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Novel pathogen-specific primers for the detection of *Agrobacterium vitis* and *Agrobacterium tumefaciens*

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Summary

To detect agrobacteria causing crown gall disease of grapevine novel virulence and oncogene specific primer combinations were tested on *Agrobacterium vitis* and *Agrobacterium tumefaciens* strains including most opine types found in grapevines. Reproducible detection of all the tested pathogens in a single reaction was only possible with multiplex PCR using mixtures of virulence-, or oncogene specific primers. A primer combination including *pehA*, *virF* and *virD2* gene-specific oligonucleotides amplified the corresponding fragments from nearly all strains included and distinguished *A. vitis* and *A. tumefaciens* strains carrying octopine or nopaline pTis and *A. vitis* vitopine strains. A second set of primers designed to amplify the T-DNA auxin genes *iaaH* and *iaaM* detected all of the tested pathogens and, as in the case of *virF*-, and *virD2*-specific primers, *A. vitis* vitopine strains formed also a distinct group. These data were further confirmed using opine synthase-, or *6b* gene-specific primers that also allowed the identification and distinction of octopine and nopaline as well as vitopine isolates of *A. vitis*. Thus, a wide range of agrobacteria occurring on grapevine were detected and identified. On the other hand, our results confirm that vitopine-type agrobacteria form a distinct group within the genus *Agrobacterium*.

K e y w o r d s : grapevine, polymerase chain reaction, Ti plasmids, opine-types, virulence genes, oncogenes.

Introduction

The crown gall disease of grapevine causes serious loss in nurseries and plantations worldwide. The disease is caused by diverse types of *Agrobacterium vitis* having octopine/cucumopine, nopaline or vitopine-type Ti plasmids (Burr *et al.* 1998, Burr and Otten 1999). The latter ones have a unique structure and T-DNA organization only distantly related to the most common octopine and nopaline type plasmids (Canaday *et al.* 1992, Gérard *et al.* 1992). Since the pathogen systemically infects its host, propagating materials do play a key role in the dissemination of crown gall (Burr *et al.* 1998). Besides *A. vitis*, *Agrobacterium tumefaciens* may also occur on grapevines as causative agent of crown gall disease (Szegedi *et al.* 2005).

The polymerase chain reaction (PCR) has widely been used to test for the presence of various plant pathogens to select clean plant material (Louws *et al.* 1999). To this end, several primer combinations have been developed to detect tumorigenic agrobacteria but their use is usually limited to few groups since the genetic diversity of the pathogen limits the efficiency of PCR. For example, the *virC*-specific primers amplify the corresponding sequences from *A. tumefaciens* (Sawada *et al.* 1995), but not from *A. vitis* (Szegedi and Bottka, 2002). Although an improved *virC*-specific primer pair detected some *A. vitis* strains (Kawaguchi *et al.* 2005), comprehensive studies including the various opine groups have not been published. Similarly, the *virD2* specific primers (Haas *et al.* 1995) detect pathogenic *A. tumefaciens* but they are not completely specific for *A. vitis* isolates as demonstrated in this paper. A recent work (Bini *et al*. unpubl.) showed that two different nucleotide sequences of the *A. vitis virD2* exist, one is related to the octopine/nopaline-types and the other to the vitopine-type plasmids, and the published primers (Haas *et al.* 1995) are not fully specific for all sequences. Other primers designed on pTiA6 *virE2* sequences amplify this region from several *A. tumefaciens* and *A. vitis* strains, but do not detect *A. vitis* vitopine strains (Szegedi and Bottka 2002, Genov *et al.* 2005 a). More recently further attempts were carried out with *iaaH*-specific primers that detected a wide range of *A. tumefaciens* and a few *A. vitis* strains (Pulawska and Sobiczewsky 2005).

In spite of several attempts, no single or multiplex primer combinations are known that are able to detect reliably all types of agrobacteria occurring in grapevine. Our aim was to carry out further studies with published and new primer combinations that allow detection and identification of any tumorigenic agrobacteria that potentially may infect grapevine in a single PCR.

Materials and Methods

Bacterial strains and DNA preparation: Strains with their origin and relevant characteristics are listed in Tab. 1.

