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Opines in crown gall tumours induced by biotype 3 isolates of *Agrobacterium tumefaciens*

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Pathogenic properties and opine markers of biotype 3 (grapevine) isolates of *Agrobacterium tumefaciens* (Smith and Townsend) Conn were compared with those of biotypes 1 and 2. By contrast with biotypes 1 and 2, grapevine isolates induced only small tumours on *Kalanchoe daigremontiana* Hamet and Perrier stems but produced large organogenic galls on decapitated shoot tips of this plant species. Grapevine agrobacteria were classified according to their opine catabolic properties and the type of opine they induced in *Kalanchoe tubiflora* Hamet and grapevine (*Vitis vinifera* L.) tumours. Crown gall tissues obtained after infection with octopine-utilizing grapevine isolates contained octopine in very large amounts compared to biotype 1 tumours. A similar quantitative difference was not observed when nopaline production of tumours incited by biotypes 1 and 2 and by grapevine isolates was examined. Tumours formed by a third class of grapevine isolates synthesized a novel opine, for which we propose the trivial name vitopine. Octopine, nopaline, agropine and vitopine were also detected in healthy tissues of plants infected with the appropriate *Agrobacterium* isolates, indicating that these opines can selectively promote the colonization of the whole plant by agrobacteria. Biotype 3 octopine isolates induced octopine synthesis at the infection sites of a crown gall-resistant grapevine hybrid, showing that pathogenic agrobacteria can transform plants without tumour formation.

INTRODUCTION

Transformation of higher plants by *Agrobacterium tumefaciens* results in two basic physiological changes: tumour formation and opine synthesis. Tumour formation is caused by

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abnormal hormonal balance which is a consequence of T-DNA (transferred DNA of Ti-plasmids) encoded phytohormone synthesis [*25*]. The opines, synthesized by crown gall tissues, are secreted by the transformed cells [*23,24*] and selectively utilized by the inciting bacteria [*30*]. Until now several opine classes have been described [*5, 30*]. Tumour tissues generally produce two types of these compounds, i.e. catabolic opines, occurring in relatively large amounts and, less abundantly, conjugative opines. The catabolic opines are utilized as carbon, nitrogen and energy sources, while the conjugative opines, besides their utilization, promote the Ti-plasmid transfer from pathogenic agrobacteria to non-pathogenic ones. For example, tumours induced by nopaline isolates of *A. tumefaciens* produce nopaline (catabolic opine) and agrocinopine A (conjugative opine). Agrobacteria inducing the synthesis of the catabolic opines agropine and mannopine can be divided into two groups according to their conjugative opines: octopine and agrocinopine C, respectively [*37*]. In this way these compounds play a specific role in nature in selective maintenance and propagation of Ti-plasmids [*30*].

Agrobacterium isolates can be divided into three biotypes according to their chromosomal markers [*18,33*]. The biotype classification does not correlate with classification based on the Ti-plasmid encoded opine markers. Of the three types, biotype 3 is associated with grapevines all over the world [*3, 22, 29, 36*]. Until now only the isolates of biotype 3 with Ti-plasmids inducing octopine producing tumours have been extensively studied. There are several basic differences between biotypes 1 and 3 octopine Ti-plasmids, including their host range [*38*], virulence [*28, 41*] and oncogenes [*2, 16, 41*], T-DNA organization [*1, 21, 40*] and the substrate range of octopine-synthase [*27*]. Furthermore, agropine and mannopine are not present in tumours of biotype 3 agrobacteria belonging to the octopine class [*21, 30*] but they contain a novel, as yet uncharacterized, opine reacting with the Pauly-reagent [*30*]. Nopaline utilizing strains belonging to biotype 3 have also been isolated [*6, 20, 29, 34*]. Nine of 44 Hungarian biotype 3 isolates did not utilize either octopine or nopaline [*34*], suggesting that this biotype is heterogeneous.

In this report we publish a comparison of biotype 3 with the well-known biotypes 1 and 2 on the basis of their opine markers. The occurence of a new opine in tumours induced by certain grapevine isolates is also described.

MATERIALS AND METHODS

Bacterial isolates

Isolates used in this work (Table 1) were maintained on YE-agar (1% glucose, 0·5% yeast-extract) supplemented with 0·5% $CaCO_3$ (pH 7·0–7·2) at 4 °C. For all experiments, 2-day-old cultures grown on the same medium at 25 °C were used.

