View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by JKI Open Journal Systems (Julius Kühn-Institut)

Vitis **41** (1), 37–42 (2002)

Detection of *Agrobacterium vitis* by polymerase chain reaction in grapevine bleeding sap after isolation on a semiselective medium

E. Szegedi[1)] and S. Bottka[2)]

[1)] Research Institute for Viticulture and Enology, Kecskemét, Hungary
[2)] Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

Summary

DNA samples prepared from 15 *Agrobacterium vitis* and one *A. tumefaciens* strains were tested in polymerase chain reaction with 4 primer pairs. The pTiC58 *virC* specific primers did not detect any *A. vitis*, while the polygalacturonase specific primers resulted in positive reactions with all grapevine strains. Octopine and nopaline strains could be distinguished from vitopine strains by *virE2* specific primers. Like for other bacterial species Triton X-100 with sodium-azide strongly increased the sensitivity of detection of *A. vitis*. Fourty-six colonies were isolated on a tartrate medium from grapevine bleeding sap collected from galled and symptomless *Vitis vinifera* cv. Cabernet Sauvignon and Riesling plants. Fourteen of them were identified by PCR as *A. vitis*, 11 of which proved to be pathogenic. Analysis of bleeding sap for *A. vitis* may become a useful diagnostic method for selection of healthy plants.

K e y w o r d s : *Agrobacterium vitis*, crown gall, polymerase chain reaction, sodium-azide, bleeding sap.

Introduction

Crown gall, caused by *Agrobacterium vitis* or less frequently by *A. tumefaciens*, is one of the most serious grape diseases in several grape-growing countries. *A. vitis* strains are classified into three taxonomical groups based on Ti plasmid-encoded opine markers, as octopine-, nopaline-, and vitopine-type strains. Of these, octopine strains are found most commonly in grapevine accounting for 60-75 % of isolates (Burr *et al*. 1998, Ridé *et al*. 2000). Since the bacterium systemically infects host plants, the disease spreads out by symptomless propagating material as well. Therefore production of *Agrobacterium*-free stock material and indexing plants for the presence of pathogens are crucial to reduce the disease development (Burr *et al.* 1998, Burr and Otten 1999).

Since the introduction of thermostable DNA polymerase and automated thermocyclers (Saiki *et al*. 1988), PCR has rapidly become a basic diagnostic and identification protocol in plant pathology as well (Henson and French 1993, Louws *et al*. 1999). Early studies to identify *Agrobacterium* with PCR used pure bacterial cultures to determine the suitability of primers which were usually designed on the basis of Ti plasmid *vir* region-, or T-DNA sequences (Schulz *et al*. 1993, Haas *et al*. 1995, Sawada *et al*. 1995). Several methods are used for template DNA preparation from pure cultures. The traditional method based on lysis of bacterial cells with SDS followed by phenol/chloroform extraction and ethanol precipitation (Haas *et al*. 1995) yields high quality DNA, but is time consuming. Lysis in boiling distilled water (Schulz *et al.* 1993, Haas *et al*. 1995, Cubero *et al*. 1999) or in alkaline solution of the non-ionic detergent Triton X-100 (Eastwell *et al*. 1995) yields template DNA which is also suitable for direct amplification reaction without any purification.

Recently PCR has been successfullly used to detect *Agrobacterium* cells directly from naturally infected plants as well (Eastwell *et al*. 1995, Kauffmann *et al*. 1996, Cubero *et al*. 1999). Due to the presence of polyphenolics and polysaccharides in plants DNA should be purified using 2-mercaptoethanol or polyvinylpyrrolidone or with ion-exchange column (Eastwell *et al*. 1995, Cubero *et al*. 1999). Eastwell *et al*. (1995) found that *in situ* lysis of bacterial cells in grapevine cuttings followed by DNA purification was more efficient to detect *A. vitis* than analysis of bacteria eluted from canes with water. This observation may be due to the strong attachment of agrobacteria to grapevine cell walls. Kauffmann *et al*. (1996) used immunocapture cultivation of plant extracts to improve the sensitivity and reliability of the method.