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Table 1

List of strains

Strain	Origin	Characteristics	Reference
Agrobacterium tumefaciens			
A348	pTiA6 in C58 chromosomal background	octopine pTi	Garfinkel *et al*. 1981
C58	Wild type from cherry, USA	nopaline pTi	Hooykaas *et al.* 1980
A281	pTiBo542 in C58 chromosomal background	L,L-succinamopine pTi	Komari *et al*. 1986
Agrobacterium vitis			
Strain	Origin (grapevine variety, location, year of isolation)*	Characteristics	Reference
AT6	*V. vinifera* cv. Ezerfürtű crown gall, Kecskemét (H), 1976	octopine pTi	Szegedi *et al*. 1988
Tm4	*V. vinifera* cv. Téli muskotály crown gall, Kecskemét (H), 1980	octopine pTi	Szegedi *et al*. 1988
AB3	*V. vinifera* cv. Alicanthe Bouchet crown gall, Balatonboglár (H), 1982	octopine pTi	Szegedi *et al*. 1988
Zw2	*V. vinifera* cv. Zweigelt crown gall, Balatonboglár (H), 1982	octopine pTi	Szegedi *et al*. 1988
B10/7	*V. berlandieri x V. riparia* Teleki 5BB crown gall, Kecskemét (H), 1982	octopine pTi	Szegedi *et al*. 1988
AT1	*V. vinifera* cv. Olimpia crown gall, Kecskemét (H), 1967	nopaline pTi	Szegedi *et al*. 1988
AT66	*V. vinifera* cv. Olimpia root xylem, Kecskemét (H), 1976	nopaline pTi	Szegedi *et al*. 1988
AB4	*V. vinifera* cv. Alicanthe Bouchet crown gall, Balatonboglár (H), 1982	nopaline pTi	Szegedi *et al*. 1988
Rr4	*V. vinifera* cv. Riesling crown gall, Balatonboglár (H), 1982	nopaline pTi	Szegedi, unpublished
Ni1	*V. vinifera* cv. Narancsízű crown gall, Kecskemét (H), 1980	nopaline pTi	Szegedi *et al*. 1988
CG49	Grapevine isolate, USA	nopaline pTi	Otten *et al*. 1996
S4	*V. vinifera* cv. Sárfehér crown gall, Orgovány (H), 1981	vitopine pTi	Szegedi *et al*. 1988
Sz1	*V. vinifera* cv. Pinot gris crown gall, Helvécia (H), 1981	vitopine pTi	Szegedi *et al*. 1988
NW221	Grapevine crown gall, Neustadt/ Weinstrasse (D) (variety and date: unknown)	vitopine pTi	Bien *et al*. 1990
SF93	*V. vinifera* cv. Sárfehér crown gall, Orgovány (H), 1993	vitopine pTi	Szegedi and Bottka 2002
F2/5	Grapevine crown gall, Pretoria (RSA) (variety and date: unknown)	avirulent strain	Staphorst *et al*. 1985 Burr *et al*. 1997

*Wild type *Agrobacterium vitis* strains were isolated from Hungary (H), except NW221 and F2/5 which are from Germany (G) and Republic of South Africa (RSA), respectively.

Additionally, 83 *A. vitis* and 12 *A. tumefaciens* isolates were considered for this study at the Phytobacteriology laboratory in Bologna (Tab. 2). Most of them were obtained from grapevine tumors collected in various Italian and European regions. Bacteria were grown on glucose/yeast-extract (Szegedi *et al.* 2005) or on YMA medium (Miller *et al.* 1990) at 27 °C for 48 h. To prepare template DNA cell suspensions ($A_{600\ nm}$ = 0.1, approx. 10^8 CFU/ml) were lysed in Triton X-100/sodium-azide buffer (Abolmaaty *et al.* 2000) or in 0.1% Tween 20 by heating the samples at 95 °C for 10-15 min. Lysates were centrifuged and used directly for PCR or stored at -20 °C.

Primer design and DNA sequence determination: Primers, annealing temperatures and the length of amplified fragments are listed in Tab. 3. The new, *virF*-specific primers $VIRFF_1$/$VIRFR_2$ were designed to amplify this region (accession number: AF044200) of nopaline and octopine-types of *A. vitis*. In order to detect vitopine strains *virD2*-specific primers $VIRD2S4F_{716}$/ $VIRD2S4R_{1036}$ based on the *A. vitis* S4 sequence (http://agro.vbi.vt.edu) were also constructed. The PGF/PGR primers amplifying the chromosomal polygalacturonase gene *pehA* (Szegedi and Bottka 2002) were used to identify *A. vitis* strains and to distinguish them from *A. tumefaciens*. For detection and amplification of diverged *iaaH* genes DNA sequences from seven different *A. tumefaciens* (C58, A6 and P022) and *A. vitis* (Tm4, AG162, CG474 and S4) strains were aligned and two conserved regions separated by about 400 bp were chosen for primer design (iaaHF2/iaaHR1 and iaaH-F10/iaaH-R10 primer pairs).

Table 2

List of strains assayed at the Phytobacteriology Laboratory in Bologna

Strain	Origin (reference)	Opine*	PG	virF	virD2
Tumorigenic *Agrobacterium vitis*					
IPV-BO 5161 to 5168, IPV-BO 5171, 5172, 5280, 5366, 6244, 6299, 6564, 6570, 6572, 6573	Italy	O	+	+	-
IPV-BO 5763, 5766	Moldavia	O	+	+	-
CG102	USA (Bazzi *et al.* 1988)	O	+	+	-
Tm4	Hungary (Szegedi *et al*. 1988)	O	+	+	-
IPV-BO 1861-5, 2152, 5159, 5160, 5232, 5235, 5237, 5238, 5291, 5293, 5296, 5297, 5299, 5362, 5363, 5365, 5378, 5394, 5397, 6088, 6247, 6256, 6263, 6268, 6566, 6571, 6593, 6596, 6623, 6624, 6625	Italy	V	+	-	+
FC_2/14	Italy (Bazzi *et al*. 1987, Schulz *et al.* 1993)	V	+	-	+
IPV-BO 5372	Italy	O/V	+	+	+
AB4	Hungary (Szegedi *et al*. 1988)	N	+	+	-
CG49	USA (Otten *et al.* 1996)	N	+	+	-
Non-tumorigenic *A. vitis*					
CG523	USA		+	-	-
IPV-BO 5233, 5240, 5281, 5292, 5367, 5370, 5379, 5386, 5387, 5391, 5392, 5393, 5395, 6066, 6079, 6489, 6577, 6580, 6584, 6586, 6594, 6595, 6597, 6598, 6322	Italy		+	-	-
Agrobacterium tumefaciens					
IPV-BO 5236, 5295, 6073, 6074	Italy	ND	-	-	-
IPV-BO 6048	Montenegro	ND	-	-	-
IPV-BO 5823, 5824	Italy	O	-	+	-
Ach5, B6	Petit and Tempé 1985	O	-	+	-
C58, T37	Hooykaas *et al.* 1980	N	-	-	-
Bo542	Komari *et al*. 1986	L	-	-	-