Plant material and pathogenicity tests

Plants were kept in a glasshouse at 23–28 °C under natural light conditions. *Agrobacterium* isolates were tested on *Vitis vinifera* L. cv. Narancsizü, *Kalanchoe daigremontiana* Hamet and Perrier and on *Kalanchoe tubiflora* Hamet. For biotype 1 octopine and biotype 2 succinamopine isolates, which were not pathogenic on *V. vinifera* cv. Narancsizü (unpubl. obs.), *V. vinifera* cv. Pearl of Zala was used. *Agrobacterium* isolates representing each biotype and opine group were tested on the grapevine hybrid A4/42 which showed resistance to *A. timefaciens* AT-1 [*35*].

Pathogenicity tests were carried out as described earlier [*34, 35*] but *K. tubiflora* plants were kept at a lower temperature (18–22 °C) for a week following infection because the tumour induction process by biotype 3 isolates is thermosensitive on this species [*6*].

In vitro *tumour induction*

Decapitated shoot tips of sterile *V. vinifera* cv. Narancsizü, *Nicotiana plumbaginifolia* Viviani and *Solanum aviculare* Forst plants were inoculated with the appropriate *A. tumefaciens* isolates basically as described by Hemstad & Reisch [*15*]. After incubation for 4 weeks, the tumours were subcultured three times, at 3-week intervals, on hormone-free B-5 medium [*13*] supplemented with 500 μg ml^{-1} carbenicillin to obtain bacterium-free tissues. The tumours were then transferred onto the same medium without carbenicillin and used for opine assays. Non-transformed calluses were also initiated from stem segments of this grapevine variety on B5 medium containing 1·0 μg ml^{-1} naphthaleneacetic acid and 0·1 μg ml^{-1} benzyladenine.

Detection of opines

All isolates were screened on grapevine and on *K. tubiflora*. For opine tests approximately 50 mg of tumour tissue sample was homogenized in 50 μl of distilled water in an Eppendorf-tube and centrifuged for 3 min; 4 μl of the supernatant was used for paper electrophoretic separation of opines for which the formic acid/acetic acid buffer (pH 1·6) was used as in earlier experiments [*26*]. The pH 2·8 buffer was prepared by titrating 0·1 M acetic acid with NaOH. The pH 9·2 buffer was prepared by titrating 5 g l^{-1} NH_4CO_3 with NH_4OH.

Octopine and nopaline were detected as described earlier [*26*]. For their identification pure compounds (from Calbiochem AG) were used as standards. Agropine and mannopine were identified by their specific staining with alkaline silver-nitrate reagent as used by Czakó & Márton [*11*], by their relative electrophoretic mobilities according to Petit *et al.* [*31*], and compared with tumour samples of authentic agropine and mannopine inducing reference strains Ach-5 and B-6 [*30*]. For the Pauly-reagent (diazotized sulphanilic acid [*39*]) positive opine, tumours of *A. tumefaciens* K 305 and K 308 were used as authentic sources ([*30*], M. E. Tate, pers. comm.). For reversed silver nitrate staining [*8*] the dried papers were dipped into 0.01% solution of acetone in glucose (200 μl of 5% glucose in distilled water was added to 100 ml of acetone) and dried before the silver-nitrate staining procedure.

Opine utilization

Octopine and nopaline utilization has been described [*34*]. For the unknown compound the method of Chilton *et al.* [*8*], basically unmodified was used. Ten grams of crown gall tissue induced by isolate S-4 on *K. tubiflora* was homogenized in 30 ml of AB minimal medium [*7*], supplemented with 0·2% yeast-extract and centrifuged. The pH of the tumour extract was adjusted to 7·2 with 3% KOH and the extract was filter-sterilized. A 1-ml sample was inoculated with 10 μl of bacterial suspension (10^8 cells ml^{-1} in 0·8% sterile NaCl solution) and incubated for 4 days at 25 °C. A 50-μl portion of the incubation mixture was centrifuged and subjected to paper electrophoresis.