It has been shown by Lehoczky (1968) that bleeding sap of infected grapevines contained approximately 7.0-15.4 x 10^3 *Agrobacterium* cells per ml. Later Lehoczky's results were confirmed by Burr and Katz (1983) who also found pathogenic agrobacteria from sap samples collected from bleeding grapevines. For the detection of *A. vitis* we have compared a set of *Agrobacterium* specific primers and various rapid template DNA preparation protocols. Next we have tested the suitability of bleeding sap analysis for the diagnosis of *A. vitis*.

Material and Methods

B a c t e r i a l s t r a i n s : *Agrobacterium* strains used to test the suitability of primers are listed in Tab. 1. For DNA preparation strains were grown at 27 °C overnight in liquid medium containing 10 g·l^{-1} glucose, 5 g·l^{-1} yeast extract supplemented with AB minimal salts (Lichtenstein and Draper

Correspondence to: Dr. E. Szegedi, Research Institute for Viticulture and Enology, 6000 Kecskemét, POBox 25, Hungary. Fax: +36-76-494924.

Table 1

Agrobacterium tumefaciens and *A. vitis* strains used

Strain	Relevant characteristics	Reference
A. tumefaciens		
A348	pTiA6 in C58 chromosomal background, octopine pTi	GARFINKEL *et al*. 1981
A. vitis		
F2/5	non-virulent strain containing octopine-, and tartrate utilization plasmids	BURR *et al*. 1997, SZEGEDI *et al*. 1999
AT6	wild type, octopine pTi	SZEGEDI *et al*. 1988
Tm4	wild type, octopine pTi	SZEGEDI *et al*. 1988
AB3	wild type, octopine pTi	SZEGEDI *et al*. 1988
Zw2	wild type, octopine pTi	SZEGEDI *et al*. 1988
B10/7	wild type, octopine pTi	SZEGEDI *et al*. 1988
AT1	wild type, nopaline pTi	SZEGEDI *et al*. 1988
AT66	wild type, nopaline pTi	SZEGEDI *et al*. 1988
Ni1	wild type, nopaline pTi	SZEGEDI *et al*. 1988
AB4	wild type, nopaline pTi	SZEGEDI *et al*. 1988
CG49	wild type, nopaline pTi	OTTEN *et al*. 1996
S4	wild type, vitopine pTi	SZEGEDI *et al*. 1988
Sz1	wild type, vitopine pTi	SZEGEDI *et al*. 1988
NW221	wild type, vitopine pTi	BIEN *et al*. 1990
SF93	wild type, vitopine pTi	OTTEN *et al*. 1995

1986). For control experiments *A. tumefaciens* A348, *A. vitis* F2/5, AB3 and S4 strains were used.

Oligonucleotide primers: Four primer pairs were compared to test their suitability for the detection of *A. vitis*. Their origin and sequence data are listed in Tab. 2. Of these primers, VCF/VCR have already been published by SAWADA *et al*. (1995), the others were designed in our laboratory. Experiments were repeated at least twice with each primer pair.

Template DNA preparation: Basically two methods were used to obtain template DNA for PCR analyis. For primer evaluation pure DNA was prepared from 1 ml bacterial culture according to KADO and LIU (1981) followed by RNase treatment, a repeated phenol:chloroform extrac-

