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METABOLISM OF SOLANACEOUS ALKALOIDS IN TRANSGENIC PLANT TERATOMAS INTEGRATED WITH GENETICALLY ENGINEERED GENES

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Abstract: Transgenic hairy roots and shooty teratomas of *Atropa belladonna* L., *Nicotiana tabacum* L. and *Solanum tuberosum* L. were obtained with *Agrobacterium* Ti and Ri plasmids. The hairy roots of *A. belladonna, N. tabacum* and *S. tuberosum* accumulated tropane alkaloids, nicotine alkaloids and steroidal alkaloids, respectively. The shooty teratomas of these plant species failed to produce these alkaloids. However, the shooty teratomas had the abilities to store and metabolize the alkaloids. These results indicate that the biosynthesis and metabolism of these solanaceous alkaloids are regulated in correlation to tissue differentiation of the plants.

INTRODUCTION

The biosynthesis of some plant secondary metabolites is tissue specific[1]. Recent molecular genetic technology is allowing the progressive elucidation of the molecular mechanism underlying this precise regulation of secondary metabolism. However, the majority of these control mechanisms remains to be clarified.

For the study of the biosynthesis and production of plant secondary metabolites, Ti and Ri plasmids of *Agrobacterium* are useful tools as vector

systems for the transfer of foreign genetic information. They have ability to induce uncontrolled root, shoot and teratoma proliferation[2].

The Ri (root inducing) plasmid present in *A. rhizogenes* induces autonomously proliferating rooty teratomas, so-called "hairy roots", in some dicot plants. This is due to the integration into the plant nuclear genome and expression of a certain DNA fragment, called T-DNA (transferred DNA), from the Ri plasmid[3]. Hairy roots grow rapidly without the addition of any exogenous plant-growth regulators. A number of reports of the successful production of specific secondary metabolites have appeared[4-7]. A biosynthetic study involving a deuterium-labelled precursor in alkaloid-producing hairy roots was reported recently[8]. A report on the enzymes responsible for alkaloid biosynthesis in hairy roots has also appeared[9]. Hairy roots have the advantages of a fast growth rate and concomitant high biosynthetic ability as to specific secondary metabolites. Nevertheless, the successfully obtained products are strictly limited to those which are normally produced in the roots of differentiated plants. Thus, it is necessary to examine modified hairy roots, *i. e.*, green hairy roots, or, alternatively, transformed shooty teratomas for the production of metabolites normally biosynthesized in the green parts of plants.

The Ti (tumor inducing) plasmid in *A. tumefaciens* causes crown gall disease, in which an unorganized proliferated plant tumor forms at the site of infection by the bacterium[10]. This is also due to the integration and expression of the T-DNA fragment of ca. 20 kb in length from the Ti plasmid into the chromosomal DNA of a plant cell. Three oncogenes within the T-DNA are responsible for this phenotype. These oncogenes encode enzymes responsible for the synthesis of two plant growth regulators, auxin and cytokinin, in transformed plant cells[11].

Crown gall and derived cell suspension cultures incited with wild Ti plasmids have been used for the production of some specific secondary metabolites (Table 1). It can be assumed that endogenous phytohormones produced by the action of oncogenes in T-DNA can affect the secondary metabolism as well as morphological changes[13].

Transformed shooty teratomas of higher plants can be induced with several strains of *A. tumefaciens* (Table 1). These transgenic organ cultures are also useful for the *de novo* synthesis and biotransformation of some specific metabolites, which are usually produced or biotransformed in leaves and/or green stems of differentiated plants. The induction of shooty teratomas has been performed with mutants of the Ti plasmid lacking the genes for auxin biosynthesis[16] and with the shoot-forming Ti plasmid, pTiT37[17,18].

Table 1 Biosynthesis and biotransformation of secondary products by crown galls and shooty teratomas induced with Ti plasmids.