TABLE 1
List of bacterial isolates and their relevant characteristics

Agrobacterium tumefaciens	Biotype	Characteristic opine markers[a]	Source[b]
Ach-5	1	Octopine,agropine,	L.M.
B-6		mannopine [*30*, *T.W.*]	S.S.
OD-50			L.B.N.
B-23			I.M.
C-58	1	Nopaline,	L.M.
T-37		agrocinopine A [*30*]	L.M.
A 281	1	L,L-Leucinopine L,L-succinamopine, agropine, mannopine, agrocinopine C [*5*, *9*, *10*, *30*, *32*]	M.-D.C.
1	2	Nopaline [*33*, *T.W.*]	S.S.
7			S.S
Eu6	2	D.L-succinamopine	S.S.
AT-181		[*4*, *8*]	S.S.
AT-6	3	Octopine,	J.L.
Hm-1		Pauly-reagent positive	GI
Tm-4		opine [*27*, *30*, *34*, *T.W.*]	GI
AB-3			GI
Zw-2			GI
B-10/7			GI
K 305			M.R.
K 308			M.R.
AT-1	3	Nopaline [*34*, *T.W.*]	J.L.
AT-66			J.L.
NI-1			GI
AB-4			GI
S-4	3	Vitopine [*34*, *T.W.*]	GI
Sz-1			GI
Sz-2			GI

[a]References are given in brackets; *T.W.* = this work.
[b]L.M. = L. Márton (Szeged, Hungary), S.S. = S. Süle (Budapest, Hungary), L.B.N. = L. B. Nicolaevna (Odessa, U.S.S.R.), I.M. = I. Malenin (Pleven, Bulgaria), M.-D.C. = M.-D. Chilton (Ciba-Geigy Co., Research Triangle Park, U.S.A.), J.L. = J. Lechoczky (Budapest Hungary), M.R. = M. Ryder, (Glen Osmond, Australia) and GI = grapevine isolates of our laboratory (Kecskemét).

RESULTS

Pathogenicity and tumour morphology

All isolates listed in Table 1 were pathogenic on *K. tubiflora* stems where they induced indifferentiated tumours. Biotype 3 isolates incited slow growing tumours on *K. daigremontiana* stems in only a proportion of inoculation sites. On the other hand, these isolates generally induced large organogenic tumours on decapitated *K. daigremontiana* shoot tips. The morphology of these tumours was basically different from those induced by biotypes 1 and 2 since they developed column-like organs (nopaline isolates) or leaves