Table 2

Oligonucleotide primers used to characterize the *Agrobacterium* strains

Name	Sequence data (nucleotide position)	Length of the amplified fragment	Reference*
A. tumefaciens pTiC58 *virC* gene specific primers			
VCF/VCR	5'-ATCATTTGTAGCGACT-3' (1289-1273) and 5'-AGCTCAAACCTGCTTC-3' (560-575)	730 bp	SAWADA *et al*. 1995
A. vitis CG49 polygalacturonase gene specific primers			
PGF/PGR	5'-GGGGCAGGATGCGTTTTTGAG-3' (679-699) and 5'-GACGGCACTGGGGCTAAGGAT-3' (1144-1124)	466 bp	HERLACHE *et al*. 1997 and this study
A. tumefaciens pTiA6 *virE*2 gene specific primers			
VirE2PF/VirE2PR	5'-CGTGCTGCCGTCTCTACA-3' (960-977) and 5'-ACTGAACGCGATCCCACA-3' (1712-1695)	753 bp	WINANS *et al*. 1987 and this study
A. vitis pTiS4 vitopine synthase gene specific primers			
VisF/VisR	5'-CCGGCCACTTCTGCTATCTGA-3' (2192-2212) and 5'- CCATTCACCCGTTGCTGTTATT-3' (2752-2731)	561 bp	CANADAY *et al*. 1992 and this study

* The VCF/VCR oligonucleotide primer sequences were published by SAWADA *et al*. (1995), other primers were designed in our laboratories on the basis of the published sequence data.

tion and ethanol precipitation. Finally DNA was dissolved in 200 µl sterile distilled water and stored in aliquots at -75 °C. For PCR analysis 1 µl DNA was used.

Since the routine use of PCR requires simple rapid protocols to obtain template DNA from a large number of samples we compared three direct lysis methods based on the heat treatment of cells. Bacterial suspensions (OD_{600}=1.2) were incubated for 10 min at 95 °C (i) in distilled water, (ii) in 1.0 % (v/v) Triton X-100, (iii) in 1.0 % (v/v) Triton X-100 containing 0.25 % (w/v) sodium-azide (ABOLMAATY *et al.* 2000) followed by centrifugation at 5700 *g* for 5 min. Then 30 µl of the supernatants were analysed in a 0.7 % (w/v) agarose gel stained with 1.0 $mg \cdot l^{-1}$ ethidium-bromide after electrophoresis to compare the relative amount of DNA recovered.

Next serial dilutions were made in sterile distilled water from suspensions of *A. vitis* AB3, AT1 and S4 strains containing $5x10^8$, $2.5x10^8$, $1.0x10^8$, $5x10^7$, $1x10^7$, $5x10^6$, $1x10^6$ and $5x10^5$ cells per ml. These suspensions were divided into 450 µl aliquots supplemented with 50 µl of (i) distilled water, (ii) 10 % (v/v)Triton X-100, and (iii) 10 % (v/v) Triton X-100 containing 2.5 % (w/v) NaN_3 (ABOLMAATY *et al.* 2000). Then samples were heated for 10 min at 95 °C in a water bath followed by centrifugation at 5700 *g* for 5 min. For PCR analysis carried out with the PGF/PGR primer pair 2 µl of the supernatants were used.

B l e e d i n g s a p s a m p l e s : Grapevine bleeding sap was collected in April 2000 from 18-year-old symptomless and tumor bearing *Vitis vinifera* cvs Riesling and Cabernet Sauvignon. Three one-year-old canes per plant were cut and the bleeding sap was collected into sterile Eppendorf tubes and used directly or stored at -75 °C. To detect *A. vitis* the following three methods were tested: (i) a 2-5 µl sap sample was added directly to the PCR reaction, (ii) a 2.0 ml sap sample was centrifuged and the pellet was resuspended in 50 µl distilled water with 1 % Triton X-100 and heated for 10 min at 95 °C; (iii) for further enrichment of bacteria a 50 µl sap sample was streaked onto tartrate plates (AB minimal medium with 0.5 % (w/v) L(+)tartrate and 2.5 $mg \cdot l^{-1}$ bromothymolblue) and incubated at 27 °C for 7 d. Then a half loop of colonies resembling *Agrobacterium* was suspended in 0.9 ml of sterile distilled water, supplemented with 0.1 ml 10 % (v/v) Triton X-100 and processed as described above. For the PCR analysis the polygalacturonase specific primers were used. Positive colonies were retested with pathogen-specific primers and inoculated onto *Kalanchoe tubiflora* plants for virulence tests.