* O: octopine, N: nopaline, V: vitopine, L: leucinopine, ND: not determined

DNA sequence of the fragment amplified from octopine strain *A. vitis* AB3 was determined on both strands using the above primers. To determine the full length coding sequence of *iaaH* genes amplified from *A. vitis* nopaline strains AT1 and AB4 additional primers were designed for both directions using the most homologous published DNA sequence, TA-*iaaH* from *A. vitis* pTiTm4 (accession number: X56185). Since TA-*iaaH* is a pseudogene interrupted by IS*866* first the wild type gene was reconstructed. For amplification of the gene two primer pairs were used (iaaH-p1/iaaHR1 and iaaHF2/iaaH-end) and the sequence of the amplified products was determined on both strands using the above and additional primers (Hend2, Hend4, Hinv3, Hinv4, see Tab. 3). DNA sequences have been registered in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers AM745117, AM745118. Two primers (S4iaaM5/S4iaaM3) were designed to amplify an 800 bp fragment of the *iaaM* gene (accession no.: M91609) of the vitopine strain *A. vitis* S4. PCR analyses using primers designed on octopine-, nopaline-, and vitopine synthase genes (Bini *et al*. unpubl., Szegedi and Bottka 2002) as well as *6b*-genes of *A. vitis* (Tab. 3) were also performed to identify the opine-type of each strain.

Table 3

Primers used for PCR and sequence analysis

Name (forward/ reverse)	Primer specificity	Sequence	Length of amplified fragment	Annealing temperature used during PCR	Reference
Primers used for strain detection and identification					
PGF/ PGR	Polygalacturonase gene of *A. vitis* CG49 (accession number: U73161)	5'GGGGCAGGATGCGTTTTTGAG3' 5'GACGGCACTGGGGCTAAGGAT3'	466 bp	54 °C	SZEGEDI and BOTTKA 2002
VirD2A/ VirD2C, or VirD2F	*virD2* gene of *A. tumefaciens* and *A. rhizogenes* (see ref.)	5'ATGCCCGATCGAGCTCAAGT3' 5'TCGTCTGGCTGACTTTCGTCATAA3' 5'CTGACCCAAACATCTCGGCTGCCCA3'	224 bp 338 bp	50 or 54 °C	HAAS *et al.* 1995
$VirFF_1$/ $VirFR_2$	*virF* gene of *A. vitis* octopine and nopaline pTi (accession number: AF044200)	5' ATG AGA AAT TCG AGT TTG CAT GAT G 3' 5' TCG TGA TGG GTA TAC GCT ACG 3'	382 bp	60 °C	This work
$VirD2S4F_{716}$/ $VirD2S4R_{1036}$	*virD2* gene of *A. vitis* S4 vitopine pTi (http://agro.vbi.vt.edu)	5' GAC CGC AAA ACC TGC CAG 3' 5' GAG CCT GTA TTG ACG ATG TC 3'	320 bp	60 °C	This work
OCTF/ OCTR	Octopine synthase gene of *A. vitis* pTiTm4 (accession no.: U83987)	5' GAA TAT GAG AAA TCC GTC TCG 3' 5' ACT CAG AGC TCG TGG CCT TG 3'	475 bp	50 or 52 °C	BINI *et al.* unpublished
NOPF/ NOPR	Nopaline synthase gene of *A. vitis* pTiAB4 (accession no.: X77327)	5' GCA AAC GTA AGT GTT GGA TC 3' 5' CAA GCG AAT ACT CGA GAC G 3'	394 bp	50 or 52 °C	BINI *et al.* unpublished
TF/TR	*6b* gene of *A. vitis* octopine Ti plasmid pTiTm4 (accession no.: U83987)	5'TGGCCGAAATTGTTTACTTCCACCC3' 5'CTATG CCGAAAGACGGCTTGACCCT3'	520 bp	58 °C	SZEGEDI *et al.* 2005
NF/NR	*6b* gene of *A. vitis* nopaline Ti plasmid pTiAB4 (Accession no.: X77327)	5'TTAACCCAAATGAGTACGATGACGA3' 5'TTATTTCGGTACTGGATGATATTAG3'	570 bp	54 °C	SZEGEDI *et al.* 2005
SF/SR	*6b* gene of *A. vitis* vitopine Ti plasmid pTiS4 (accession no.: M91608)	5'TGGCGGTACCGAGATGGGCTGTTCG3' 5'TTAAGCAGAATTAGGACATGAGCCC3'	620 bp	62 °C	This work
iaaH-F2/ iaaH-R1	*iaaH* gene of *Agrobacterium* T-DNA (see Material and Methods)	5'ACATGCATGAGTTATCGTTTGGAAT3' 5'GCATCAAGGTCATCGTAAAAGTAGGT3'	420 bp	54 °C	This work
iaaH-F10/ iaaH-R10	*iaaH* gene of *Agrobacterium* T-DNA (see Material and Methods)	5'GGAAACATGCATGAGTTATCGTT3' 5'CCACATCAGCATCAAGGTCATC3'	424 bp	54 °C	This work
S4iaaM5/ S4iaaM3	*iaaM* gene of the vitopine Ti plasmid pTiS4 (accession no.: M91609)	5'CGCGTCCCCGTTTACACTA3' 5'CGAGATCGCGCTTCAAGAT3'	800 bp	54 °C	This work
VisF/ VisR	vitopine synthase gene of *A. vitis* vitopine Ti plasmid pTiS4 (accession no.: M91608)	5'CCGGCCACTTCTGCTATCTGA3' 5'CCATTCACCCGTTGCTGTTATT3'	561 bp	54 °C	SZEGEDI and BOTTKA 2002
Sequencing primers					
iaaH-p1/ iaaH-end	Forward and reverse primers for sequencing of the *iaaH* gene of *A. vitis* AT1 and AB4	5'GGAAATTCCCTCCAATAATCGC3' 5'CAAGCAGATGTTTTGATTTTGGG3'	-	-	This work
Hend2/ Hend4	Forward and reverse primers for sequencing the 3' part of *iaaH* gene of *A. vitis* AT1 and AB4	5'CTTGGCCTGAAGGATTGACG3' 5'AATTCGTAGTCCCGATGTAGCG3'	-	-	This work
Hinv3/ Hinv4	Reverse and forward primers for sequencing the *iaaH* gene of *A. vitis* AT1 and AB4	CGCAGCAGCCACACCAC CACCGCCGGAATCATAGC	-	-	This work

