−); *M* MarkerGeneruler 1 kb DNA Ladder Plus (Fermentas)

Table 3 Real-time PCR assays with *A. vitis* and *A. tumefaciens* strains

Primers		Opine-type	Strains
VIRD59F26/ VIRD59R122	VIRD62F23/ VIRD62R135		
A. vitis			
+	−	V	IPV-BO 1861-5, FC_2/14, IPV-BO 5159, IPV-BO 5160; IPV-BO 5232, IPV-BO 5237, IPV-BO 5238, IPV-BO 5398, IPV-BO 6082, IPV-BO 6086, IPV-BO 6088, IPV-BO 6187R
−	+	O	IPV-BO 5161 to IPV-BO 5172, IPV-BO 5231, IPV-BO 5278, IPV-BO 5279, IPV-BO 5389, IPV-BO 5761, IPV-BO 5763, IPV-BO 5766, IPV-BO 5807, IPV-BO 5808, IPV-BO 5810, IPV-BO 5811, IPV-BO 5820, IPV-BO 5881, IPV-BO 5898, IPV-BO 6048, IPV-BO 6068, IPV-BO 6172, IPV-BO 6186, Tm4, IPV-BO 6207 to IPV-BO 6214
−[a]	+	N	AB4, CG49
A. tumefaciens			
−	−	N	C58, T37
−	−	O	Ach5, B6

+ PCR amplification, − no PCR amplification, *N* nopaline, *O* octopine, *V* vitopine

[a] Weakly positive for AB4

Sequencing of *virD*2 gene of *A. vitis* strains and PCR assay

A partial sequence for vitopine-type-strains and another one for octopine and nopaline-type *A. vitis* strains were determined for the *virD2* gene (Fig. 2), and two primer pairs were designed for real-time PCR assays, VIRD59F26–VIRD59R122 from the former sequence type and VIRD62F23–VIRD62R135 from the latter (Table 2). The 53 *A. vitis* strains were assayed with these *virD2* primers. All vitopine-type strains were amplified by the first primer pair. The

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IPV-BO 5159    1  CTCTTCCGCATTGTGCCAGGGAGGTGGGACGAAAGACCCTCCAACAAATTATTAATCAATT
IPV-BO 5160    1  ....-............................-............................
IPV-BO 5162    1  -.GC.T..A........TA..-..C..A..T...-...A.T..G..GG..........G.G
IPV-BO 5163    1  -.GC.T..A........TA..-..C..A..T...-...A.T..G..GG..........G.G
Tm4            1  -.GC.T..A........TA..-..C..A..T...-...A.T..G..GG..........G.G
CG49           1  -.GC.T..A........TA..-..C..A..T...-...A.T..G..GG..........G.G
AB4            1  -.GC.T..A........TA..-..C..A..T...-...A.T..G..GG..........G.G

IPV-BO 5159   62  GGAATATCTGTCCCGGAAGGGCACCTTGGAGCTGCAGCGTTCAGCACGGCATCTCGATATT
IPV-BO 5160   62  ............................................................
IPV-BO 5162   62  .........C...A.........AG........C.G..TC.....C.....C.........
IPV-BO 5163   62  .........C...A.........AG........C.G..TC.....C.....C.........
Tm4           62  .........C...A.........AG........C.G..TC.....C.....C.........
CG49          62  .........C...A.........AG........C.G..TC.....C.....C.........
AB4           62  .........C...A.........AG........C.G..TC.....C.....C.........

IPV-BO 5159  123  TTCGTACCACCGGATAATATCCGCGATCTCGCCCGAAGTTGGATTCAAGAAACCGGGAC
IPV-BO 5160  123  ............................................................
IPV-BO 5162  123  CCGC....G..T...C....TAC.AGGT.T..T..G..C...G...TG..G.....A.A
IPV-BO 5163  123  CCGC....G..T...C....TAC.AGGT.T..T..G..C...G...TG..G.....A.A
Tm4          123  CCGC....G..T...C.A..TAC.AGGT.T..T..G..C...G...TG..G.....A.A
CG49         123  CCGC....G..T...C.A..TAC.AGGT.T..T..G..C...G...TG..G.....A.A
AB4          123  CCGC....G..T...C.A..TAC.AGGT.T..T..G..C...G...TG..G.....A.A
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Fig. 2 Alignment of *virD2* sequences of *A. vitis* strains. The similarity of nucleotide sequences between vitopine-type sequences and octopine/nopaline-type sequences is 70%. The EMBL accession numbers for nucleotide sequences: AM490794 for strain IPV-BO 5159 vitopine-type and AM490795 for strain IPV-BO 5162 octopine-type. Strains IPV-BO 5159, 5160: vitopine-type; strains IPV-BO 5162, IPV-BO 5163, Tm4: octopine-type; strains CG49, AB4: nopaline-type

octopine and nopaline-type strains were amplified by the second one (Table 3 and Fig. 3). *Agrobacterium tumefaciens* strains were not amplified, assayed with either primer set (Table 3). Alignment of the two *virD2* gene sequences of *A. vitis* with those of *A. rhizogenes*, strain A4 (accession number X12867) and *A. tumefaciens* octopine-type, strain A6, (accession number AF242881) and nopaline-type, strain C58 (accession number NC_003065) present in GenBank, showed a higher homology for the *virD2* sequences from vitopine strains (82% and 78%) than for the *virD2* sequences from octopine/nopaline strains (76% and 75%).

