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# Opines in naturally infected grapevine crown gall tumors

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## Summary

**Crown gall tumors collected from naturally infected plants of 7 grapevine varieties were analysed for the presence of opines. Eighty-five of the tested 90 samples contained known *Agrobacterium vitis*-induced opines. Octopine was the most common, it was found in 50 samples. Twenty-eight crown galls contained nopaline and 8 were vitopine positive. There was only one tumor that contained two opines, nopaline and vitopine. Five samples were negative for the opines tested. The presence of *A. vitis* was confirmed in most of these tumors by PCR analysis. The opine assay may provide a simple diagnostic protocol to distinguish healthy callus tissues from crown galls as well as for the indirect identification of *Agrobacterium* infection.**

K e y w o r d s : *Agrobacterium vitis*, *Vitis vinifera*, octopine, nopaline, vitopine, polymerase chain reaction.

## Introduction

Grapevine crown gall, caused by *Agrobacterium vitis* and occassionally by *A. tumefaciens* is a common disease of grapevine in several countries (BURR *et al*. 1998, BURR and OTTEN 1999). Crown gall tumors produce specific amino acid derivatives, called opines, determined by the inducing bacterium. These compounds selectively promote the distribution of the inducing bacteria and their Ti plasmids in nature (DESSAUX *et al*. 1992, 1998). *A. vitis* isolates have been classified into octopine-, nopaline- and vitopine groups on the basis of their opine markers (PAULUS *et al*. 1989). Approximately 60-75 % of isolates belong to the octopine group, nopaline-type strains occur at a frequency of about 20-30 %, while vitopine-type *A. vitis* represent 5-10 % of grapevine isolates (BURR *et al*. 1998, RIDÉ *et al*. 2000). Octopine and nopaline are condensation products of arginine and pyruvate, and arginine and α-ketoglutarate, respectively (DESSAUX *et al*. 1993), while vitopine is a conjugate of glutamine and pyruvate (CHILTON *et al*. 2001). Octopine- and nopaline type Ti plasmids are common in the genus *Agrobacterium*, but vitopine Ti plasmids have exclusively been found in *A. vitis* (grapevine) isolates.

Most opine studies have been carried out using pure bacterial cultures inoculated onto test plants to induce crown gall and opine synthesis. Little is known about the occurrence and distribution of opines in tumors derived from natural infections. MOORE *et al*. (1997) analysed 7 aerial grapevine galls, one of which contained nopaline and 6 tumors contained a silver-chelating compound that was similar to (or identical with) vitopine. We have tested 90 crown gall tumors collected from 5 distinct grape growing regions in Hungary to get an insight into the natural occurrence of opines in grapes.

## Material and Methods

P l a n t m a t e r i a l : Samples showing the characteristic symptoms of crown gall tumors were collected in July and August 2002 from aerial, wooden parts of grapevines (7 various cultivars) from 5 distinct regions. Samples were freshly analysed or occasionally stored at -20 °C for no longer than 2-3 weeks until analysis.

O p i n e a n a l y s i s : Approximately 100 mg of tumor tissue was homogenized in 200 µl distilled water and centrifuged at 5,000 *g* for 5 min; 9 µl of the sample was spotted onto Whatmann 3 MM paper in 3 µl aliquots and in two replicates. Electrophoretic separation was carried out in formic acid/acetic acid/water (3:6:91, v/v/v) buffer. Papers were completely dried after electrophoresis and then stained with phenantrenequinone to detect octopine and nopaline (OTTEN and SCHILPEROORT 1978) or with silver-nitrate/alkaline glucose reagent to detect vitopine (SZEGEDI *et al*. 1988). The detection limit for the phenantrenequinone positive opines is 80 ng (SZEGEDI *et al*. 1989) and they were reproducibly identified from 2-3 µl samples prepared as described above (data not shown). The minimal detectable amount of the silver-chelating vitopine is about 5 µg (unpubl.).

D N A e x t r a c t i o n a n d P C R a n a l y s i s : Total DNA containing plant and bacterial nucleic acids were purified with the CTAB protocol. A 100-120 mg piece of tumor sample was frozen in liquid nitrogen then homogenized in 1 ml lysis buffer containing 2 % (w/v) CTAB, 200 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1 % (w/v) polyvinylpyrrolidone and 1 % β-mercaptoethanol (STEIN *et al*. 2001) and incubated at 65 °C for 40 min. After centrifugation at 6,000 *g* for 5 min the supernatant was transferred to a new tube and extracted with one volume chloroform:isoamyl-alcohol (24:1) followed by centrifugation. A 0.5 ml volume of the supernatant was transferred to a new tube and nucleic acids were precipitated with 0.4 ml isopropanol at room temperature. DNA was pelleted by centifugation at 8,000 *g* for 5 min and washed twice with 1 ml of 70 % ethanol. Finally, the dried pellet was redissolved in 100 µl sterile water and stored at -20 °C until use; 1 µl of this sample was used for PCR reactions carried out with the PGF and PGR primers generating a 466 bp fragment as previously described

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(SZEGEDI and BOTTKA 2002). This primer pair is specific for the polygalacturonase-gene located on the bacterial chromosome (HERLACHE *et al*. 1997) and detects any *A. vitis* strains irrespective of their virulence.