Plant species	*Agrobacterium*-Ti plasmid	Type of culture	Specific metabolites	Reference
Cinchona ledgeriana	*A. tumefaciens* A6	Gall cell suspension	Quinoline alkaloids	12
Bidens sp.	Ti plasmid	Gall	1-Phenylhepta-1,3,5-triyne and other polyines	13
Lupinus polyphyllus *L. hartwegii*	*A. tumefaciens* DSM 30150	Cell suspension from gall	Isoflavonoid glucosides orobol-3'-7-O-diglc and genistein-4'-7-diglc	14
Digitalis lanata	pTiC58, pTiB6S3, pTiA6NCtmr-338::Tn5	Gall	Cardenolides	15
Nicotiana tabacum	pGV3845 (*aux*⁻)	Shooty teratoma	Biotransformation of nicotine to nornicotine	16
	pGV3304 (*cyt*⁻)	Rooty teratoma	Nicotine, nornicotine	
Mentha citrata	pTiT37	Shooty teratoma	Mint oil terpenes	17
M. piperita	pTiT37	Shooty teratoma	Mint oil terpenes	18

The system of Ti- and Ri-*Agrobacterium* can also be useful as a vector system for the transfer of genetically engineered foreign genes into plants to cause the metabolic modification of secondary metabolism. We are currently studying the biosynthesis and production of secondary metabolites in organ cultures[19,20] and in transgentic tissues integrated with foreign genes[16,21,22]. In the present study, we established transgenic organ cultures of *Atropa belladonna* L., *Nicotiana tabacum* L. and *Solanum tuberosum* L., and examined the accumulation and transformation of the solanaceous alkaloids within these tissues.

RESULTS

Transgenic *Atropa belladonna*.

A. belladonna produces tropane alkaloids, typically hyoscyamine (1) and scopolamine (3). Hairy roots were induced with *A. rhizogenes* harboring an Ri plasmid, pRi15834. Shooty teratomas of *A. belladonna* were induced with *A. tumefaciens* (pGV2215) (Table 3, Experimental section). pGV2215 is a substitution mutant (*aux*$^-$), in gene 2 of T-DNA, of an octopine-type Ti plasmid, pTiB6S3, and causes shooty teratomas in tobacco.

1 was produced at the concentrations of 0.14 mg/g fresh weight of tissue and 0.86 mg/l medium on 7 days culture. These values are almost comparable with those reported previously[23]. The alkaloids were accumulated in higher concentrations in the medium than in hairy root tissues. The shooty teratomas incited with pGV2215, on the contrary, failed to accumulate any alkaloids at all.

To examine the abilities of shooty teratomas as to the storage and biotransformation of 1, the shooty teratomas were incubated with exogenous 1 in liquid B5 medium. As shown in Fig. 1 , the concentration of 1 in the medium decreased rapidly within one day to 10% of the initial concentration. Consequently, the concentration in the shooty teratoma tissues increased and then decreased gradually. The total alkaloidal fractions in shooty teratomas cultured for 7 and 14 days with exogenous 1 were examined by gas chromatography. Scopolamine (3) detected in the alkaloidal fraction amounted to

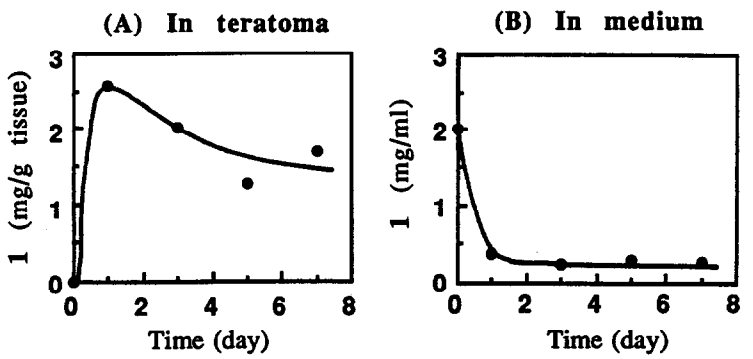
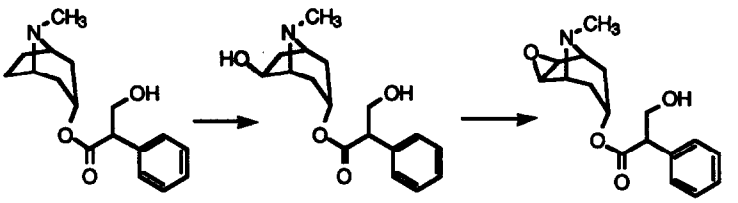


