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Biotransformation of nicotine alkaloids by tobacco shooty teratomas induced by a Ti plasmid mutant

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ABSTRACT

Tobacco shooty or rooty teratomas and hairy roots were induced by *Agrobacterium tumefaciens* (pGV 3845), *A. tumefaciens* (pGV 3304) and *A. rhizogenes* (pRi 8196), respectively. The tobacco alkaloids, nicotine, nornicotine and anatabine, were produced in hairy roots and in rooty teratomas but not in shooty teratomas. However, the shooty teratomas have the ability to accumulate the alkaloids and to biotransform nicotine to nornicotine. These were established by co-culture experiments incubating hairy roots and shooty teratomas in a same dish and by biotransformation experiments with shooty teratomas.

INTRODUCTION

Three phytohormone-related genes present on the T-DNA of a Ti plasmid are directly involved in Crown gall tumor formation induced by *Agrobacterium tumefaciens* (Inzé et al. 1987, Weiler and Schröder 1987). The auxin-like effect can be ascribed to the combined activities of T-DNA gene *1* and gene *2*. Gene *1* encodes a tryptophan-2-monooxygenase and gene *2* a indoleacetamide hydrolase (Inzé et al. 1984, Schröder et al. 1984, Thomashow et al. 1984, Van Onckelen et al. 1986). The cytokinin-like activity is due to T-DNA gene *4*, which encodes an isopentenyl transferase for cytokinin biosynthesis (Akiyoshi et al. 1984, Barry et al. 1984). The Ti plasmid mutants in gene *1* and/or *2* (Shi⁻, Shoot inhibition) or in gene *4* (Roi⁻, root inhibition) induce shooty teratomas or rooty teratomas, respectively, on some host plants (Leemans et al. 1982, Joos et al. 1983).

The Ri plasmid of *A. rhizogenes* induces hairy root disease which also results in the development of a kind of rooty teratoma on many host plants (De Cleene and De Ley 1981). The features of Ti and Ri plasmids are similar in several ways, for example, in T-DNA transfer and opine synthesis (Chilton et al. 1982, Willmitzer et al. 1982), although the precise mechanism of hairy root formation is not yet clear.

Several attempts have been successful in producing plant secondary metabolites by hairy root cultures (Flores et al. 1987, Hamill et al. 1987). These metabolites are normally biosynthesized only in root tissue of differentiated plants. However, no studies have been reported on the production and biotransformation of plant secondary metabolites by shooty teratomas incited by Ti plasmid mutants.

In the present study, we have induced shooty teratomas and hairy roots on tobacco and examined the production and biotransformation of nicotine alkaloids by these transformed tissues.

MATERIALS AND METHODS

Plasmids and bacteria

The plasmid pGV 3845 is a substitution mutant of gene *1* constructed by Van Haute et al. (1983). pGV 3304 is an insertion mutant of gene *4* (Joos et al. 1983). These plasmids are in the cured *A. tumefaciens* C58C1 Rif^R. *A. tumefaciens* C58C1 Rif^R lacking a Ti plasmid was used as a negative control for transformation. *A. rhizogenes* (pRi 8196) is a mannopine-type strain and used for hairy root induction. Bacteria were maintained on YEB agar plate (Vervliet et al. 1975) supplemented with appropriate antibiotics if necessary. Before infection, they were cultured in liquid minimal A medium (Miller 1972) at 28℃ for 2 days.

Induction of teratomas and hairy roots

Leaf discs of *Nicotiana tabacum* cv. Petit Havana SR1 were transformed by the method of De Block et al. (1987), without any selective agents. After 3-4 weeks, the teratomas and hairy roots were excised from leaf discs and maintained on B5 medium (Gamborg et al. 1968) containing 3% sucrose, 0.8% agar, pH 5.7, and 200 mg/l cefotaxime (Claforan, Hoechst). Hairy roots were cultured in liquid B5 medium with 3% sucrose, pH 5.7, and 200 mg/l cefotaxime. Tissues were transferred to fresh medium every 2 weeks. Cefotaxime could be omitted after 3-4 passages of transfer.