## Results and Discussion

Ninety grapevine crown gall tumors collected from 7 grapevine varieties grown in 5 distinct regions were assayed for opines (Table). Eighty-five samples contained opines described in *A. vitis*-induced tumors (SZEGEDI *et al*. 1988, PAULUS *et al*. 1989, RIDÉ *et al*. 2000). Of these 50 (58.8 % of the opine positive samples) were octopine positive, 28 (32.9 %) were nopaline positive and 8 (9.4 %) were vitopine positive. Almost all of these tumors derived from natural infections contained one single opine. There was only one tumor which produced two opines, nopaline and vitopine (Fig. 1, lane 10). The observed frequencies in the distribution of octopine, nopaline and vitopine are in agreement with previously published data obtained with pure cultures of isolated *A. vitis* strains on test plants (BURR *et al*. 1998, RIDÉ *et al*2000). Five samples did not contain any detectable opines. They might have been non-tumorous callus tissues caused by natural or mechanical wounding, or they were induced by agrobacteria belonging to rare opine groups.

All of the Cabernet Sauvignon, Lakhegyi mézes and Teréz crown galls uniformly contained octopine. Similarly, only nopaline was detectable in the 12 Kármin tumor samples. Further studies should be carried out to find out if the given type of agrobacteria prefer these varieties, or if natu-

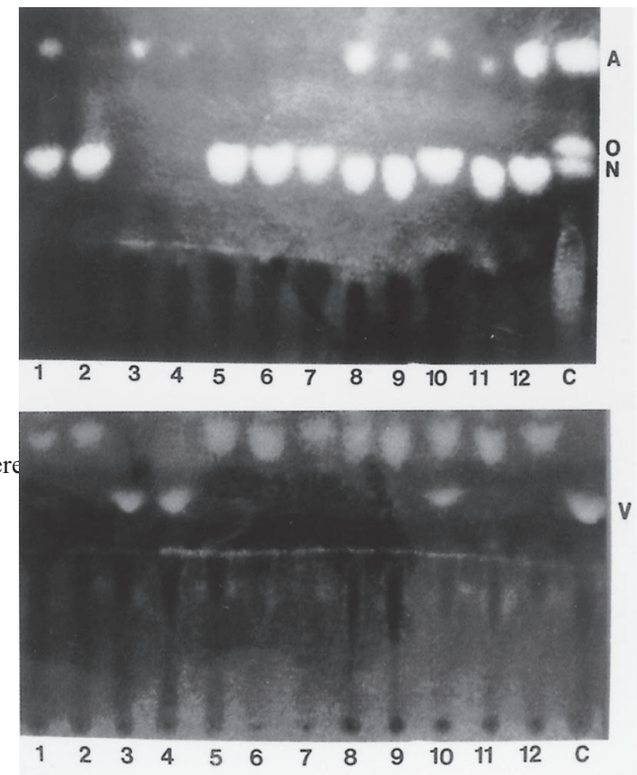


Fig. 1: Opine and PCR analysis of 12 crown galls collected from cv. Ezerfürtû. Upper panel: Phenantrenequinone-stained paper that detects nopaline in samples 1, 2 and 5-12. Lane C: 2µg each of pure arginine (A), octopine (O) and nopaline (N). Lower panel: Silver-nitrate/alkaline glucose-stained paper showing the presence of vitopine in samples 3, 4 and 10. Lane C: standard vitopine (V) tumor sample induced on tobacco with *Agrobacterium vitis* S4. Lanes 1-12 are identical tumor samples in both panels.