Specificity of primers on *virD2* gene for detection of *A. vitis* with real-time PCR assay

The primers VIRD59F26–VIRD59R122 and VIRD62F23–VIRD62R135 for real-time PCR were assayed for specificity with various bacteria, including strains of *A. tumefaciens* (4), *Erwinia amylovora* (5), *Erwinia carotovora* (1), *Erwinia billingae* (1), *Erwinia tasmaniensis* (1), *Erwinia* sp. from South Africa (1), *Erwinia* spp. (6), *Xanthomonas campestris* pvs. (2), *Pseudomonas syringae* pv. *syringae* (1), *Bacillus subtilis* (1) and eight endophytic grapevine bacteria (EGB), vacuum-extracted from canes according to the method of Bazzi et al. (1987). Specific signals were detected only for *A. vitis* strains, whereas *A. tumefaciens* strains were not amplified, or the other bacterial strains tested.

We also combined primers VIRD59F26–VIRD59R122 and VIRD62F23–VIRD62R135 in the same PCR assay (multiplex PCR) to detect vitopine and octopine/nopaline groups of *A. vitis* strains simultaneously. All *A. vitis* strains tested gave specific signals and were unambiguously detected, whereas in some cases *A. tumefaciens* strains and the other bacterial species gave signals above the water control (data not shown).

Detection of *A. vitis* from grapevine tumours and sensitivity threshold of the real-time PCR assay

Application of DNA purification columns reduced inhibition of Taq DNA polymerase by plant compounds in real-time PCR experiments. Amplification signals were detected from both extracts of tumours from experimentally-inoculated grapevines with the vitopine strain IPV-BO 5159 and from dried and necrotic tumour tissues of naturally infected grapevines. Bacterial colonies isolated from tumour samples of naturally infected grapevines were shown to

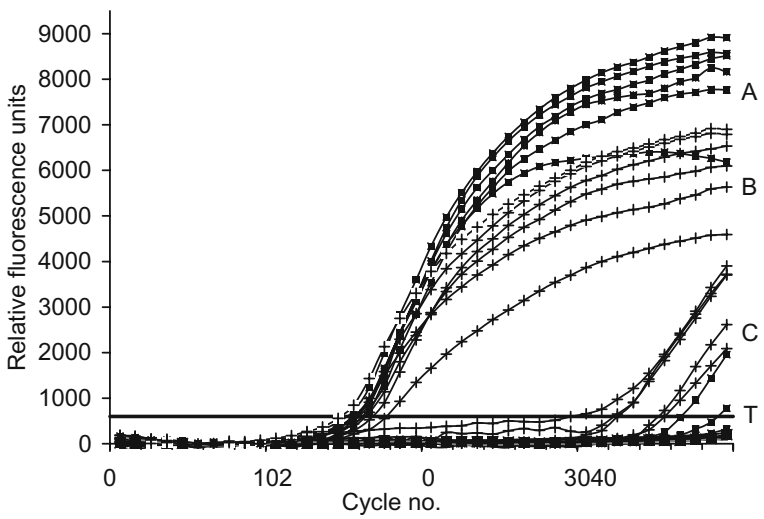

Fig. 3 Real-time PCR with SYBR Green I and primers VIRD59F26–VIRD59R122 and VIRD62F23–VIRD62R135 for specific amplification of *A. vitis* strains. Region *A* (*filled squares*): Amplification plots of the vitopine-type strains IPV-BO 6082, 6086, 6088, 6187R, 5159 and FC2/14 using primers VIRD59F26–VIRD59R122; Region *B* (*plus signs*): amplification plots of octopine-type strains IPV-BO 6172, 6186, Tm4 and nopaline-type strains AB4 and CG49 using primers VIRD62F23–VIRD62R135; Region *C* (*plus signs* and *filled squares*): non specific amplification plots of strains in Region *A* using primers VIRD62F23–VIRD62R135 and in Region *B* using primers VIRD59F26–VIRD59R122. *T*: Threshold

be octopine-type with standard PCR and OCT F/R primers (Table 2).

The sensitivity of the real-time PCR assay was in the range of one to ten cells per 50 µl reaction volume. Plate counting showed that from 200 mg of natural tumour tissue, it was possible to isolate 10^4–10^6 cfu ml^{-1}.