Production and transformation of tobacco alkaloids

The teratomas and hairy roots were cultured on agar or in liquid B5 medium as described above at 25℃ under illumination (2000-3000 lux, 16hr/day). The tissue (200 mg fresh weight) was homogenized with 300 μl of H_2O, 30 μl of 0.1 N HCl and 30 μg of 4-methylquinoline. After centrifugation, the acidic supernatant was extracted with 300 μl of ethyl acetate. The organic layer was discarded after centrifugation. To the aqueous layer, K_2CO_3 powder was added to saturation, and then the alkaloids were extracted with 300 μl of ethyl acetate. To the separated organic layer, 30 μl of 10% acetic acid in acetone was added to make the solution acidic. This was evaporated *in vacuo*. The tobacco alkaloids were identified by gas chromatography/mass spectrometry (Shimadzu GC-MS QP1000 system) and quantified by GC (2% OV-17, 2m, 140℃) using 4-methylquinoline as an internal standard.

Opine assays

Nopaline assay was carried out as described previously (Otten and Schilperoort 1978). Mannopine was detected as reported (Petit et al. 1983).

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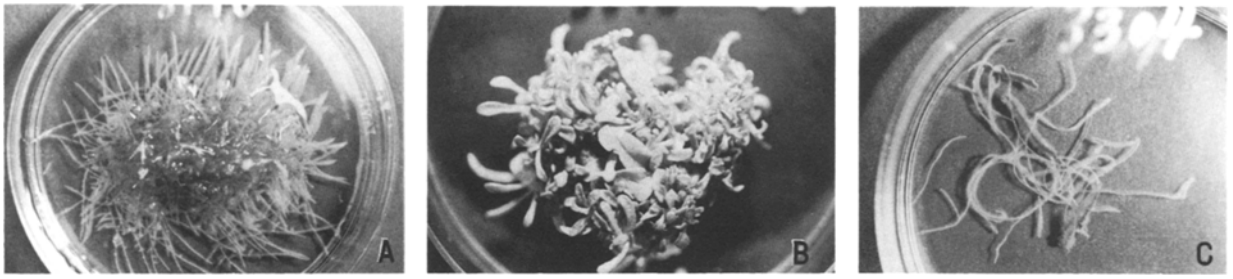

Fig. 1 (A) Hairy roots induced by *A. rhizogenes* (pRi 8196). (B) Shooty teratomas induced by *A. tumefaciens* (pGV 3845). (C) Rooty teratomas induced by *A. tumefaciens* (pGV 3304).

Results

Transformation by Agrobacterium species

Leaf discs infected with *A. tumefaciens* (pGV 3845 or pGV 3304) gave shooty teratomas or rooty teratomas, respectively, 3-4 weeks after infection. However, leaf discs treated with *A. tumefaciens* C58C1 Rif^R, a cured strain, gave no calli or teratomas. The discs infected with *A. rhizogenes* (pRi 8196) induced proliferated hairy roots 2-3 weeks after infection. These transformed tissues grew rapidly without the addition of any phytohormones (Fig. 1). The transformation state was confirmed by the detection of nopaline or mannopine (Fig. 2).

Production of alkaloids

The transformed hairy roots produced nicotine, nornicotine and anatabine during 14 days culture (Table 1, Exp. 1). These alkaloids were identified by gas chromatography/mass spectrometry (nicotine M^+ m/z 162, nornicotine M^+ 148, anatabine M^+ 160). Substantial amounts of alkaloids were accumulated in the medium as well as in the cells. However, the shooty teratomas induced by pGV 3845 failed to synthesize any alkaloids, although the bio-mass production was high. The rooty teratoma induced by pGV 3304 produced a low amount of alkaloids, presumably because of the unsuitable culture condition for rooty teratomas.

Co-culture of transformed root and shoot tissues in vitro

It is generally believed that the tobacco root is the organ responsible for biosynthesis of nicotine and that the shoot is the site of storage and biotransformation of nicotine and related alkaloids (Dawson 1948, Waller and Dermer 1981). The results described above also support this idea. We carried out co-culture experiments with transformed root and shoot tissues *in vitro*. That is, the hairy roots and shooty teratomas were co-cultured in liquid B5 medium in the same petri dish, and 2 weeks later, the alkaloids in each tissue were analyzed (Table 1). This kind of experiment is possible, because the transformed tissues have the ability to maintain their morphology without addition of exogeneous hormones. Results from these studies show that the alkaloids were distributed in shooty teratomas more than in hairy roots. The alkaloidal amounts in the medium were decreased as compared to those found in experiments in which only hairy roots were present. The combined amounts of nicotine, nornicotine and anatabine were highest in shooty teratomas. These phenomena were essentially reproducible within the 3-days experiment (Table 1, Exp. 2), although the amounts in shooty teratomas were lower than those of 14-days experiment 1 and the alkaloid concentrations in the medium were similar for hairy roots and for co-culture, unlike in 14-days experiment. These results indicated that shooty teratomas do not have the ability for *de novo* synthesis of tobacco alkaloids but are capable of storage and biotransformation of alkaloids synthesized and released by hairy roots.