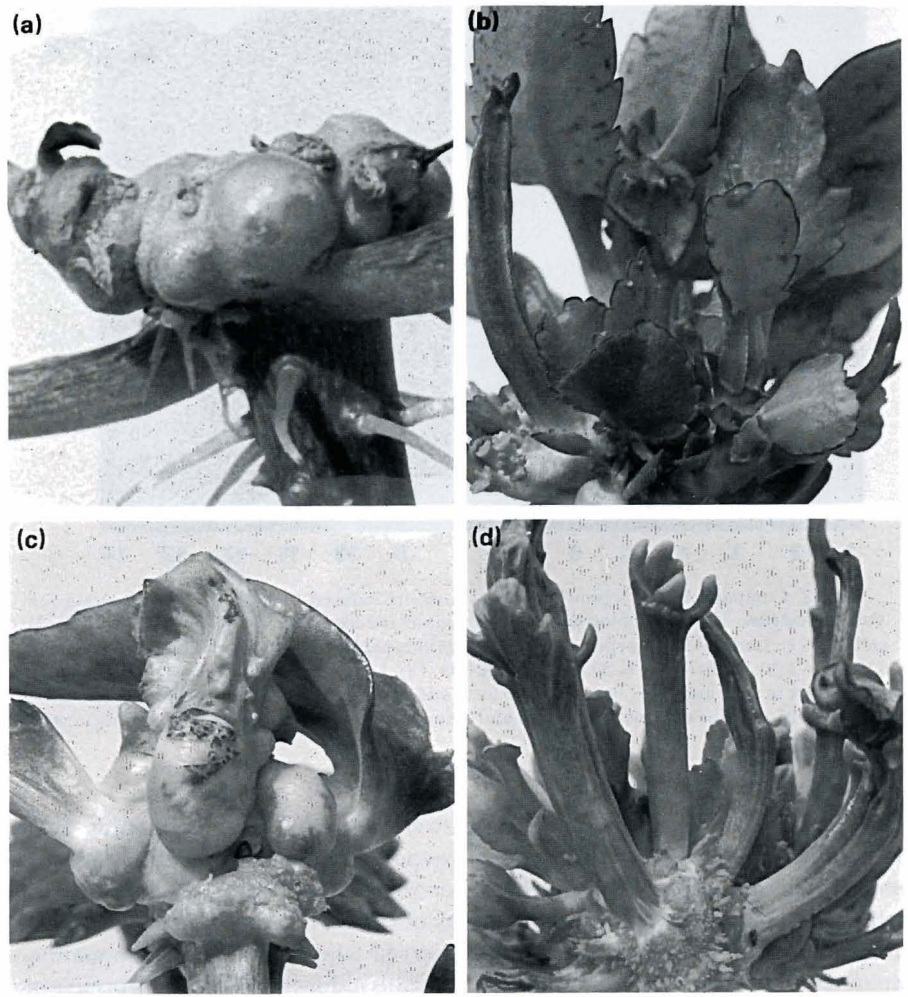

FIG. 1. Morphological differences of *Kalanchoe daigremontiana* shoot tip tumours induced by biotype 1 and 3 isolates. Plants infected with *Agrobacterium tumefaciens* B-6 (a), Tm-4 (b), T-37 (c) and AT-66 (d) were photographed 2 months after infection.

(octopine isolates, see Fig. 1). Organ differentiation was never observed on grapevine tumours.

The A4/42 grapevine hybrid, in common with hybrids from other crosses between *Vitis amurensis* Ruprecht with *Vitis vinifera* showed resistance to biotype 1 octopine isolates Ach-5 and B-6, biotype 1 nopaline isolate C-58, biotype 2 nopaline isolates 1 and 7, and to our biotype 3 isolates AT-1 [*35*], AT-66, Tm-4 [Fig. 3(a)] AB-3, S-4 and Sz-1.

Opine assays

Tumours of biotype 1 octopine isolates OD-50 and B-23 contained agropine and mannopine in high levels similar to the tumours of isolates Ach-5 and B-6. Galls induced

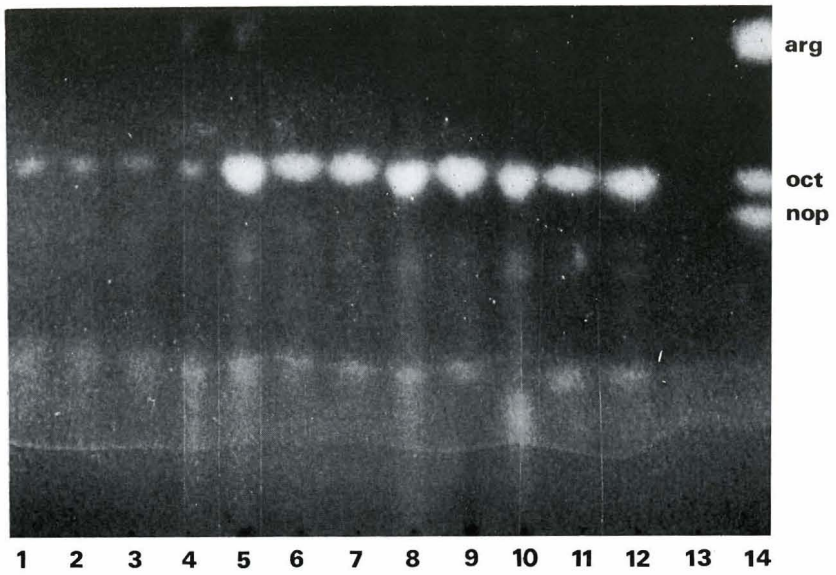

FIG. 2. Comparison of octopine content of tumours induced by biotype 1 and 3 strains on *Kalanchoe tubiflora*. Lanes 1–4: biotype 1 tumour samples induced by Ach-5 (1), B-6 (2), OD-50 (3) and B-23 (4). Lanes 5–12: biotype 3 tumour extracts induced by AT-6 (5), Hm-1 (6), Tm-4 (7), AB-3 (8), Zw-2 (9), B-10/7 (10), K 305 (11) and K 308 (12). Lane 13: non-infected plant extract; lane 14: mixture of pure arginine (arg), octopine (oct) and nopaline (nop) standards. The quantitative difference in octopine content of biotype 1 and 3 tumours was approximately 20-fold (determined by dilution).

by Hungarian biotype 3 octopine isolates were agropine and mannopine negative and contained the Pauly-positive opine corresponding to earlier findings [*21, 30*]. All tumours induced by these grapevine agrobacteria contained large amounts of octopine which was present only in traces in tumours of the biotype 1 octopine class (Fig. 2). Similar quantitative differences were not observed when the amount of nopaline was compared in grapevine and in *K. tubiflora* crown galls induced by isolates, representing all three biotypes.