Discussion

A. vitis is a diverse bacterial species based on its genomic organization. Further research is still necessary to reconstruct the evolutionary history of ancestor genetic structures due to various processes such as deletions and insertions. Different oncogene arrangements give rise to T-DNA variants, clearly related to opine-types. The chromosomal background of this bacterial species also varies, and it is highly correlated with the type of Ti plasmid carried by strains (Burr and Otten 1999). In this study, the genetic diversity of *A. vitis* was examined to provide useful insights for its differentiation and detection. PCR-based assays are well suited for characterization of *A. vitis* strains in relation to the opine-type. Procedures for opine analysis, such as high voltage paper electrophoresis (HVPE) (Otten and Schilperoort 1978; Szegedi 2003), require fresh tumour tissue, which is not always available. We designed novel primers capable of discriminating octopine and nopaline *A. vitis* strains: these primers, combined with known primers from the vitopine synthase gene (Szegedi and Bottka 2002), provided information about the presence of different opine synthesis genes in the T-DNA of *A. vitis* strains.

Of 50 bacterial isolates assayed, 38 and 12 (76% and 24%) were identified as octopine- or vitopine-type, respectively; none of the isolates was referred to the nopaline-type. Such results are not in accordance with those of Burr et al. (1998) for strains of different origin where nopaline-types were reported (30%) to exceed vitopine ones (10%). In a recent publication, Gevov et al. (2006) reported that all but one of *A. vitis* strains from Bulgaria analysed by PCR, harboured the octopine/cucumopine-type of Ti plasmid and only one carried the vitopine-type. The prevalence of certain opine-type strains in a given viticultural area must be proven by statistically validated data on a higher number of isolates and properly planned competition experiments under controlled conditions.

Partial sequencing of the *A. vitis virD2* gene identified two different sequences, one for vitopine strains and another one for octopine and nopaline-type strains. The *A. vitis* octopine and nopaline-type vir regions are very similar, if not identical, as demonstrated by restriction enzyme analysis by Otten and De Ruffray (1994). This is confirmed by the present analysis. Alignment of the two different *virD2* gene sequences of *A. vitis* with those of *A. rhizogenes* and *A. tumefaciens virD2* genes present in GenBank, respectively, showed a higher homology for the *virD2* sequences from vitopine strains than for the *virD2* sequences from octopine/nopaline strains. These results may explain why primers by Haas et al. (1995), designed by alignment of *A. tumefaciens* and *A. rhizogenes virD2* sequences, gave better PCR amplification of vitopine *A. vitis* than octopine/nopaline strains, as observed in our laboratory and by Szegedi (2006, pers. comm.). The novel primers designed for each sequence type and reported in this study allowed an unambiguous discrimination between vitopine and octopine/nopaline strains, in real-time PCR assays; moreover, the assays were species-specific since no amplification bands occurred with *A. tumefaciens* strains.

An important goal in crown gall diagnosis is the direct detection of the pathogen in tumours, by-passing the lengthy procedures for direct isolation. In this study, we purified *A. vitis* DNA from grapevine tumours and applied the samples to real-time PCR assays. Amplification of nucleic acids extracted from plant material can be limited by plant compounds inhibiting the DNA polymerase, especially polyphenolics (De Boer et al. 1995). A genomic DNA extraction kit was used to purify bacterial DNA from grapevine tumours. Applied directly in real-time PCR assays, amplification signals were detected with a sensitivity threshold ranging from one to ten cells per assay. The present PCR protocol could be improved by even more specific molecular assays such as TaqMan probes. In the recent work of Weller and Stead (2002), a rapid real-time PCR assay was developed for the detection of rhizogenic *Agrobacterium* based on TaqMan chemistry, reaching a sensitivity threshold of 10 cfu ml^{-1}. Moreover, the complete sequence of *virD2* gene for octopine and nopaline *A. vitis* strains, not yet available, could be compared with the corresponding sequence of the vitopine strain S4 (see: http://agro.vbi.vt.edu) to

identify shared regions to design specific primers identifying all *A. vitis* strains by real-time PCR.

Another interesting aspect emerging from our work concerns the survival of *A. vitis* cells in the dried and necrotic tumours from naturally infected grapevines used in the experiments. It is commonly believed that isolation of the pathogen is made most easily from white to cream-coloured gall of young, actively growing galls (Moore et al. 2001). In our experimental approach, we provided evidence that the pathogen can be detected in dried and in necrotic grapevine tumour tissues (10^4–10^6 cfu ml^{-1}). This further supports the finding that *A. vitis* is able to persist as a saprotroph in decaying grapevine tissues maintaining its Ti plasmid (Burr et al. 1995). Decaying tumour tissue contributes to the spread of the pathogen and is important for epidemiological and diagnostic purposes.

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