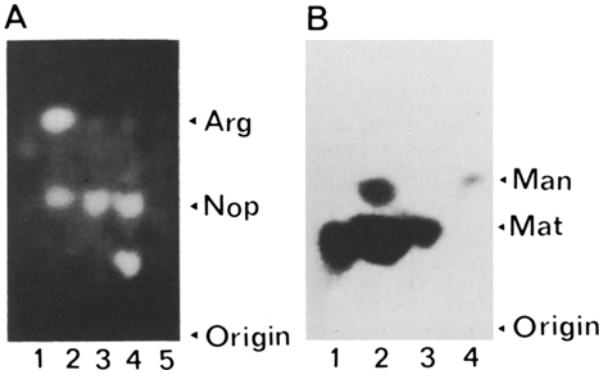

Fig. 2 (A) Nopaline detection in the teratomas. Lane 1: colour marker, lane 2: standard arginine (Arg) and nopaline (Nop), lane 3: shooty teratoma induced by pGV 3845, lane 4: rooty teratoma induced by pGV 3304, lane 5: control SR1 plant. (B) Mannopine detection in hairy roots. Lane 1: control SR1 plant, lane 2: hairy root induced by pRi 8196, lane 3: standard mannitol (Mat), lane 4: standard mannopine (Man).

Biotransformation of nicotine by shooty teratomas

To confirm the activities of storage and biotransformation in shooty teratomas, we have studied the metabolism of exogenously added nicotine by incubating it with shooty teratomas in the liquid B5 medium. As shown in Fig. 4, the nicotine content in the medium decreased rapidly. The concentration of cellular nicotine increased dramatically within 1 day and then decreased gradually. Nornicotine in the tissue increased slowly during the first 4 days and then decreased. These results clearly suggest that extracellular nicotine was taken up in the shooty teratomas and then demethylated to nornicotine in the teratoma cells.

DISCUSSION

The production of secondary metabolites by transformed hairy root cultures is now recognized as an efficient way for the *in vitro* production of useful compounds (Hamill et al. 1987, Flores et al. 1987, and the papers cited therein). For example, nicotine is produced by hairy root cultures of *N. rustica* (Hamill et al. 1986) and other *Nicotiana* species (Parr et al. 1987). Our present study has confirmed the high

Table 1. The production and biotransformation of tobacco alkaloids by hairy roots, teratomas and reconstitution systems[1].

System	Bio-mass production (fold) (g)[2]	Alkaloids in tissue (μg/g fr. wt.) (μg)[3] Nicotine	Nornicotine	Anatabine	Alkaloids in medium (μg/ml medium) (μg)[3] Nicotine	Nornicotine	Anatabine
Exp. 1 (14-days culture)							
HR (8196)[4]	1.82	24.7	94.6	2.2	5.4	36.4	2.0
	(4.43)	(109.4)	(419.1)	(9.7)	(75.6)	(509.6)	(28.0)
ST (3845)[5]	5.66	-	-	-	-	-	-
	(19.4)						
RT (3304)[6]	1.35	4.6	3.8	-	-	-	-
	(0.75)	(3.5)	(2.9)				
Co-culture[7]							
HR (8196)	1.28	2.8	18.1	0.6	0.8	5.6	0.4
	(2.79)	(7.8)	(50.5)	(1.7)	(8.0)	(56.0)	(4.0)
ST (3845)	3.34	20.6	138.7	4.3			
	(6.62)	(136.4)	(918.2)	(28.5)			
Exp. 2 (3-days culture)							
HR (8196)	0.83	45.3	56.8	2.8	6.9	13.6	0.5
	(2.23)	(101.0)	(126.7)	(6.2)	(103.5)	(204.0)	(7.5)
Co-culture							
HR (8196)	0.87	7.1	26.2	trace	2.8	13.5	0.4
	(3.08)	(21.9)	(80.7)		(42.0)	(202.5)	(6.0)
ST (3845)	1.07	8.4	16.3				
	(3.62)	(30.4)	(59.0)				

