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Research Note

Isolation of *Agrobacterium vitis* from grapevine propagating material by means of PCR after immunocapture cultivation

MARTINA KAUFMANN, H. H. KASSEMEYER and L.OTTEN

K e y w o r d s : Agrobacterium vitis, Vitis vinifera L., crown gall, detection of latent infections, PCR, immunocapture cultivation.

Introduction: Crown gall is the most important bacterial disease of grapevine (*Vitis vinifera* L.) throughout the world. It is caused by *Agrobacterium vitis* (OPHEL and KERR 1990), syn. *Agrobacterium tumefaciens* Smith & Townsend biovar 3 (KERR and PANAGOPOUOS 1977). Pathogenicity is due to the occurrence of large plasmids (pTi). A part of this plasmid (T-DNA) is transferred into the plant nuclear DNA during infection (CHILTON *et al.* 1977). Subsequent expression of T-DNA genes results in an overexpression of auxins and cytokinins, finally leading to plant tumor formation and the production of tumor-specific compounds, so-called opines, that serve as nutrients for *A. vitis*. According to the production of different types of opines, three *A. vitis* strains (octopine, nopaline and vitopine) can be distinguished (SZEGEDI *et al.* 1988).

Transmission of *A. vitis*, that is adapted to living in the vascular system of grapevine plants, occurs by vegetative propagation of infected cuttings (BURR and KATZ 1982, TARBAH and GOODMAN 1986). Since vineyard soils have been excluded as a source of infection with *A. vitis*, spreading of the disease can be prevented by utilization of pathogen-free propagation material (GOODMAN *et al.* 1986, TARBAH and GOODMAN 1986, OPHEL *et al.* 1988, JÄGER *et al.* 1989).

Previous identification of *A. vitis* by cultivation and subsequent examination of the cultures did not lead to reliable results due to the formation of similar cultures by other bacteria. No conclusive statement about the pathogenicity could be drawn (STELLMACH 1991). Evidence for pathogenicity can be obtained by inoculation of *A. vitis* cultures into shoots of *Nicotiana glauca*, leading to tumor formation after 3-6 weeks (MOORE 1989). Recently the use of molecular techniques based on the recognition of one region of the T-DNA, namely colony hybridisation (JÄGER 1988) and PCR (DONG *et al.* 1992, SCHULZ 1993) made the reliable detection of pathogenic strains of *A. vitis* possible. However, due to the occurrence of secondary compounds, the direct detection of *A. vitis* in grapevine extracts is not yet possible. Since cultivation of *A. vitis* from grapevine extracts is hampered by overgrowth of accompanying fungi and bacteria, the purpose of this study was to develop a practical method to detect *A. vitis* in grapevine-propagating material by immunocapture cultivation.

Dr. H. H. KASSEMEYER, Staatliches Weinbauinstitut Freiburg, Merzhauserstraße 119, D-79100 Freiburg, Germany.
Fax: (761) 401 6570.

Materials and methods: M a t e r i a l e x a m i n e d : For detection of *A. vitis* in grapevine propagating material field-grown *Vitis vinifera* cv. Kerner was used. Plants with fleshy tumors, that gave positive results in a previous opine test as well as symptom-less plants, that gave negative results in these tests, were utilized.

E x t r a c t i o n o f p l a n t s a p f r o m g r a p e v i n e c a n e s : The bark on the basal part of freshly cut 2-year-old canes was removed and the canes were washed with 70 % ethanol to reduce surface microflora and air-dried. After discarding the first cut, a 1-2 cm section of the cane was cut with a sterile scalpel into thin slices, put immediately into a mortar containing 4 ml of 0.9 % NaCl, ground with a pestle and pressed out after 10 min with a garlic press.

P r o d u c t i o n o f a n t i s e r u m : A 1 ml aliquot of *A. vitis* strain H16/6 (nopaline type) grown in 3DG-medium (BRISBANE and KERR 1983) was spun down 10 min at 15000 rpm (24,000 *g*), resuspended in 0.9 ml 0.9 % NaCl and mixed with 0.6 ml IFA (incomplete Freund's adjuvant, Sigma, Deisenhofen). Antiserum was raised in rabbits on inoculation with this mixture. The serum was obtained from weekly bleedings and antibodies purified by affinity chromatography with protein A-Sepharose CL-4B (Pharmacia, Freiburg).