P C R c o n d i t i o n s : The reactions were carried out in a PTC-150-HB thermal cycler (MJ Research, Inc., Watertown, USA) in 25 µl final volumes, in 1x *Taq* polymerase buffer prepared with 5 % DMSO (v/v), 1.5 mM $MgCl_2$, 0.4 µM of each primer, 200 µM of each dNTP, 1.25 unit Taq polymerase and 1.0 or 2.0 µl template DNA. The amplification was started with an initial denaturation step at 94 °C for 1 min, followed by 30 cycles at 92 °C for 1 min, 54 °C for 1 min and 72 °C for 1.5 min. Finally the reaction was completed with an extension step at 72 °C for 3 min. Then samples were mixed with 6 µl loading buffer containing 10 $\mu g \cdot ml^{-1}$ ethidium bromide and loaded onto 1.5 % (w/v) agarose gel made in Tris-acetate buffer (pH 8.0) for electrophoretic separation of amplified fragments.

Results

P r i m e r e v a l u a t i o n : We have compared 4 primer pairs (Tab. 2) to test their suitability to detect *A. vitis*. The pTiC58 *virC* specific primer pair (VCF/VCR) detected only the *A. tumefaciens* A348 strain used as a positive control, and none of the *A. vitis* strains (Tab. 3). On the other hand, the PGF/PGR primer pair which was designed to detect the chromosomally localized polygalacturonase gene, amplified the specific fragment from all *A. vitis* DNA tested. The *virE2* specific primer pair (VirE2PF/VirE2PR) amplified the 753 bp region from the control strain A348 as well as from *A. vitis* octopine-, and nopaline-, but not from vitopine strains. Vitopine-type strains were selectively identified with vitopine synthase gene *(vis)* specific oligonucleotide primers. The same results were obtained with pure DNA and with heated bacterial cell suspensions prepared in 1.0 % Triton X-100.

T a b l e 3

Evaluation of primers used to detect *Agrobacterium vitis* strains

Agrobacterium strain (opine type of pTi)*	Primer pairs VCF/ VCR	PGF/ PGR	VirE2PF/ VirE2PR	VisF/ VisR
A348 (o)	+	-	+	-
F2/5 (o)	-	+	-	-
AT6 (o)	-	+	+	-
Tm4 (o)	-	+	+	-
AB3 (o)	-	+	+	-
Zw2 (o)	-	+	+	-
B10/7 (o)	-	+	+	-
AT1 (n)	-	+	+	-
AT66 (n)	-	+	+	-
Ni1 (n)	-	+	+	-
AB4 (n)	-	+	+	-
CG49 (n)	-	+	+	-
S4 (v)	-	+	-	+
Sz1 (v)	-	+	-	+
NW221 (v)	-	+	-	+
SF93 (v)	-	+	-	+

* o: octopine-, n: nopaline-, and v: vitopine type Ti plasmid. Strain F2/5 contains an *inc*Rh1 non-Ti plasmid encoding the utilization of octopine (SZEGEDI *et al.* 1999).

S e n s i t i v i t y o f t h e c e l l l y s i s m e t h o d s : Since the traditional DNA isolation techniques are rather time consuming or require the use of toxic organic solvents, the large-scale use of PCR to detect *Agrobacterium* is limited. Therefore we tested three rapid lysis protocols based on a simple heat treatment in distilled water, in 1 % Triton X-100, or in 1 % Triton X-100 with 0.25 % NaN_3 to obtain template DNA for PCR. Agarose gel analysis of samples prepared from *A. tumefaciens* A348 and *A. vitis* AB3, AT1 and S4 strains showed that DNA could only be detected if cells were lysed in the presence of NaN_3 (Fig.1).