Fig. 1 Uptake of 1 by shooty teratomas of *A. belladonna*. The shooty teratomas (100 mg) were incubated in 1.5 ml of liquid B5 medium, 3% sucrose, pH5.7, and 2mg/ml of 1. The alkaloids were analyzed by gas chromatography. Data are from duplicate incubations.



hyoscyamine(1) 6β-hydroxyhyoscyamine(2) scopolamine(3)

Scheme 1

0.25% of the initial amount of **1** fed (200 μg/ml). These results indicated that shooty teratomas of *A. belladonna* have the abilities to store tropane alkaloids and to oxidatively biotransform **1** (Scheme 1).

Transgenic *Nicotiana tabacum*.

Hairy roots, shooty teratomas and crown galls of *N. tabacum* were obtained by infection with various *Agrobacterium* cells harboring Ri plasmid, Ti plasmid and binary Ti vectors (pGSGluc1 and pBI121), which contain chimeric NPT-II (neomycin phosphotransferase II) and Gus (β-glucuronidase) genes.

The production of nicotine alkaloids was observed in hairy root cultures. However, no production of alkaloids was observed in either shooty teratomas or crown galls of *N. tabacum*. We co-cultured hairy roots and shooty teratomas for 3 and 14 days (Fig. 2). On the co-culture of hairy roots and shooty teratomas, the concentrations of the alkaloids in hairy roots and the liquid medium decreased concomitantly with increases in their concentrations in shooty teratomas. These results together with the previously reported data[16] on the transformation of nicotine to nornicotine in tobacco shooty teratomas showed that the shooty teratomas of *N. tabacum* are able to accumulate and biotransform nicotine alkaloids (Scheme 2), but not to biosynthesize the alkaloids *de novo*.

N. tabacum is easy to be transferred with foreign genes by means of *Agrobacterium* and to be regenerated to mature fertile transgenic plants. We have obtained transgenic tobacco tissues integrated with chimeric genes for NPT-II and Gus on binary vectors. The contents of tobacco alkaloids, nicotine (**4**), nornicotine (**5**), anabasine (**6**) and anatabine (**7**), in these transgenic tissues were

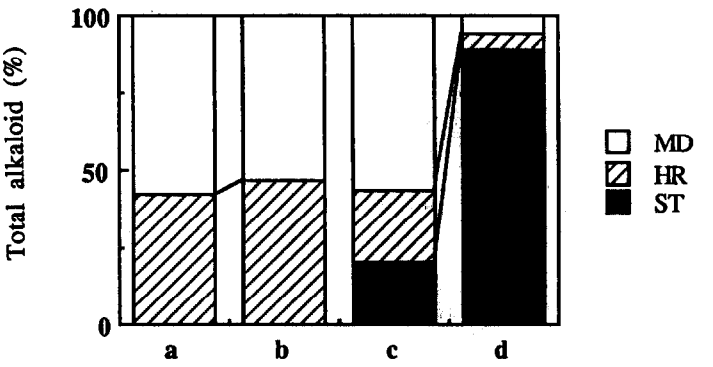
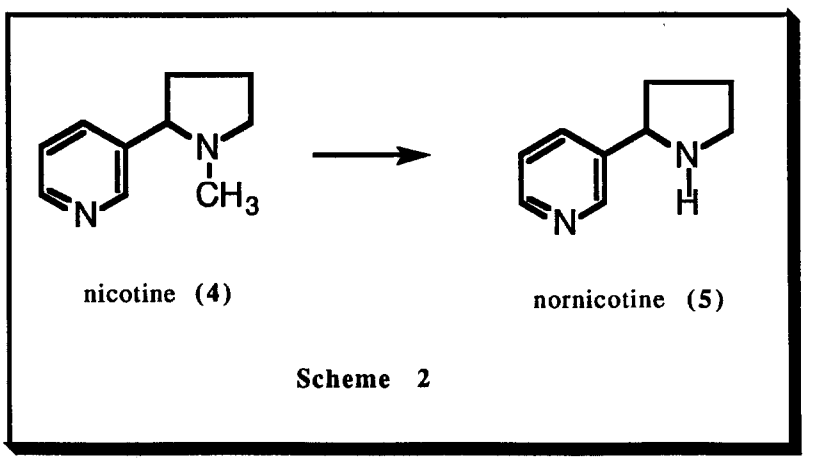