Tissues surrounding the infection sites of the crown gall-resistant A4/42 grapevine hybrid inoculated with biotype 3 octopine and nopaline isolates were also analysed. Nopaline was not detectable in the wound callus formed at the infection sites, but the presence of octopine was clearly demonstrated in these tissue extracts [Fig. 3(b)].

When non-tumorous stem samples of *K. tubiflora* plants infected with biotype 3 octopine isolates were analysed octopine was clearly detectable in the healthy parts of the infected plants two months after their inoculation (Fig. 4). Agropine, mannopine and nopaline, respectively, were also present not only in tumours but also in the healthy stem parts of plants infected with the appropriate *A. tumefaciens* isolates.

The opine marker of the novel group

In tumours induced by S-4, Sz-1 and Sz-2 isolates no known opines were detectable. By reversed silver-nitrate staining a new specific substance was found both in grapevine and

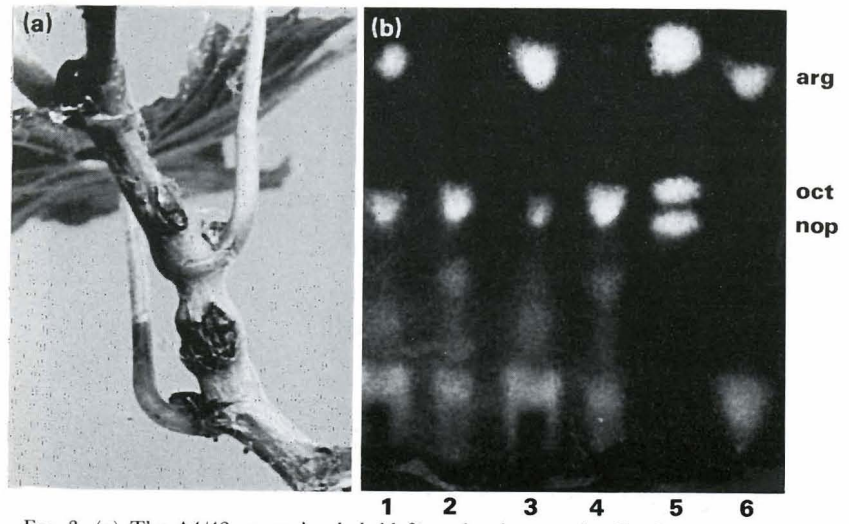

FIG. 3. (a) The A4/42 grapevine hybrid formed only wound callus but no tumour tissue after infection with *Agrobacterium tumefaciens* (Tm-4 infected plant 2 months after inoculation). (b) Detection of octopine in the wound callus formed at the infection sites of the crown gall-resistant A4/42 hybrid. Lanes 1 and 3: wound callus extracts (20 µl) of AB-3 and Tm-4 infected plants, respectively. Lanes 2 and 4: AB-3 (2) and Tm-4 (4) tumour samples (4 µl) from *Vitis vinifera* cv. Narancsizü. Lane 5: mixture of pure arginine (arg), octopine (oct) and nopaline (nop) standards. Lane 6: non-inoculated A4/42 stem extract (20 µl).