Subsequently the same lytic conditions were compared in PCR using serial dilutions of bacterial suspensions (see

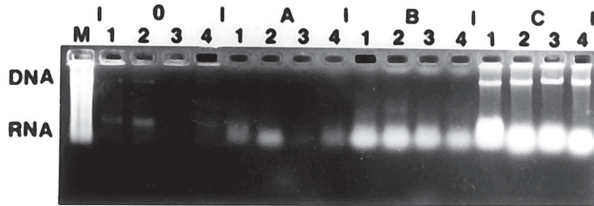

Fig. 1: Comparison of cell lysis methods for DNA isolation. Bacterial suspensions were heated for 10 min at 95 °C in distilled water (panel A), or in 1.0 % Triton X-100 (B), or in 0.25 % (w/v) NaN_3 prepared in 1.0 % Triton X-100 (C). Panel 0 shows non-heated control in distilled water. Lane M: 5 µg sheared chicken DNA, lane 1: *Agrobacterium tumefaciens* A348, lane 2: *A. vitis* AB3, lane 3: *A. vitis* AT1, lane 4: *A. vitis* S4.

Material and Methods). The detection limit of the heating method in distilled water and Triton X-100 was approximately 10^5 cells per reaction with PGF/PGR primers. The Triton X-100 solution was slightly more efficient than distilled water alone (Fig. 2, lanes 3 and 4 in panel A and B). Considering that usually 150-200 cells per reaction are detected by PCR (BURR and OTTEN 1999, CUBERO *et al.* 1999), this relatively high detection limit indicates that only a small proportion of cells are lysed under these conditions. Therefore, the use of the simple boiling method in distilled water or Triton X-100 is appropriate only for pure cultures and isolated colonies. To increase the sensitivity of detection we have tested the effect of NaN_3 with Triton X-100 (ABOLMAATY *et al.* 2000). This solution markedly increased the recovery rate of DNA also in the case of *Agrobacterium* (see above). The detection limit for *A. vitis* AB3 in PCR was reduced by NaN_3 at least by two orders of magnitude, since 10^3 cells per reaction still resulted in an intensive band (Fig. 2, panel C, lane 8). The same results were obtained if these experiments were repeated with the nopaline strain AT1 and with the vitopine strain S4 (data not shown).

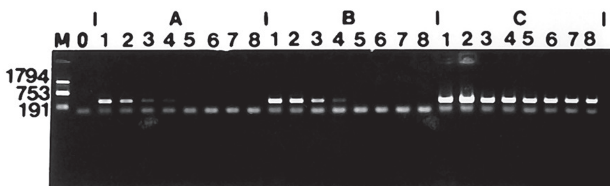

Fig. 2: Effect of NaN_3 on the efficiency of PCR detection of *Agrobacterium vitis* strain AB3. M: size marker, 0: negative control without template DNA. Lanes 1-8 contain DNA preparation from $1x10^6$, $5x10^5$, $2x10^5$, $1x10^5$, $2x10^4$, $1x10^4$, $2x10^3$ and $1x10^3$ cells per reaction, respectively. Cells were lysed in distilled water (panel A), in 1.0 % Triton X-100 (B), or in 0.25 % (w/v) NaN_3 prepared in 1 % Triton X-100 (C) and tested in PCR with the PGF/PGR primer pair.

B l e e d i n g s a p a n a l y s i s : For rapid detection of *A. vitis* we have analysed more than 80 samples, but the pathogen was never found if bleeding sap or bacterial cell suspensions concentrated by centrifugation of fresh bleeding sap samples (2-5 µl) were added directly to the PCR reaction mixture. Therefore for further studies bleeding sap samples were streaked onto tartrate plates to isolate single colonies. The number of tartrate utilizing colonies was rather variable among samples, it usually varied from $4x10^2$ to $2x10^3$ cells per ml. Single colonies were randomly selected for PCR using the polygalacturonase specific primer pair. We have investigated 46 tartrate utilizing colonies, 14 of which were positive in PCR (Fig. 3). Six colonies resulted in non-specific amplification (data not shown), 26 colonies were negative to these primers. None of the 14 positive colonies reacted with the *virE2* specific primers, but 11 colonies induced tumors on *Kalanchoe tubiflora* plants showing that a relatively high proportion of PGF/PGR positive *A. vitis* isolates is pathogenic. These tumors contained vitopine and representative isolates (three colonies were tested) gave positive reactions with the VisF/VisR primers in PCR (data not shown). Results are summarized in Tab. 4.