Fig. 2 The production and uptake of total nicotine alkaloids by hairy roots and shooty teratomas of *N. tabacum*. **a**, Hairy root culture for 3 days; **b**, hairy root culture for 14 days; **c**, co-culture of hairy roots and shooty teratomas for 3 days; **d**, co-culture of hairy roots and shooty teratomas for 14 days. **MD**, medium; **HR**, hairy roots; **ST**, shooty teratomas. Shooty teratomas did not accumulate any alkaloids when incubated by themselves.



determined (Fig. 3). Crown galls and shooty teratomas again failed to produce any alkaloids. In the transgenic regenerated plants with a disarmed Ti plasmid (pGV2260) and a wild Ri plasmid (pRi15834), higher concentrations of alkaloids were accumulated in leaves than in roots. This indicated the transfer of the alkaloids from roots to leaves, as suggested by our results above and previous studies[16,24]. The alkaloid concentrations were higher in the regenerant (005)

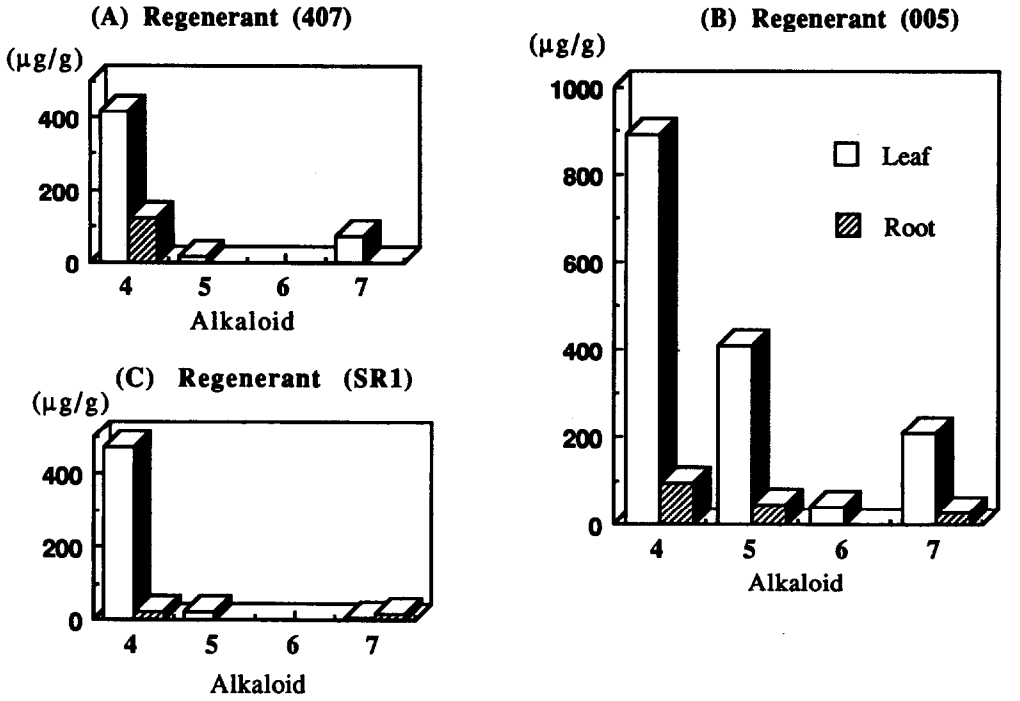


Fig. 3 Distribution of tobacco alkaloids in *N. tabacum* regenerants transformed with *Agrobacterium*. (A) Regenerant 407 with A. *tumefaciens* (pGV2260;pGSGluc1); (B) regenerant 005 with *A. rhizogenes* (pRi15834;pGSGluc1); (C) non-transformed control, SR1.

from hairy roots than those in the normal regenerant (407) with the disarmed Ti plasmid. This could be due to the morphological phenotypic changes, *e. g.*, dwarfism and short internodes, of the regenerant from hairy roots as reported[25].