T a b l e

Opine content and detection of *Agrobacterium vitis* in naturally infected grapevine tumors

| Grapevine variety (site)* | Number of tested samples | Sum of opine + tumors | Oct + | Nop + | Vit + | Opine - | Sum of A. vitis (PCR) + tumors | Opine + PCR + | Opine - PCR + | Opine + PCR - | Opine - PCR - |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cabernet Sauvignon (A) | 6 | 6 | 6 | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 0 |
| Merlot (A) | 12 | 10 | 5 | 0 | 5 | 2 | 10 | 10 | 0 | 0 | 2 |
| Ezerfürtü (B) | 12 | 12 | 0 | 10** | 3** | 0 | 11 | 11 | 0 | 1 | 0 |
| Kármin (C) | 12 | 12 | 0 | 12 | 0 | 0 | 12 | 12 | 0 | 0 | 0 |
| Ezerjó (C) | 12 | 11 | 9 | 2 | 0 | 1 | 12 | 11 | 1 | 0 | 0 |
| Cabernet franc (C) | 12 | 12 | 8 | 4 | 0 | 0 | 11 | 11 | 0 | 1 | 0 |
| Lakhegyi mézes (D) | 12 | 12 | 12 | 0 | 0 | 0 | 9 | 9 | 0 | 3 | 0 |
| Teréz (E) | 12 | 10 | 10 | 0 | 0 | 2 | 11 | 9 | 2 | 1 | 0 |
| Sum (%)*** | 90 (100) | 85 (94.4) | 50 (55.5) | 28 (31.1) | 8 (8.8) | 5 (5.5) | 79 (87.7) | 76 (84.4) | 3 (3.3) | 9 (10) | 2 (2.2) |

* Identical capitals mean the same vineyard site (5 distinct regions A....E).
** One tumor contained both nopaline and vitopine.
*** Percentages were calculated from total number of samples.
Abbreviations: oct = octopine, nop = nopaline and vit = vitopine.

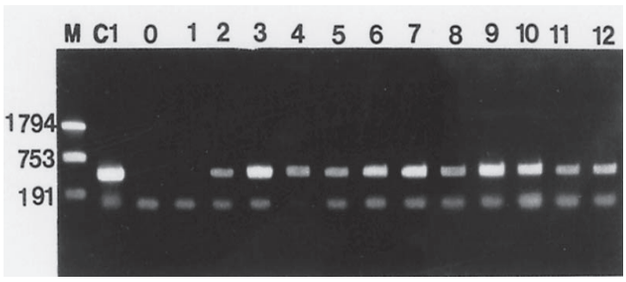


Fig. 2: Detection of the *Agrobacterium vitis* specific sequence (466 bp) by PCR in the DNA preparations of cv. Ezerfürtü crown galls. Lane M: size markers (in base pair), lane C1: *A. vitis* AB3 DNA used as control, lane 0: DNA-free sample. Lanes 1-12: tumor DNA samples identical with those shown in Fig. 1.

ral infections have a common origin. Tumors of cvs Merlot and Ezerfürtü (Fig. 1) contained octopine or vitopine, while the Ezerjó and the Cabernet franc crown galls produced octopine or nopaline (Table).

To show the presence of *A. vitis* in the tested tumors, total DNA samples were assayed by PCR using a polygalacturonase specific primer pair (Szegedi and Bottka 2002). Using this primer we could amplify the specific fragment in most, but not in all samples (Fig. 2). *A. vitis* was detected in 79 (87.7 %) of the 90 DNA preparations. The correlation between biochemical (opine) and molecular (PCR) assays was shown in 76 tumors (84.4 % of total sample number). Nine opine positive crown galls did not respond to PCR. These unexpected negative PCR results were possibly caused by remaining polysaccharide contamination in DNA preparations which inhibit the *Taq* polymerase, but the potential occurrence of *A. tumefaciens* which cannot be detected by the primers used here should also be considered. Three plant extracts did not contain detectable opine but their DNA preparations were positive in PCR. Since the primer pair used is specific for the chromosomally encoded polygalacturonase gene that does not directly contribute to the virulence of *A. vitis* (Herlache *et al*. 1997), these positive results have probably been caused by the occurrence of non-virulent bacterial cells. Two samples that morphologically resembled crown gall tumors were negative in both tests (Table) showing that they derived from wound callus formation.

The application of opine-tests provides a simple and rapid diagnostic method to test plant material on a large scale for the presence of *Agrobacterium* and to distinguish crown gall tumors from healthy callus tissues formed after natural or mechanical woundings. Calli of various sizes are frequently formed at fresh wounds, *e.g.* on graftings at the basal part, at the nodes due to disbudding and at the grafting junction. Damage of phloem inhibits auxin translocation that may result in a hormone accumulation just above the wound. This usually led to an intensive callus formation resembling crown gall. The presence or absence of opines in these calli clearly indicate if they are crown gall tumors or healthy tissues. On the other hand, this simple protocol can also help to determine the origin of infection that has recently been shown by comprehensive studies using isolated *Agrobacterium* strains (Pionnat *et al*. 1999).

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