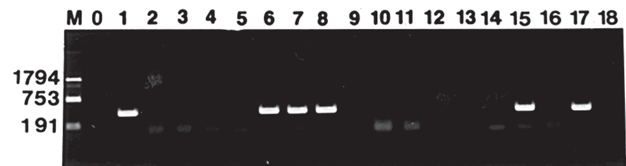

Fig. 3: Identification of *Agrobacterium vitis* from the tartrate utilizing bacterial colonies isolated from bleeding sap. M: size marker, 0: negative control without template DNA, lane 1: *Agrobacterium vitis* AB3 DNA used as positive control. Lanes 2-18 are randomly selected colonies grown on tartrate as sole carbon source. Lanes 6-8, 15 and 17 show *A. vitis*.

Discussion

In order to detect *A. vitis* we compared a set of primers designed on the basis of chromosomal or Ti plasmid sequence data. Surprisingly, the *virC* specific primers did not amplify the corresponding fragment from *A. vitis* DNA preparations; however SAWADA *et al.* (1995) as well as CUBERO *et al.* (1999) found that these sequences are common in nearly all pathogenic agrobacteria. The reason for our failure to detect *A. vitis* by VCF/VCR primers is unknown, most probably it is due to differences in the strains used. On the other hand, the polygalacturonase gene specific primers PGF/PGR detected all *A. vitis* strains tested. These observations are in agreement with previous results (EASTWELL *et al.* 1995). Since this chromosomal gene is common in pathogenic and non-pathogenic *A. vitis* strains we tested *virE2* specific primers as well to distinguish virulent strains. This primer pair detected the octopine-, and nopaline-, but not the vitopine type of pathogenic strains. The latter strains were identified by *vis* gene-specific oligonucleotides (CANADAY *et al.* 1992). Although we have not found a primer pair which can detect all pathogenic *A. vitis*, for routine use the PGF/PGR primers can be used since most (11 out of 14) colonies found in bleeding sap were pathogenic.

For large scale application of PCR it is necessary to have a simple rapid protocol to obtain template DNA. Thus we compared distilled water, Triton X-100 and NaN_3 with Triton X-100 to lyse *A. vitis* cells. NaN_3 in combination with Triton X-100 highly increased the efficiency of the recovery of template DNA as well as the sensitivity of PCR detection as previously described for *Escherichia coli* and other human pathogenic bacteria (ABOLMAATY *et al.* 2000). We have found that this simple protocol was similarly sensitive as

Table 4

Detection of *Agrobacterium vitis* from bleeding sap

Sample (number of plants tested)	No. of tested colonies	No. of PGF/PGR positive colonies	No. of pathogenic colonies	Positive/total number of plant samples
Riesling, symptom-free (4)	5	0	0	0/4
Riesling, infected (6)	16	4	4	1/6
Cabernet Sauvignon, symptom-free (2)	10	7	4	1/2
Cabernet Sauvignon, infected (4)	15	3	3	1/4

the traditional DNA isolation methods including SDS-lysis, organic extraction and ethanol precipitation (data not shown). Therefore we now prefer this method as a routine protocol.

Detection of the pathogen directly from plant samples is quite difficult due to the low number of bacterial cells or due to the presence of PCR inhibitors such as polyphenols and polysaccharides. To overcome these difficulties template DNA should be isolated with multi-step protocols, or with the application of DNA purification columns (EASTWELL *et al*. 1995, CUBERO *et al*. 1999, LLOP *et al*. 1999). Enrichment of the bacterial population from plant samples in a semi-selective medium may also increase the efficiency of detection as described for *Pseudomonas* strains (PENYALVER *et al.* 2000). Although analysis of whole bacterial populations may result in non-specific amplifications as well, we preferred to use single colonies selected on tartrate plates for template DNA preparation. Using this method we could detect pathogenic *A. vitis* in 3 of 16 grapevines tested. Altogether 14 colonies were identified as *A. vitis* of which 11 were pathogenic. Our results confirm previous observations published by LECHOCZKY (1968) and BURR and KATZ (1983) concerning the occurrence of *A. vitis* in bleeding sap.