1. Hairy roots and teratomas were cultured in liquid B5 medium, 3% sucrose, pH 5.7. The alkaloids were extracted and analyzed by gas chromatography as described in MATERIALS AND METHODS. Data are the means of duplicate incubations.
2. The weight of tissues after incubation for 14 days (Exp. 1) and 3 days (Exp. 2).
3. Total amount of each alkaloid in the tissue or in the medium.
4. Hairy roots induced by *A. rhizogenes* (pRi 8196).
5. Shooty teratomas induced by *A. tumefaciens* (pGV 3845).
6. Rooty teratomas induced by *A. tumefaciens* (pGV 3304).
7. Co-culture experiments culturing hairy roots and shooty teratomas in the liquid medium in the same dish.

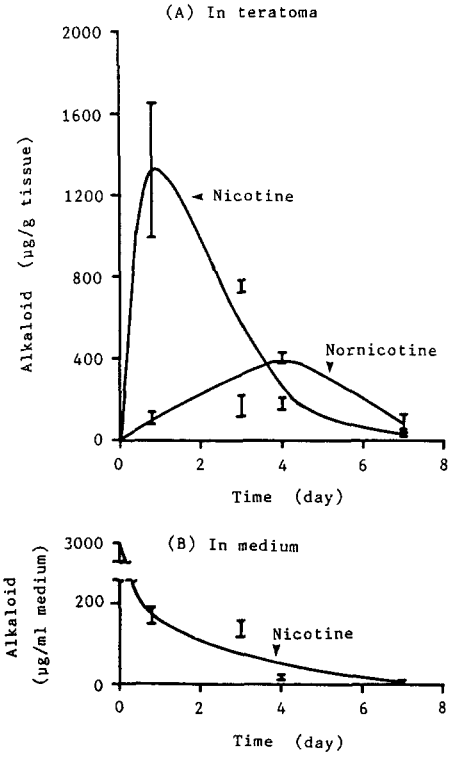

Fig. 3 Biotransformation of nicotine by shooty teratomas. The shooty teratomas (1.5 g) were incubated in 15 ml of liquid B5 medium, 3% sucrose, pH 5.7, and 3000 μg/ml of nicotine. The alkaloids were analyzed by gas chromatography. Data are from duplicate incubations.

production of nicotine and related alkaloids by tobacco hairy roots. The production capacity is almost comparable to that reported previously (Hamill et al. 1986, Parr et al. 1987). It has also been shown that the alkaloids are excreted to the medium in rather high amounts.

In addition, the present study indicates that the shooty teratomas transformed by a Ti plasmid mutant of gene *1* lack the *de novo* biosynthetic activity of the alkaloids but have the abilities of storage and biotransformation of the alkaloids. It has been reported that cell-free extracts of *N. alata* leaves, themselves alkaloid-free, catalyzed the nicotine-nornicotine interconversion (Schröter 1966). Leete (1968) has also shown that excised shoots of *N. glauca* converted ^{14}C-nicotine to ^{14}C-nornicotine but not to anabasine.

There are several lines of evidence in the literature supporting the close relationship between cell differentiation and the various features of secondary metabolism, *i. e.* biosynthesis, transport, accumulation, transformation, degradation, release etc. (Luckner 1985). For example, lupin alkaloids in *Leguminosae* are synthesized in the green parts of the plant (Wink 1987, Saito et al. submitted) and accumulate in other parts, such as in roots. Tropane alkaloids in *Solanaceae* are produced in roots and transported to leaves (Liebisch and Schütte 1985). In this context, the transformed shooty and rooty teratomas are interesting for the study of the relationship between cell differentiation and secondary metabolism. In particular, shooty teratomas of any plant species would be useful for the biotransformation of exogenous compounds which are not only originated from plants but also from microorganisms and even from artificial organic synthesis. The teratoma cultures may also be an efficient material for the production of plant metabolites which are normally biosynthesized in shooty tissues of differentiated plants. Work on these lines is now going on in our laboratories.

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