PCR analysis: The specificity of $VIRFF_1$/$VIRFR_2$ and $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$ primers was checked using 12 *A. tumefaciens* strains and 35 unidentified isolates originated from grapevine tumors and xylem extracts (Tab. 2). The PGF/PGR primers were also used to identify *A. vitis*. PCR amplification was carried out in a reaction volume of 25 µl containing 1X buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 0.3 µM for primers $VIRFF_1$/$VIRFR_2$ and $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$, 0.4 µM for primers PGF-PGR, 5% DMSO, 0.02U/µl DNA polymerase (GoTaq Flexi DNA polymerase, PROMEGA) and 5 µl of template DNA prepared in 0.1 % Tween-20. PCR experiments were performed in a thermal cycler "Gene Amp PCR System 2400" (Applied Biosystems) according to the following conditions: pre-denaturation 94°C 1 min; 40 x 94 °C 1 min, 60 °C 1 min, 72 °C 1 min; final extension 72 °C 5 min. A similar protocol was used to analyze biosuspensions obtained from vacuum washed grapevine cuttings (Bazzi *et al*. 1987). PCRs with virD2A/C-, virD2E-, *iaaH*-, *iaaM*- and *6b*-specific primers were carried out in 25 µl volumes in 1X *Taq* polymerase buffer prepared with 5 % (v/v) DMSO, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP, 1.25 unit of *Taq* polymerase and 1 µl of template DNA prepared in Triton X-100/sodium-azide buffer. The DNA amplification was started with an initial denaturation at 94 °C for 1 min followed by 32 cycles at 92 °C for 1 min, 50-62 °C (Tab. 3) for 1 min and 72 °C for 1.5 min. The reaction was completed with a final elongation step at 72 °C for 3 mins. Samples were analyzed after electrophoretic separation in ethidium-bromide stained 1.5 % (w/v) agarose gels.

Sensitivity of multiplex BIO-PCR assays: *A. vitis* strains IPV-BO 5162, CG49 and IPV-BO 5159, octopine-, nopaline- and vitopine-type respectively, were grown on YMA medium (27 °C for 24 h). Each bacterial culture was used to prepare tenfold dilutions in vacuum flushed xylem extracts from healthy grapevine cuttings, starting from an initial suspension of approx. $1x10^8$ CFU/ml (A_{600nm} = 0.1). One hundred µl of 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated on RS medium in duplicate and incubated at 27 °C for 4-5 days. Bacterial colonies were collected in 1 ml of sterile distilled water (SDW) by plate washing, added with Tween 20 to a final concentration of 0.1 %, and lysed at 95 °C for 15 min. Five µl suspensions were used for Multiplex BIO-PCR.

Isolation of agrobacteria from field tumors: Crown galls derived from natural infections were collected from *Vitis vinifera* 'Kunleány', 'Riesling' and 'Ezerfürtű' cultivars and surface sterilized in 1 % commercial Chlorox followed by washing with sterile water. The outer part was removed and approximately 50 mg pieces of tumors were homogenized in 100 µl sterile water. This sample was used for isolation of bacteria on glucose/yeast-extract medium and for the determination of opine content (Szegedi *et al*. 2005). Purified colonies were tested for 3-ketolactose production and tartarate utilization (Szegedi *et al*. 2005). *A. vitis* was identified by PCR using the PGF/PGR primers followed by identification of octopine, nopaline and vitopine strains with *6b* specific primers (Tab. 3).