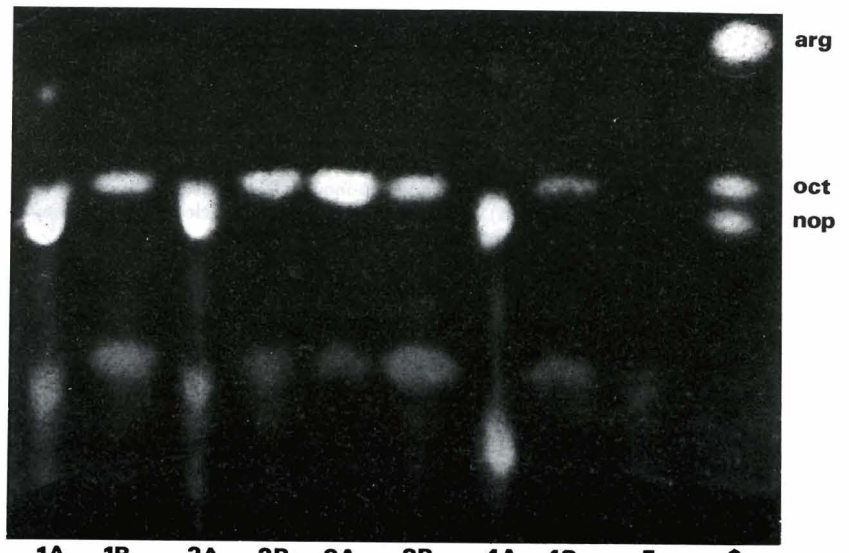

FIG. 4. Presence of octopine in healthy tissues of infected *Kalanchoe tubiflora* plants. Lanes 1A, 2A, 3A and 4A show extracts (4 µl) of tumour tissues induced by *Agrobacterium tumefaciens* AT-6, AB-3, Zw-2 and B-10/7, respectively. Lanes 1B, 2B, 3B and 4B show extracts (12 µl) from non-transformed stem parts of plants infected with AT-6 (1B), AB-3 (2B), Zw-2 (3B) and B-10/7 (4B). Lane 5: 12 µl sample from non-inoculated plant. Lane 6: mixture of pure arginine (arg), octopine (oct) and nopaline (nop) standards.

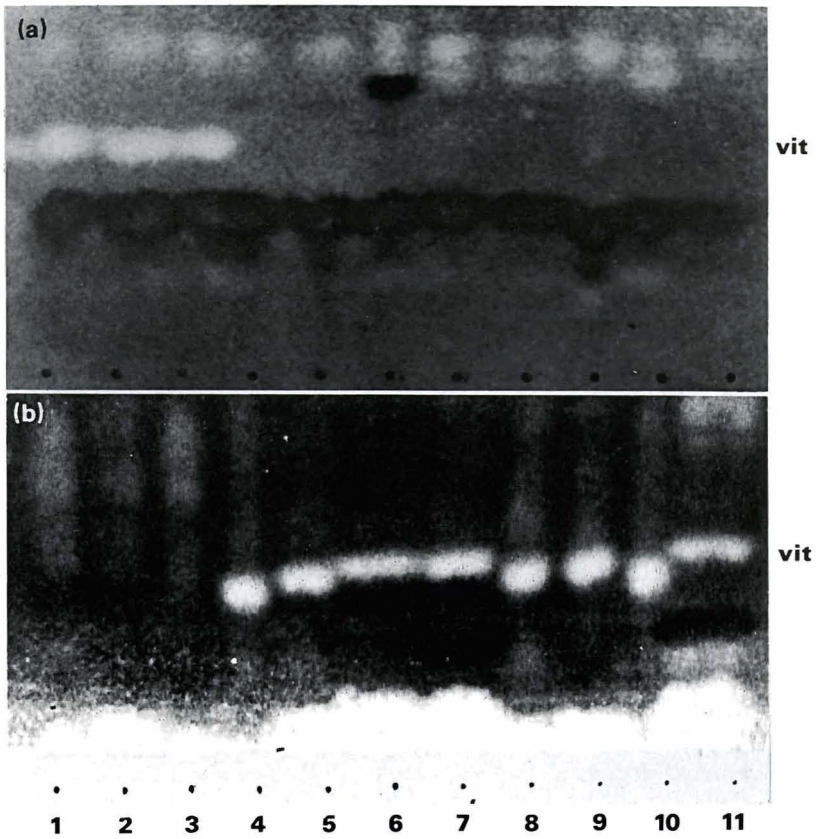

FIG. 5. (a) Detection of the new opine in extracts of primary tumours induced on *Kalanchoe tubiflora*. (b) Utilization of the new opine from S-4 tumour extract by various *Agrobacterium tumefaciens* isolates. Lanes 1–11: inoculations with *A. tumefaciens* S-4 (1), Sz-1 (2), Sz-2 (3), Eu6 (4), AT-181 (5), Ach-5 (6), C-58 (7), 1 (8), AB-3 (9), AT-1 (10) and non-inoculated sample (11). Succinamopine in Eu6 and AT-181 tumour samples [on (a)] was not detectable under these electrophoretic conditions (pH 1·6) because of interference with reducing substances. vit = vitopine.

in *K. tubiflora* crown galls, which was not present in non-infected plant extracts nor in tumours incited by isolates inducing known opines [Fig. 5(a)]. This compound was also readily detectable in healthy stem tissues of *K. tubiflora* plants infected with these agrobacteria indicating that it is secreted and translocated in plants. Sterile crown gall tissues induced by *A. tumefaciens* S-4, Sz-1 and Sz-2 on *V. vinifera* cv. Narancsizü also contained the new specific substance but it was not present in non-transformed calluses and shoots of this grapevine variety. Bacterium-free tumour lines of *N. plumbaginifolia* and *S. aviculare* synthesizing the new compound were also successfully established. The opine nature of this substance was proved by assaying its catabolism. Isolates S-4, Sz-1 and Sz-2 catabolized the new opine completely while it remained non-degraded in samples inoculated with isolates inducing known opines [Fig. 5(b)].