Transgenic *Solanum tuberosum*.

Tuber discs of *S. tuberosum* were infected with *Agrobacterium* harboring the Ti or Ri plasmid. Hairy roots were induced with pRi15834, crown galls with pTiC58, and shooty teratomas with pGV3132, which is an insertion mutant (*aux*$^-$) of gene 2 of a nopaline type pTiC58.

Hairy roots produced steroidal alkaloids, solanine (ca. 0.1 mg/g fresh weight) and chaconine (ca. 0.1 mg/g fresh weight). These alkaloids were accumulated in the medium but not in the tissue. However, crown galls and shooty teratomas never biosynthesized these alkaloids.

DISCUSSION

In the present study, we examined morphologically different transgenic tissues of *A. belladonna, N. tabacum* and *S. tuberosum for* their abilities to synthesize and transform typical solanaceous alkaloids. These plant species are susceptible to infection by *Agrobacterium,* which leads to hairy roots, crown galls and shooty teratomas, as summarized in Table 2. In each case, accumulation of the characteristic alkaloids of each plant species was only observed in hairy roots, but never in crown galls or shooty teratomas. However, shooty teratomas have the abilities to oxidatively transform the alkaloids, *e.g.*, **1** to **3** and **4** to **5** in *A. belladonna* and *N. tabacum*, respectively. These results confirm earlier work suggesting that these solanaceous alkaloids are synthesized *de novo* in the roots of the differentiated plants and then transported to the leaves where they are accumulated and oxidatively transformed.

However, a root culture of *Hyoscyamus niger* has the high activity of bioconversion of **1** to **2** and **3**. Recently, the enzyme responsible for this bioconversion was purified from the root culture of this plant[26] and a molecular biological study has been started on this interesting enzyme[27]. It would be important to clarify the molecular bases of the cell differentiation and the metabolic differentiation of secondary metabolism.

The induction of shooty teratomas with *Agrobacterium* Ti plasmids has been reported for several plant species[28-32]. However, only a limited number of reports have been published on the synthesis of secondary products in shooty teratomas[16-18]. Shooty teratomas, having the abilities to biosynthesize and biotransform specific secondary products, could be useful systems for the molecular study of the regulation of biosynthesis, in particular, in correlation to cell differentiation. Recently, we obtained transgenic *N. tabacum* integrated with a cytochrome P-450 gene from rabbit livers to change metabolite pattern in transgenic plants[33]. We observed phenotypic changes of transgenic tobacco and changes of the alkaloid pattern. Research along these lines, *e.g.*, manipulation of secondary metabolism through foreign gene expression, should be promising in the future.

Table 2 Transformation with *Agrobacterium*, and production and biotransformation of solanaceous alkaloids.

Plant species	Crown gall		Hairy root		Shooty teratoma		
	Trans.[a]	Prod.[b]	Trans.	Prod.	Trans.	Prod.	Biotrans.[c]
A. belladonna	Yes pTiC58 pTiB6S3	No	Yes pRi15834	Yes 1,	Yes pGV2219	No	Yes 1 to 3
N. tabacum	Yes pTiC58 pTiB6S3	No	Yes pRi15834 pRi8196	Yes 4, 5, 6, 7	Yes pV3845 pGV2215	No	Yes 4 to 5
S. tuberosum	Yes pTiC58	No	Yes pRi15834	Yes solanine, chaconine	Yes pGV3132	No	N.D.[d]

[a]Genetic transformation with Ti and Ri plasmids. [b]Production of alkaloids. [c]Biotransformation of alkaloids. [d]Not determined.

EXPERIMENTAL

Bacteria and plasmids.

The bacteria and plasmids used in this study are listed in Table 3. *Agrobacterium* was maintained on YEB agar plates[34], supplemented with appropriate antibiotics if necessary. Before infection, bacteria were cultured in liquid minimal A medium[35] a 28°C for 2 days.