Extraction of bacterial cells from grapevine material: Symptomless grafted vines (188 plants) were grouped in 30 samples, 5-6 of each, prefrozen to favor the release of bacterial cells from xylem of rootstocks were vacuum flushed (Bazzi *et al*. 1987, Stover *et al*. 1997). The collected xylem fluids were concentrated by centrifugation (11,000 rpm, 15 min) and pellets resuspended in 200 µl of SDW. One hundred µl of such concentrated samples were plated on RS medium and incubated at 27 °C for 6-7 days. *A. vitis*-like colonies were picked up for identification. The remaining bacterial film was collected through plate washing with SDW and 1 ml of this suspension plus Tween 20 (0.1 % final concentration) was heated at 95 °C for 15 min and diluted 1:10 for BIO-PCR analysis.

Pathogenicity tests: Virulence of *Agrobacterium* isolates was tested on sunflower and tomato stems. Strains CG49 (nopaline pTi) and CG523 (avirulent) were used as positive and negative controls, respectively. Plants were kept in the greenhouse (2 7°C, 70 % RH). Results were recorded 4 weeks after wound inoculation.

Results

In the first step we have analysed a representative set of *A. vitis* strains using the virD2A/virD2C and virD2A/virD2F primer combinations that were published as universal primers for the detection of tumorigenic agrobacteria (Haas *et al*. 1995). At 50 °C annealing temperature virD2A/virD2C primers amplified the corresponding fragment from all the examined strains (data not shown), although these results were not always reproducible. When the annealing temperature was increased to 54 °C we could only detect *A. tumefaciens* and vitopine strains of *A. vitis*. Similarly, virD2A/virD2F detected only these strains at both annealing temperatures while *A. vitis* nopaline strains showed a non-specific amplification only (Fig. 1). These observations show that the published primers sequences are not fully complementary to the *virD2* genes of the tested strains. Thus, further attempts were carried out using multiplex PCR to find primers that are able to detect a wide range of strains in a single reaction.

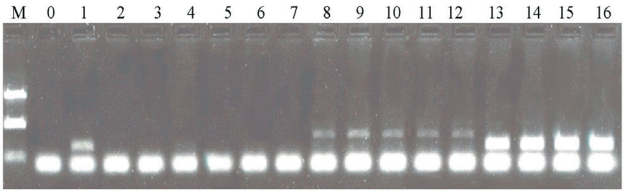

Fig. 1: PCR analysis of *Agrobacterium vitis* with virD2A/virD2F primers. **M**: size markers (1794, 753 and 191 bp), 0: DNA-free sample, **1**: *A. tumefaciens* A6 DNA (positive control), **2**: *A. vitis* F2/5 (avirulent control), **3** to **7**: *A. vitis* AT6, Tm4, AB3, Zw2 and B10/7 (octopine strains), **8** to **12**: *A. vitis* AT1, AT66, N1, AB4 and CG49 (nopaline strains), **13** to **16**: *A. vitis* S4, Sz1, NW221 and SF93 (vitopine) strains yielding a specific amplification product. The virD2A/virD2F primers amplified a faint, non-specific products from the DNAs of *A. vitis* nopaline strains (lanes 8-12). The common band present in all samples is a non-specific product probably generated by primer annealing.

Multiplex PCR with primer pair $VIRFF_1$/$VIRFR_2$ detected octopine and nopaline but did not detect vitopine isolates of *A. vitis*. Opposite results were obtained using $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$ primers that amplified the corresponding sequences from vitopine strains only. The *pehA* gene-specific PGF/PGR primers were also involved in the reaction to identify *A. vitis* (Szegedi and Bottka 2002) and to distinguish them from *A. tumefaciens* (Fig. 2). The opine types of each strain were determined in parallel using primers designed according to the octopine, nopaline and vitopine synthase gene sequences of *A. vitis* (Bini *et al*. unpubl., Szegedi and Bottka 2002). Out of the 83 *A. vitis* strains analysed in this study, 57 carried *vir* gene sequences according to the results of multiplex PCR (Tab. 2). Of the 57 *vir* positive isolates, 22 had octopine-type, only the two control strains CG49 and AB4 had nopaline-type, and 32 had vitopine-type pTis. One isolate, named IPV-BO 5372 was positive for the presence of both *virD2* and *virF* sequences (Fig. 2) and also for both octopine-, and vitopine synthases. Of the *A. tumefaciens* strains assayed with multiplex PCR, octopine strains Ach5 and B6 were positive for *virF* but not for *virD2* whereas nopaline strains C58 and T37 as well as the leucinopine strain Bo542 were negative for both amplicons. Of the seven *A. tumefaciens* strains isolated in our laboratory (Tab. 2), only IPV-BO 5823 and 5824 were positive for *virF* but not for the *A. vitis* S4 *virD2*-gene specific primers. Moreover, all of them gave positive results with Haas' VIRD2A/C primers. They were also probed with *A. vitis* octopine-, and nopaline synthase specific primers designed on the basis of corresponding genes of *A. vitis* (Tab. 3) decreasing the annealing temperature to 50°C for OCTF/OCTF primers (Bini *et al*. unpubl.). Strains IPV-BO 5823 and 5824 were found to be octopine-type, confirming the results obtained with multiplex PCR (Tab. 2) whereas the remaining *A. tumefaciens* isolates IPV-BO 5236, 5295, 6048, 6073, 6074 were negative for either opine gene.