The new opine is cationic at pH 1·6 but migrates toward the anode at pH 2·8. Its relative electrophoretic mobilities to picrate anion are −0·32 at pH 1·6, +0·52 at pH 2·8, and +0·76 at pH 9·2. Compared with succinamopine and leucinopine (mixtures of synthetic D,L- and L,L-diastereomers) as standards, the relative electrophoretic mobilities of this novel opine at pH 1·6 are 1·40 and 1·08, respectively, showing that it is not identical with the similarly detectable known opines [*8, 9*]. This fact was further confirmed by assaying these isolates for leucinopine utilization in comparison to reference strain A281 [*9*]. Only strain A281 grew on minimal medium (AB) containing 0·2% leucinopine as carbon source, but isolates S-4, Sz-1 and Sz-2 did not.

DISCUSSION

We present further evidence that the oncogenic properties of biotype 3 (grapevine) isolates of agrobacteria are different from those belonging to biotypes 1 and 2 [*2, 16, 41*]. Biotype 3 isolates were only weakly pathogenic on *K. daigremontiana* stems in contrast to biotypes 1 and 2. On the other hand, they induced large shooty tumours on decapitated shoot tips of this plant species with a morphology different from that of crown galls of biotypes 1 and 2. The genetic and physiological background of this phenomenon is unknown. The observed morphology might be due to the effect of new types of oncogenes in these strains.

As described earlier [*1, 2, 16, 21, 27, 28, 38, 40, 41*], the biotype 3 octopine isolates are basically different from the corresponding biotype 1 agrobacteria. Here we present an extension of those results by showing that tumours induced by grapevine isolates of the octopine class contain large amounts of octopine in contrast to crown galls induced by the well-known biotype 1 agrobacteria. On the other hand, octopine does not induce the conjugative activity of Ti-plasmids of biotype 3 isolates ([*30*]; Otten, unpublished results), and therefore seems to play exclusively a catabolic role in this particular *Agrobacterium*–plant interaction.

Quantitative differences in nopaline contents were not observed in tumours produced by isolates of biotypes 1, 2 and 3 known to induce this substance. Up to now, three properties are known in which the Ti-plasmids of grapevine isolates inducing nopaline differ from those of biotype 1 and 2 agrobacteria, i.e. agrocin 84 resistance ([*20, 29*] and our unpublished observations), host range [*19, 20, 34*] and the particular tumour morphology on *K. daigremontiana* shoot tips.

Three isolates S-4, Sz-1 and Sz-2 which could not be allocated to the known opine classes induced the synthesis of a new specific reversed $AgNO_3$ positive compound. Since it fulfils the opine criteria (specific production by the transformed plant tissues and selective degradation by the inciting *A. tumefaciens* isolates, (see [*30, 37*]) this compound can be considered as a novel opine. We propose the trivial name vitopine for the novel opine, since it is induced by agrobacteria isolated from grapevine (*Vitis vinifera*).

The presence of opines in healthy parts of infected plants shows that opines are not only secreted [*23, 24*] but also translocated in plants. Thus, these crown gall-specific compounds can selectively promote the growth of pathogenic agrobacteria in the whole plant since these pathogens invade their host systemically [*36*].

Previous results [*14,17*] showed that *A. tumefaciens* can transform certain monocotyledonous plant species without tumour formation. On a dicotyledonous plant, the grapevine hybrid A4/42, infection with the appropriate biotype 3 *Agrobacterium* isolates was not followed by tumour growth but octopine was detectable in the wound callus at

the inoculation sites. Factors determining this particular form of *Agrobacterium*–host plant interactions are unknown. These observations suggest that a wider range of plant species can be considered for genetic engineering using Ti plasmid vectors than the known susceptible hosts [*12*].

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