Plant transformation with *Agrobacterium*.

Leaf discs (ca. 1cm x 1cm) of sterile shoot cultures of *A. belladonna* and *N. tabacum* maintained on the medium containing Murashige and Skoog salts at half-strength, 1% sucrose and 0.7% agar were infected with *Agrobacterium* essentially as reported previously[36]. For the transformation of *S. tuberosum*, sterile tuber discs treated with 1% NaOCl were infected with *Agrobacterium*. The transformed tissues (hairy roots, crown galls and shooty teratomas) were excised from tissues and cultured in B5 agar medium[37] with 3% sucrose, pH 5.7, and 200mg/l Claforan™ (Hoechst). Claforan™ could be omitted after 3-4 passages of transfer to new medium. The established transformed tissues were cultured on B5 medium with 3% sucrose, with 0.7% agar for crown galls and shooty teratomas,

and without agar for hairy roots. Regenerated *N. tabacum* plants from the hairy roots and the leaf discs infected with *Agrobacterium* harboring a disarmed Ti plasmid were obtained as described elsewhere[38].

Table 3 Bacterial strains and plasmids.

Bacterial strains	Resistant marker	Opine	Specification
A. tumefaciens C58C1	Rif^r	-	Nononcogenic derivative of C58, cured of pTiC58
A. rhizogenes	Rif^r	-	
Plasmids			
pTiB6S3	-	Octopine	Wild oncogenic
pTiC58	-	Nopaline	Wild oncogenic
pGV2215[a]	Km^r	Octopine	*Aux*⁻ pTiB6S3 substitution mutant of gene 2, shooty
pGV2219[a]	-	Octopine	*Aux*⁻ pTiB6S3 deletion mutant of gene 2, shooty
pGV3132[b]	Sp^r,Sm^r	Nopaline	*Aux*⁻ pTiC58 insertion mutant of gene 2, shooty
pGV3845[b]	Sp^r,Sm^r	Nopaline	*Aux*⁻ pTiC58 susbstitution mutant of gene 1, shooty
pGV2260[c]	Cb^r		T-DNA of pTiB6S3 replaced with pBR322
pRi8196	-	Mannopine	Wild virulent
pRi15834	-	Agropine	Wild virulent
pGSGluc1[d]	Cb^r,Sp^r,Sm^r	-	Binary vector containing NPT-II and Gus under the control of TR promoters
pBI121[e]	Km^r	-	Binary vector containing NPT-II and Gus under the control of Nos and CaMV 35S promoters

[a]Details given in ref. 41. [b]Details given in ref. 31. [c]Details given in ref. 42. [d]From Plant Genetic Systems, Gent, Belgium. [e]Details given in ref. 43.

Molecular genetic and biochemical analyses of transformed tissues.

The transgenic states of the established cultures were confirmed by DNA blot hybridization and/or opine assays. DNA blot hybridization of the transformed tissues against the T-DNA fragments was carried out as described previously[21,22]. As the marker of transformation with *Agrobacterium*, the production of opines, specific amino acids synthesized in transformed tissues with *Agrobacterium*, were examined. Nopaline and octopine were assayed by paper electrophoresis as reported[39]. Agropine and mannopine were detected by silver staining after paper electrophoresis as described previously[40]. The activities NPT-II and Gus were determined as described previously[21,22].

Biosynthesis and biotransformation of alkaloids.

For the biosynthesis and biotransformation of the alkaloids, the transgenic tissues were cultured in B5 liquid medium at 25°C under light (2000 lx, 16 hr/day). Total alkaloidal fractions were obtained from the cultured tissues and the medium as described previously[16,19,20].

For the tropane alkaloids, the alkaloidal fraction was treated with N,O-bis(trimethylsilyl)acetamide and then analyzed by gas chromatography (2% OV-17, 2m, 250°C), with sparteine as an internal standard. The extraction and determination of tobacco alkaloids were carried out as reported elsewhere[16]. The alkaloidal fractions of the tissues and the medium of *S. tuberosum* were analyzed by TLC (Kieselgel $60F_{254}$, 0.25 mm, Merck) using a mixture of acetic acid and ethanol (1:3).

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