The multiplex PCR protocol with primer pairs $VIRFF_1$/$VIRFR_2$, $VIRD2S4F_{716}$/ $VIRD2S4R_{1036}$ and PGF/PGR was also successfully used for the detection of *A. vitis* from symptomless cuttings. Out of 30 samples, 9 gave positive results for *pehA* and *virD2*, 3 for *pehA* and *virF*, 8 for all of the three genes, 8 only for *pehA* and 2 were negative for all genes. Of the bacterial isolates picked up from RS plates inoculated with vacuum washed samples of symptomless grapevine cuttings, 24 isolates were identified as *A. vitis* after multiplex PCR, but only 4 of them were found to carry either *virF* or *virD2* sequences (data not shown).

Sensitivity of the multiplex BIO-PCR protocol was assayed using bio-suspensions from RS plates with known concentrations of octopine-, nopaline-, and vitopine-type of *A. vitis* cells. Such experiments showed that after enrichment on RS, *A. vitis* DNA was successfully detected up to a threshold of 10 CFU/ml for the octopine-type IPV-BO 5162, 15 CFU/ml for the nopaline-type CG49 and 15 CFU/ml for the vitopine-type IPV-BO 5159. *A. vitis* strains which were positive for *virD2* or *virF* sequences in multiplex PCR assay were also virulent on sunflower and tomato plants. *A. vitis* strains which were only positive for the presence of *pehA* but not for the two *vir* sequences proved to be avirulent on these plants.

In further experiments we have tested a combination of T-DNA auxin biosynthesis gene specific primers designed on the basis of available sequence data. Primer pairs iaaHF2/iaaHR1 and iaaH-F10/iaaH-R10 are specific for the *iaaH* gene of *A. tumefaciens* and *A. vitis* octopine and nopaline strains, the S4iaaM5/S4iaaM3 is specific for the *iaaM* gene of *A. vitis* vitopine strains. A combination of these *iaaH* and *iaaM* specific primers indeed amplified the appropriate region from all laboratory strains tested including pathogenic agrobacteria representing the three opine types of *A. tumefaciens* as well as octopine, nopaline and vitopine strains of *A. vitis* (Fig. 3).

Interestingly, iaaH-F10/iaaH-R10 amplification from DNA samples of *A. vitis* AT6, AB3 and Zw2 resulted in a fragment about 170 bp larger than from other samples (data not shown). Sequence analysis of the isolated PCR fragment of *A. vitis* AB3 showed that the amplification indeed started from the conserved site that was used for design of the forward primer but the sequence contained an extra repeated region that was complementary to the reverse primer 171 nt from the the 3' end. There were six nucleotide mismatches between the reverse primer and the AB3 sequence at the original primer site of iaaH-R10. The appearance of the extra 3' sequence suggests that there was a secondary primer site for the reverse primer downstream

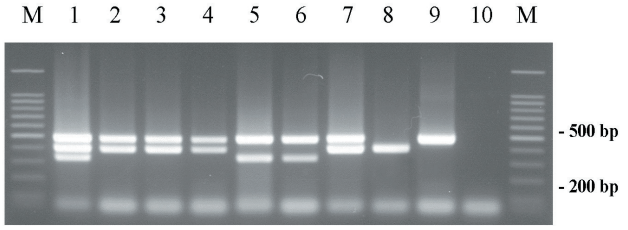

Fig. 2: Multiplex PCR with primer pairs $VIRFF_1$/$VIRFR_2$+ $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$ (382 bp and 320 bp, respectively) PGF/PGR (466 bp) using pure cultures of *Agrobacterium vitis* and *Agrobacterium tumefaciens*. **Lane 1**: *A. vitis* IPV-BO 5372 (octopine and vitopine); **lane 2**: *A. vitis* Tm4 (octopine); **lane 3**: *A. vitis* AB4 (nopaline); **lane 4**: *A. vitis* CG49 (nopaline); **lane 5**: *A. vitis* IPV-BO 5159 (vitopine); **lane 6**: *A. vitis* FC_2/14 (vitopine); ***lane 7***: *A. vitis* CG102 (octopine); **lane 8**: *A. tumefaciens* Ach5 (octopine); **lane 9**: *A. vitis* F2/5 (non tumorigenic); **lane 10**: H_2O, negative control. M: 100 bp ladder, PROMEGA.

