Investigations Implying the Invalidity of Octopine as a Marker for Transformation by *Agrobacterium tumefaciens*

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The presence of the rare free amino acid octopine in different plant material was tested in order to confirm its validity as a specific marker for the expression of bacterial genetic information in tumors produced by infection with *Agrobacterium tumefaciens*. This guanidine derivative is present in auxin-autotrophic fast growing callus cultures of *Crepis capillaris*, and *Nicotiana tabacum* var. "White Burley", all transformed by infection with *Agrobacterium tumefaciens*. In auxin-heterotrophic callus cultures and in healthy plant material of the same species octopine could not be found. In all *A. tumefaciens* strains including the avirulent No. 55 tested this guanidine derivative was present. However, since octopine was found in fast growing "habituated" cultures of *N. tabacum* var. "White Burley" and, in small amounts in root tips of pea and bean seedlings and not in a slow growing "transformed" strain T5 of *Crepis*, it cannot be considered as a specific marker for the expression of genetic material from the transforming bacteria.

Introduction

We use as characterization of the tumor state in plants auxin-autotrophy. Auxin-autotrophy can be achieved either through "habituation" [1] or through "transformation" [2]. In the case of transformation by means of infection with *Agrobacterium tumefaciens* genetic material is probably incorporated from the bacterium in the plant cell, *cf.* the hybridization experiments by Schilperoort *et al.* [3]. Morel *et al.* [4] found the rare guanidine derivatives octopine and nopaline in tumors of different plants, produced by infection with *A. tumefaciens*. In these tumors the presence of one or the other guanidine derivative seems to be independent of both the plant and external factors, but dependent upon the type of bacterium used for transformation. Moreover, the observation that these guanidine derivatives could not until now be found either in "normal" heterotrophic callus cultures or in *in vitro* cultures of tumors lacking *A. tumefaciens* infection, supported the assumption that in plant tumors, as in some animal virus tumors, the transformation by *A. tumefaciens* is due to the incorporation of foreign genetic material which, to some extent transcribed and translated, leads to bacterium specific products.

The free amino acids octopine and nopaline would be a very useful marker for the expression of genetic material from the transforming bacterium, if they are exclusively present in tumors induced by *A. tumefaciens*, because the use of these guanidine derivatives as markers would be more advantageous than the detection of *A. tumefaciens* DNA through complicated hybridization experiments. It is in order to confirm the validity of these amino acids as markers that we report and discuss in this paper the results of electrophoretic and chromatographic investigations on specific guanidine derivatives in plant material. *In vitro* callus cultures of *Crepis capillaris* and *Nicotiana tabacum* var. "White Burley" as well as root tips and hypocotyls of young seedlings from different plants were chosen for study. We looked also for guanidine derivatives in different strains of *A. tumefaciens*, some virulent and one avirulent.

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Material and Methods

The *Crepis capillaris* transformed (crown-gall) callus cultures were produced by infecting inflorescence stalks with *Agrobacterium tumefaciens* strain 542 (s. later) [5]. The sterilized explants were cultivated from the beginning *in vitro* on medium without auxin and kinetin (basal medium of Murashige and Skoog [6]). All the lines (named TA, TD, TG, T5) which grew three years on this medium were autotrophic for auxin and kinetin. TA, TD, TG had a rapid growth but T5 grew slowly and was the only one to produce chlorophyll in light [5]. The heterotrophic callus cultures of *Crepis capillaris* were started 5 years ago and were cultured on a basic medium of Murashige and Skoog with 6 mg/l

NAA and 0.6 mg/l kinetin (named D2w). Transformed (named Tc and Tc_n) and habituated (named Ta and Ta_n) cultures of *Nicotiana tabacum* var. "White Burley" [7, 8] were initiated about 25 years ago in Gautheret's laboratory, and are growing on Kl medium [9] without hormones (Tc and Ta were obtained from Gautheret's laboratory in 1965, Tc_n and Ta_n in 1969). Young seedlings of pea (*Pisum sativum* "Rivalin", van Waveren Pflanzenzucht, GmbH., Göttingen), bean (*Phaseolus vulgaris* var. "Pinto", Brown's Best, Chester B. Brown Co., Morril, Nebraska) and sunflower (*Helianthus annuus* "Hohe Sonnengold", Benary, Hann. Münden) were raised from about 1000 g dry seeds each. After a 10 min sterilization in 10% Na-hypochlorite and 5 min in 0.5% $HgCl_2$ they were washed in sterile water and placed in sterile petri dishes on humid filter paper in the dark. 24 hours later the root tips and after two or three days the hypocotyls were cut off.

The bacterial strains have the following origin: B6: Institut für Bakteriologie der "Biologischen Bundesanstalt für Land- und Forstwirtschaft", Berlin. 542: idem. 55: Was derived from 542 as an avirulent mutant, by treatment with *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine by L. Schilde-Rentschler. In all other tested properties it is indistinguishable from the virulent strain 542 (pers. communication). 1001: National Collection of pathol. plant bacteria, England.