I m m u n o c a p t u r e c u l t i v a t i o n : The polyclonal antibodies were diluted 1 : 500 with coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, 0.02 % NaN_3). 1 ml of this solution was filled into a 1-ml reaction tube and incubated at 4 °C overnight. Subsequently the tubes were washed 3 times with 2 ml wash buffer (0.14 M NaCl, 1.47 mM KH_2PO_4, 8.0 mM $NaH_2PO_4 \cdot 12\ H_2O$, 2.7 mM KCl, 0.02 % NaN_3, 0.05 % Tween 20) and once with sterile water. Coated tubes could be stored at -20 °C. The coated tubes were incubated with 1 ml of the extracted plant sap at 4 °C overnight and subsequently washed 3 times with wash buffer and once with sterile water. After addition of 1 ml 3DG-medium, the tubes were incubated at 28 °C for 2-3 d. As negative control, extracted plant sap was added to non-coated tubes and treated in the same way.

D N A i s o l a t i o n : Since DNA isolation from *A. vitis* cultures is a rather time-consuming step, we initiated lysis of the bacteria by heat treatment (SCHULZ 1993): *A. vitis* cultures were spun down for 10 min at 15,000 rpm (24,000 *g*), resuspended in 500 µl PBS (0.14 M NaCl, 1.47 mM KH_2PO_4, 8.0 mM $NaH_2PO_4 \cdot 12\ H_2O$, 2.7 mM KCl, 0.02 % NaN_3) and repelleted. After addition of 20 µl sterile water, the bacterial pellet was resuspended by vortexing. The suspension was transferred to 0.5-ml reaction tubes. The tubes were boiled in a thermocycler (Crocodile III, Appligene) for 10 min at 95.0 °C to lyse bacteria.

After cooling cell debris was spun down for 5-10 min at 7000 rpm (5000 *g*) and 15.5 µl of the clear supernatant was used for PCR.

Polymerase chain reaction: A 693-bp part of the octopine 6 b gene Ti-plasmid-pTiTm4 (L. OTTEN, unpublished, accession number X56185) was amplified using the specific primers 4905(5´GACTTTACTGCTGCT GCCCCGGCGAACTATGCCGAAAGACGGCT 3´, 45 nt) and 4906 (5´GAAGTAAATACAAAGCTATCCCGGGC TTAAGATGACGGTAGCC 3´, 43 nt) using the following reaction mix: 2 µl 10 x reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 9.0), 2 µl tenfold diluted DNA polymerization mix (dATP, dGTP, dCTP, dTTP, each 20 mM, Pharmacia, Freiburg), primer 4905 (50 µM) + primer 4906 (50 µM) each 2 µl, 0.1 µl *Taq* DNA polymerase (5 U/µl, Pharmacia, Freiburg) and 15.5 µl bacterial lysate. Overlaying with mineral oil was not necessary, because the thermocycler was equipped with a heated lid. Sterile water was used as negative control, while 50 ng Tm4-plasmid DNA was the positive control. Temperature cycling was conducted in a Crocodile III thermocycler (Appligene) with the following cycling parameters: 4 min at 94 °C (initial denaturation) followed by 30 cycles each consisting of 45 s at 94 °C (denaturation), 45 s at 55 °C (annealing) and 1 min at 72 °C (extension). Amplification concluded with 4 min at 72 °C (final extension). Amplified PCR products (7 µl aliquots) were analyzed by gel electrophoresis in a 1.5 % agarose gel. As length marker, 1.5 µg of the DNA molecular weight marker VIII (Boehringer Mannheim) was used. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg/ml) for 1 min. Bands were visualized with a UV transilluminator (312 nm) and the gels were photographed.