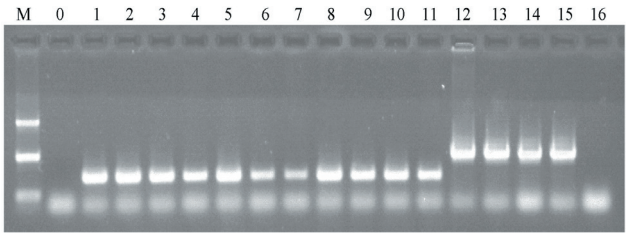

Fig. 3: PCR analysis of *Agrobacterium* strains with the iaaH-F2/iaaH-R1+S4iaaM5/S4iaaM3. Order of samples: size markers (**M**: 1794, 753 and 191 bp), DNA-free control (**0**), *A. tumefaciens* A348 (**1**), C58 (**2**) and A281 (**3**), *A. vitis* octopine strains AT6 (**4**), Tm4 (**5**), AB3 (**6**) and Zw2 (**7**), *A. vitis* nopaline strains AT1 (**8**), AB4 (**9**), Ni1 (**10**) and Rr4 (**11**), *A. vitis* vitopine strains S4 (**12**), Sz1 (**13**), NW221 (**14**) and SF93 (**15**), and the avirulent *A. vitis* F2/5 (**16**).

from the conserved target region that matched probably better with the iaaH-R10 primer sequences in the above strains. Sequence determined from strain AB3 showed 99 % identity (only one mismatch) to the TB-*iaaH* sequence described from *A. vitis* plasmid pTiTm4 (accession number: AF126447).

Using the iaaHF2/iaaHR1 primers DNA sequences from *A. vitis* nopaline strains AT1 and AB4 were also determined. The Ti plasmid of AB4 has been well characterized (Otten and De Ruffray 1994); although sequence data for the *iaaH* gene are lacking it was shown that a large region of the AB4 T-DNA has a perfectly conserved restriction map compared to the Tm4 TA-region. Since the new sequences showed 99 % identity to the TA-*iaaH* pseudogene of *A. vitis* Tm4 interrupted by IS*866* additional primers were designed on the basis of this sequence to amplify and determine the complete coding sequence of *iaaH* gene from strains AT1 and AB4. Both sequences proved to be almost identical showing only two mismatches to the sequence of Tm4 TA-*iaaH* pseudogene through the coding region. The new sequences represented wild type genes since there was no IS*866* insertion in either of them.

To test the suitability of these results obtained with the *iaaH*-, and *iaaM*-specific primers further 34 *A. vitis* colonies were isolated from natural crown galls. All colonies were 3-ketolactose negative, utilized tartarate and reacted positively in PCR with the *A. vitis* specific PGF/PGR primers. To test the presence of oncogenes all isolates were challenged in separate reactions with *6b* specific primers to identify Ti plasmid types. Although these primers are specific for the various *A. vitis* pTis, they were not combined in multiplex PCR due to their different optimal annealing temperatures (Tab. 3). Colonies derived from a given tumor always contained the appropriate Ti plasmid, e.g. those isolated from octopine crown galls contained octopine pTi, colonies isolated from nopaline tumors always contained nopaline pTi, etc. From these isolations 8 octopine, 8 nopaline and 6 vitopine strains were selected and tested with *iaa*-specific primers in multiplex PCR. As in the previous experiments, the iaaHF2/iaaHR1 pair detected all new octopine and nopaline isolates and the S4iaaM5/S4iaaM3 pair detected all new vitopine isolates (data not shown). These data confirm the suitability of the oncogene-specific primers described here for the detection in a single reaction mix of all pathogenic agrobacteria that may potentially occur in grapevines.

Discussion

For practical purposes, it is important to detect pathogenic agrobacteria present in grapevine propagation materials in a single reaction mix. The genetic diversity of *A. vitis* and *A. tumefaciens* occurring in grapevine (Paulus *et al*. 1989, Irelan and Meredith 1996, Momol *et al.* 1998, Ridé *et al*. 2000, Szegedi *et al*. 2005, Genov *et al*. 2006 b) highly limits the efficiency of detection. To overcome these difficulties we have tested several published and newly designed primers to detect agrobacteria in a single PCR assay. Although the virD2A/C primer detected all agrobacteria at 50 °C annealing temperature, at 54 °C only *A. tumefaciens* and *A. vitis* vitopine strains were identified. On the other hand, virD2A/C frequently yielded irregular results with *A. vitis* octopine and nopaline strains. These observations suggest that the *virD2* sequences in *A. vitis* octopine and nopaline pTis show weak homology to the identical *A. tumefaciens virD2* regions. On the other hand, both *virD2* primer combinations (A/C and A/E, Haas *et al.* 1995) were highly specific for *A. vitis* vitopine strains.

Since no single primer pair was found that would reproducibly detect all tumorigenic agrobacteria further attempts were carried out with multiplex PCR including virulence and oncogene-specific oligonucleotides. In the first set of experiments the published *pehA* specific PGF and PGR primers (Szegedi and Bottka 2002) were combined with novel primers designed for *virF* and *virD2* sequences. The *virF* gene is a host range factor that occurs on the octopine-type Ti plasmids of *A. tumefaciens* and octopine-, and nopaline-type Ti plasmids of *A. vitis* (Schrammeijer *et al*. 1998). The protein product (VirF) of this gene is transferred into plant cell during transformation and contributes to the deproteinization of T-DNA/VirE2 complex prior to its integration into the plant nuclear DNA (Tzfira *et al.* 2004). Multiplex PCR with these primers proved to be suitable to unambiguously detect all the assayed *A. vitis* strains and to partially discriminate among their different pTi plasmids, since *virF*-specific primers detected all octopine and nopaline strains whereas *virD2* gene primers detected all vitopine strains. The *pehA*-specific sequences allowed to distinguish *A. vitis* from *A. tumefaciens* (Eastwell *et al.* 1995, Szegedi and Bottka 2002). Moreover, only octopine type *A. tumefaciens* isolates were detected with multiplex PCR since *virF* is not present in nopaline type strains (Schrammeijer *et al.* 1998). Among the 12 *A. tumefaciens* strains of different origin analysed with multiplex PCR, 7 were isolated in our laboratory and initially erroneously considered as tumorigenic *A. vitis* using VIRD2A/VIRD2C primers (Haas *et al.* 1995). The negative response of multiplex PCR with the novel *A. vitis* specific primer sets, supported by positive 3-ketolactose test (data not shown), revealed the correct identity of these strains. Our results suggest that Haas' PCR protocol is only reliable for detection of *A. tumefaciens* but not for all *A. vitis* strains.