They were cultivated for these analyses on complete medium (Difco nutrient broth 8 g/l, Difco yeast extract 1 g/l, saccharose 5 g/l, pH 7) at 27 °C.

Extraction

In vitro cultures: Approximately 25 g of 3 – 4 week old callus were frozen with CO_2 and broken in a mortar and then in a coffee mill (Braun). The material was thawed at 4 °C and centrifuged 20 min at 20,000 × *g*. The supernatant was precipitated with 3 vol. boiling absolute ethanol and centrifuged 20 min at 20,000 × *g*. The alcohol containing supernatant was concentrated. (This procedure is a modification of a method which was personally communicated to us by Dr. Tempé, C.N.R.A., Station de Physiologie Végétale, Versailles).

In vivo material: Approximately 25 g young leaves or 5 g root tips or hypocotyls were treated in the same way as the callus cultures.

Bacteria: 3 l of bacterial suspension in exponential phase of growth were centrifuged for 30 min at 12,000 × *g*. For all strains tested approximately 15 ml packed bacteria were obtained. They were broken by ultrasonification (Sonifer, Branson Instr., 6 Amp.) and then centrifuged for 60 min at 65,000 × *g*. The supernatant was precipitated with 3 vol. boiling ethanol. The following operations were the same as for callus cultures. All steps prior to precipitation were carried out at 4 °C.

The dry extracts were each taken up in 1 ml 1% pyridine solution and were loaded on to a Sephadex G 10 column ca. 100 cm long and 12 mm ϕ (if the quantity of available material was very small, a shorter column was used). The eluent used was 1% pyridine in water at a flow rate of 1 ml/hour. The effluent was collected in 1 ml fractions on a fraction collector. About 24 hours later the fractions were tested with a guanidine specific staining: 0.1 ml eluate was mixed with 0.05 ml reagent A (4% naphthol, 8% Na-methylate in 50% methanol) and 0.05 ml reagent B (0.1% diacetyl in abs. methanol) (Clotten [10], modified).

Electrophoretic separation

Depending on the strength of the positive reaction of single fractions to the guanidine-specific stain as compared to a 1 mM solution of guanidine the fractions to be tested were subsequently either concentrated or diluted before applying to the thin layer cellulose chromatographic plates (E. Merck, Darmstadt). The volumes used varied between 5 and 60 λ.

Electrophoresis was carried out at 500 V for 90 min in pyridine/acetate buffer of pH 3.6 (3% glacial acetic acid, 0.4% pyridine). After drying at room temperature, the plates were sprayed gently with reagents A, B and 2 n NaOH. The intensity of staining reached its maximum in 20 min and was stable for many hours. The optimal spots were obtained with guanidine in amounts of about 0.005 μmoles. *In this way it is possible to detect a guanidine derivative present at a concentration 200 – 300 times smaller than another in the same extract, to a minimum of about 0.001 μmoles.*

During electrophoresis authentic arginine moved to the cathode approx. 12 cm (basic region), authentic octopine approx. 4 cm (neutral region). The position of a guanidine derivative was calculated as a ratio between the distances (from the start) traversed by it and by the corresponding authentic guanidine derivative.

Results

Strains TA, TD and TG of *Agrobacterium*-transformed cultures of *Crepis capillaris* [5] showed two guanidine derivatives in the neutral-basic region. The one has the position corresponding to authentic arginine, the other corresponding to authentic octo-

Table I. A comparison of the amounts of neutral and basic guanidine derivatives in some bacteria and auxin-autotrophic and heterotrophic plant tissues.

Material		Ratio of distances of spots travelled by unknown and authentic guanidine derivatives after electrophoresis *	Approx. concentr. in μm/g fresh weight mat. (for *A. tumefaciens* μmoles/ml packed bacteria)
C. capillaris	TA	x/oct = 1.01	0.02
crowngall callus		y/arg = 0.99	0.5
cult. (transf.	TD	x/oct = 0.99	0.09
with *A. tumefac.*)		y/arg = 0.99	0.2
	TG	x/oct = 0.99	0.02
		y/arg = 1.00	0.5
	T5	octopine not detected	
		y/arg = 1.00	0.3
C. capillaris		octopine not detected	
heterotroph. callus		y/arg = 0.98	
cultures			> 0.2
C. capillaris		octopine not detected	
leaves		y/arg = 0.97	> 0.3
N. tabacum var.			
"White Burley"		x/oct = 0.99	0.01
crowngall callus cult.		y/arg = 0.99	0.25
(transf. by *A. tumefaciens*) Tc + Tc_n			
N. tabacum var.			
"White Burley" spont.		x/oct = 0.99	0.01
habituated callus		y/arg = 0.99	0.2
cultures, Ta + Ta_n			
root tips of		x/oct = 0.99	0.004
bean seedlings		y/arg = 1.03	0.2
root tips of		x/oct = 0.99	0.005
pea seedlings		$x1$/oct = 0.58	0.01
		y/arg = 0.98	> 0.6
hypocotyls of		x/oct = 0.98	0.02
pea seedlings		y/arg = 0.98	0.8
Sunflower root tips		octopine not detected	
of young seedlings		y/arg = 1.00	0.6
Hypocotyls of sun-		octopine not detected	
flower seedlings		y/arg = 0.99	0.5
Agrobacterium strain		x/oct = 0.99	0.01
tumefaciens	B 6	$x2$/oct = 0.78	0.5
	virulent	y/arg = 0.99	> 0.75
		$y1$/arg = 0.94	0.75
	strain	x/oct = 0.98	0.01
	542	$x2$/oct = 0.87	0.4
	virulent	y/arg = 0.94	0.7
	strain	x/oct = 0.99	0.09
	1001	y/arg = 0.99	0.6
	virulent		
	strain	x/oct = 1.00	0.02
	55	$x2$/oct = 0.86	0.4
	avirulent	y/arg = 0.99	0.6
Escherichia coli		octopine not detected	
	strain	$x3$/oct = 0.67	0.7
	A 19	y/arg = 1.00	0.5