The isolation of bacterial colonies prior to PCR analysis, although more time consuming, seems to be a useful step to increase the reliability of the detection protocol. On the other hand, this step also eliminates the polymerase inhibitors (phenolic compounds and polysaccharides) present in plant samples. Analysis of grapevine bleeding sap for *A. vitis* may be a useful tool for preselection of pathogen-free vines or to monitor the occurrence of *A. vitis* in rootstock plantations.

Acknowledgements

We thank Ms. ERZSÉBET PUSKÁS for helpful technical assistance, Mr. ELEK GÁBRIS for photographic work. This study was supported by Grant No. 70.821/47-d3/2000 of the Hungarian Ministry of Agriculture and Rural Development.

References

ABOLMAATY, A.; VU, C.; OLIVER, J.; LEVIN, R. E.; 2000: Development of a new lysis solution for releasing genomic DNA from bacterial cells for DNA amplification by polymerase chain reaction. Microbios **101**, 181-189.

BIEN, E.; LORENZ, D.; EICHHORN, K.; PLAPP, R.; 1990: Isolation and characterization of *Agrobacterium tumefaciens* from the German vineregion Rheinpfalz. J. Plant Dis. Prot. **97,** 313-322

BURR, T. J.; BAZZI, C.; SÜLE, S.; OTTEN, L.; 1998: Crown gall of grape: Biology of *Agrobacterium vitis* and the development of disease control strategies. Plant Dis. **82**, 1288-1297.

- -; KATZ, B. H.; 1983: Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap and from vineyard soil. Phytopathology **73**,163-165.

- -; OTTEN, L.; 1999: Crown gall of grape: Biology and disease management. Annu. Rev. Phytopathol. **37**, 53-80.

- -; REID, C. L.; TAGLIATI, E.; BAZZI, C.; SÜLE, S.; 1997: Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment sites on grape cells. Phytopathology **87**, 706-711.

CANADAY, J.; GERARD, J. C.; CROUZET, P.; OTTEN, L.; 1992: Organization and functional analysis of three T-DNAs from the vitopine Ti plasmid pTiS4. Mol. Gen. Genet. **235**, 292-303.

CUBERO, J.; MARTINEZ, M. C.; LLOP, P.; LOPEZ, M. M.; 1999: A simple and efficient PCR method for the detection of *Agrobacterium tumefaciens* in plant tumours. J. Appl. Microbiol. **86**, 591-602.

EASTWELL, K.; WILLIS, L. G.; CAVILEER, T. D.; 1995: A rapid and sensitive method to detect *Agrobacterium vitis* in grapevine cuttings using the polymerase chain reaction. Plant Dis. **79**, 822-827.

GARFINKEL, D. J.; SIMPSON, R. B.; REAM, L. W.; WHITE, F. F.; GORDON, M. P.; NESTER, E. W.; 1981: Genetic analysis of crown gall: Fine structure map of the T-DNA by site-directed mutagenesis. Cell **27**, 143-153.

HAAS, J. H.; MOORE, L. W.; REAM, W.; MANULIS, S.; 1995: Universal PCR primers for the detection of phytopathogenic *Agrobacterium* strains. Appl. Environ. Microbiol. **61**, 2879-2884.

HERLACHE, T. C.; HOTCHKISS, A. T.; BURR, T. J.; COLLMER, A.; 1997: Characterization of the *Agrobacterium vitis pehA* gene and comparison of the encoded polygalacturonase with the homologous enzymes from *Erwinia carotovora* and *Ralstonia solanacearum*. Appl. Environ. Microbiol. **63**, 338-346.

HENSON, J. M.; FRENCH, R.; 1993: The polymerase chain reaction and plant disease diagnosis. Ann. Rev. Phytopathol. **31**, 81-109.