PCR results with opine synthase-specific primers showed that 22 (38 %) of the *A. vitis* isolates from Italy belonged to the octopine-, and 32 (56 %) belonged to the vitopine group. Nopaline type isolates were not found. These results are not in accordance with previously published data for strains (Burr *et al*. 1998, Ridé *et al.* 2000) and field tumors (Szegedi 2003). Formerly octopine-types were reported to be the most prevalent opine-type in *A. vitis* populations (~60 %), and nopaline types were also reported (~30%) to exceed vitopine ones (~10 %). Interestingly, one *A. vitis* strain (IPV-BO 5372) was found to be positive for both *virD2* and *virF* genes, as well as for vitopine and octopine synthase genes. Thus, we hypothesize that this strain might harbor two distinct Ti plasmids, one coding for vitopine- and the other for octopine-markers. The dual opine character of IPV-BO 5372 can be explained by the different incompatibility properties of octopine and

vitopine pTis (Szegedi *et al*. 1996). As regards *A. tumefaciens* isolated in our laboratory, which gave positive results with Haas' primers VIRD2A/C, 5 of them did not carry nopaline-, or octopine pTis thus they might belong to a different opine-type.

Multiplex BIO-PCR with *virF*, *virD2* and *pehA* gene specific primers was also used to detect tumorigenic agrobacteria in vacuum extracted xylem fluids from symptomless grapevine cuttings. Our results showed that this analytical protocol is very specific and sensitive for detection of tumorigenic *A. vitis* bacteria living within the vine (up to 10-15 CFU/ml). The assay performed on *A. vitis*-like colonies picked up on RS plates does not constantly guarantee a correct identification of tumorigenic ones, since we found that in the majority of cases (83 %) the chosen colonies isolated from apparently healthy cuttings were non-tumorigenic. Such results led us to hypothesize that only very few tumorigenic *A. vitis* cells might be present in asymptomatic grapevine materials, co-habiting with a prevailing population of non-tumorigenic cells. Our multiplex BIO-PCR protocol seems suitable to detect and identify the *A. vitis* population within the vascular system: in 8 out of 30 analyzed samples from asymptomatic grafted vines, both *virF* and *virD2* genes were detected. This finding supports the evidence that *Agrobacterium* cells bearing different opine-type plasmids may co-habit in the same vine, as observed in a previous work by Szegedi (2003) where a single tumor mass produced both nopaline and vitopine.

Additionally, we have shown that a combination of primers designed for the detection of *iaaH* and *iaaM* genes is able to identify *A. tumefaciens* octopine, nopaline and leucinopine, as well as *A. vitis* octopine, nopaline and vitopine strains in a single reaction mix. These observations were supported by sequencing of the corresponding genes of the octopine strain *A. vitis* AB3 and the nopaline strains *A. vitis* AT1 and AB4. By DNA sequence determination it was shown that the primers are specific although a longer product was amplified from some samples. Interestingly, we found homologs of both *iaaH* sequences described from octopine type strain *A. vitis* Tm4. The TA-*iaaH* and TB-*iaaH* coding sequences show 72 % identity. The PRC product from *A. vitis* AB3 was almost identical with the sequence of the TB-*iaaH* wild type gene while sequences determined from *A. vitis* nopaline strains AT1 and AB4 showed high homology to the TA-*iaaH* pseudogene carrying an IS*866* in strain Tm4. Although the *iaaH* gene is interrupted by IS*866* on the TA-DNA of pTiTm4, this insertion element lacks from the identical region of the octopine/cucumopine strain *A. vitis* Hm1 (Paulus *et al*. 1991). The newly determined sequences probably represent wild type genes since no insertion sequences were found in the coding regions.

This choice of primers published here allows the efficient detection and partial identification of pathogenic agrobacteria in a single reaction. In certain cases, a simple detection of the pathogen is not sufficient, for example when the origin of infection should be determined. For precise identification of isolated colonies distinct IS elements (Schulz *et al*. 1993), RAPD (Irelan and Meredith 1996, Llop *et al*. 2003), digestion of amplified fragments (PCR-RFLP or CAPS, Momol *et al*. 1996, Pionnat *et al*. 1999, Genov *et al*. 2006b) is used. Here we publish novel discriminative primers based on the differences in opine synthase and *6b* genes of T-DNA that make the specific detection of *A. vitis* octopine, nopaline and vitopine strains possible.

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