* x and y are the distances in mm of the spots corresponding to octopine and arginine respectively. $x1$, $x2$ and $x3$ are the corresponding distances of spots near octopine and $y1$ is that of a spot near arginine.

pine (the ratio of distances covered by the different guanidine derivatives tested are given in Table I). The tumorous culture T5, which was autotrophic for growth hormones but grew more slowly after three years of *in vitro* culture, showed only one guanidine in the basic region corresponding to authentic arginine. In heterotrophy callus cultures and in leaves of *Crepis capillaris* only arginine was detected in the neutral-basic region.

Transformed (Tc and Tc_n) and habituated (Ta and Ta_n) *in vitro* cultures of *Nicotiana tabacum* var. "White Burley" show two guanidine derivatives, arginine and octopine.

In sterile germinating root tips of pea and bean seedlings about 0.005 μmoles octopine and 0.6 μmoles of arginine (per g fresh weight) could be found. In root tips of pea seedlings about 0.01 μmoles of a third guanidine derivative was present. In hypocotyls of pea seedlings 0.02 μmoles/g octopine was found. In sunflower root tips and hypocotyls only arginine was detected.

In all tested *Agrobacterium tumefaciens* strains growing under the previously mentioned conditions the guanidine derivatives corresponding to authentic arginine and octopine were present (arginine in amounts of about 0.75 μmoles, octopine about 0.01 μmoles/ml packed bacteria). Two other guanidine derivatives, one near to arginine, the other near to octopine, were also detected in the neutral-basic region. In *Escherichia coli* extract tested for comparison no octopine could be found. Only arginine and another guanidine derivative in the neutral-basic region could be detected.

Discussion

The presence of octopine in transformed fast growing *in vitro* cultures of *Crepis capillaris* and its absence in heterotrophic callus cultures of the same species is in agreement with the results of Goldmann-Ménagé [11]. But some of our other results are not in accordance with the assumption that octopine is a specific expression of the transfer of genetic material from the bacterium to the plant cell.

1. In Ta and Ta_n we found in considerable amounts this guanidine derivative (about 0.01 μmoles octopine and 0.2 μmoles arginine per gram fresh weight material). Melchers [8] obtained the two cultures, originated from the same auxin-autotrophic culture, from Gautheret's laboratory at two

different times (1965 and 1969). The position of the spots on the plate of both and of the transformed material (Tc and Tc_n) was identical. Ta and Ta_n were not produced by infection with *Agrobacterium tumefaciens*, but were "spontaneously" habituated.

2. In sterile germinating root tips of young pea and bean seedlings we could detect small amounts of the guanidine derivative.

Although the method for amino acid separation with the Technicon-Analyser, used in Morel's laboratory, allows an exact identification of the different amino acids, two guanidine derivatives present in the same extract in concentrations differing by a factor of more than 50 cannot be simultaneously quantitatively analysed (Goldamnn-Ménagé[11], p. 258, Fig. 2). In the material which we tested octopine was sometimes present in amounts of 100 – 150 times smaller than arginine (s. Table I). In this situation octopine cannot be detected if the sensitivity of the Technicon-analyser is set to suit the amount of arginine.

The fact that this guanidine derivative is present, even in small amounts, in some *in vivo* and *in vitro* plant material not transformed by infection with *Agrobacterium tumefaciens* indicates that octopine is not exclusive to tumors obtained through such infection. The synthesis of this free amino acid can take place also in some normal plant tissues *in vivo*, and in "spontaneous" tumors. Morel *et al.*[4] could never find octopine or nopaline in *Agrobacterium tumefaciens*. In all our tested strains *including the avirulent mutant 55*, we found significant amounts of octopine (s. Results).

We think that the above mentioned differences between our results and those of Morel *et al.* have their basis in the different methods used. The sensitivity of our method is higher than the Technicon method for the arginine and octopine concentrations used by Goldmann-Ménagé. Our results do not exclude the possibility that, with more sensitive methods for determining octopine, it could be found also in plant material, where it was not detected by our analysis. On the other hand, octopine was found in material certainly not transformed by infection with *Agrobacterium tumefaciens*. Therefore, we have to conclude that octopine in plant tissues cannot be taken as a marker for the expression of genetic material from the transforming bacteria.

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