Results and discussion: After extraction of plant sap from grapevine canes and immunocapture cultivation, the cultures showed an optical density (660 nm) ranging from 0.1 to 0.3 after 2-3 d. In negative controls, no growth of bacteria had taken place. Using these immunocapture cultures, *A. vitis* (octopine-type) could be detected by PCR amplification of a 693-bp fragment of the Ti-plasmid-coded 6b gene in plants with fleshy tumors (that had given positive results in a previous opine test). Similary, a PCR product was detected at the first attempt in those symptom-less plants (that gave negative results in opine test) that were, because of their origin, expected to be infected with *A. vitis* (Figure).

Using common cultivation methods, such as plating aliquots of the extracted plant sap on 3DG-medium, and applying PCR either to a single colony, suspected to have been formed by *A. vitis*, or to the whole bacterial flora, it was often not possible to detect *A. vitis* in infected plants at the first attempt and several repetitions were necessary. The use of immunocapture cultivation appears to be more successful for the detection of *A. vitis* in grapevine-propagating material and hence for disease control.

However, before this method can be used routinely, several further studies need to be undertaken to determine reliably the sanitary status of the propagating material. For instance the optimal time during the vegetation period and the sampling location must be examined. To improve immunocapture cultivation, the production of new polyclonal antibodies using a mixture of several *A. vitis* strains from different origins should be carried out. The recognition of all possible *A. vitis* strains by these antibodies should be determined by Western blot analysis. Finally, new primers could be designed for use in the form of a "primer cocktail", leading to amplification products of different lengths for each opine type to detect all possible types in a single reaction. Although this work does not yet provide a "ready for use test", it represents a major step in reducing the importance of crown gall as a universal bacterial disease of grapevine (*Vitis vinifera* L.).

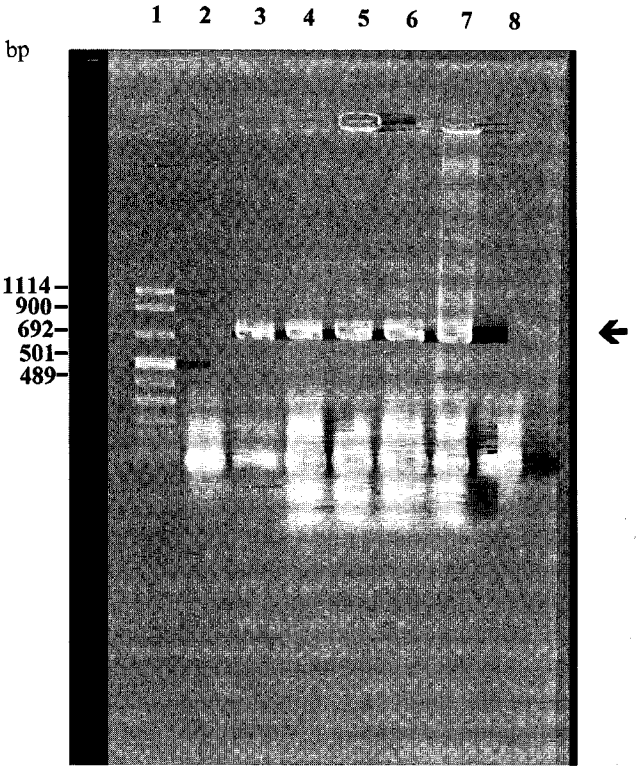

Figure: Detection of *A. vitis* (octopine type) in *Vitis vinifera* by means of PCR after immunocapture cultivation; agarose gel electrophoresis of PCR products. The arrow indicates the amplified product of 693 bp. Lanes: 1 - DNA molecular weight marker VIII (Boehringer Mannheim; 1,5 µg); 2 - negative control (H_20); 3 - positive control (Tm4-plasmid DNA); 4 - 4/17 (extract from *V. vinifera* cv. Kerner with tumors, octopine-type), single colony; 5 - 8/18 (extract from *V. vinifera* cv. Kerner with tumors, octopine-type), whole bacterial flora; 6 - 8/5 (extract from *V. vinifera* cv. Kerner with tumors, octopine-type), immuno-capture culture; 7-18/16 (extract from *V. vinifera* cv. Kerner symptomless) immunocapture culture; 8 - immunocapture culture, negative control (non-coated tubes).

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