KADO, C. I.; LIU, S. T.; 1981: Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**, 1365-1373.

KAUFMANN, M.; KASSEMEYER, H. H.; OTTEN, L.; 1996: Isolation of *Agrobacterium vitis* from grapevine propagating material by means of PCR after immunocapture cultivation. Vitis **35**, 151-153.

LEHOCZKY, J.; 1968: Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine after natural infection. Phytopathol. Z. **63**, 239-246.

LICHTENSTEIN, C.; DRAPER, J.; 1986: Genetic engineering of plants. In: D. M. GLOVER (Ed.): DNA Cloning: A Practical Approach, Vol. II., 67-119, IRL Press, Oxford-Washington.

LLOP, P.; CARUSO, P.; CUBERO, J.; MORENTE, C.; LOPEZ, M. M.; 1999: A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. J. Microbiol. Meth. **37**, 23-31.

LOUWS, F. J.; RADEMAKER, J. L. W.; DE BRUIJN, F. J.; 1999: The three ds of PCR-based genomic analysis of phytobacteria: Diversity, detection, and disease diagnosis. Ann. Rev. Phytopathol. **37**, 81-125.

OTTEN, L.; CROUZET, P.; SALOMONE, J. Y.; DE RUFFRAY, P.; SZEGEDI, E.; 1995: *Agrobacterium vitis* strain AB3 harbors two independent tartrate utilization systems, one of which is encoded by the Ti plasmid. Mol. Plant-Microbe Interactions **8**, 138-146.

- -; RUFFRAY, P. DE; MOMOL, E. A.; MOMOL, M. T.; BURR, T. J.; 1996: Phylogenic relationships between *Agrobacterium vitis* isolates and their Ti plasmid. Mol. Plant-Microbe Interactions **9**, 782-786.

PENYALVER, R.; GARCIA, A.; FERRER, A.; BERTOLINI, E.; LOPEZ, M.; 2000: Detection of *Pseudomonas savastanoi* pv. savastanoi in olive plants by enrichment and PCR. Appl. Environ. Microbiol. **66**, 2673-2677.

RIDÉ, M. ; RIDÉ, S.; PETIT, A.; BOLLET, C.; DESSAUX, Y.; GARDAN, L.; 2000: Characterization of plasmid borne and chromosome encoded traits of *Agrobacterium* Biovar 1, 2 and 3 strains from France. Appl. Environ. Microbiol. **66**, 1818-1825.

SAIKI, R. K.; GELFAND, D. H.; STOFFEL, S.; SCHARF, S. J.; HIGUCHI, R.; HORN, G. T.; MULLIS, K. B.; ERLICH, H. A.; 1988: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**, 487-491.

SAWADA, H.; IEKI, H.; MATSUDA, I.; 1995: PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium strains*. Appl. Environ. Microbiol. **61**, 828-831.

SCHULZ, T. F.; LORENZ, D.; EICHHORN, K. W.; OTTEN, L.; 1993: Amplification of different marker sequences for identification of *Agrobacterium vitis* strains. Vitis **32**, 179-182.

SZEGEDI, E.; CZAKÓ, M.; OTTEN, L.; KONCZ, C.; 1988: Opines in crown gall tumours induced by biotype 3 isolates of *Agrobacterium tumefaciens*. Physiol. Mol. Plant Pathol. **32**, 237-247.

- -; SÜLE, S.; BURR, T. J.; 1999: *Agrobacterium vitis* strain F2/5 contains tartrate and octopine utilization plasmids which do not encode functions for tumor inhibition on grapevine. Phytopathology **147**, 665-669.

WINANS, S. C.; ALLENZA, P.; STACHEL, S. E.; MCBRIDE, K. E.; NESTER, E. W.; 1987: Characterization of the *vir*E operon of the *Agrobacterium* Ti plasmid pTiA6. Nucl. Acids Res. **15**, 825-837.

Received September 14, 2001