Tobacco plants transformed with the *Agrobacterium* T-DNA gene *1* contain high amounts of indole-3-acetamide

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The T-DNA genes *1* and *2* of the Ti plasmid of *Agrobacterium tumefaciens* are involved in the biosynthesis of IAA in transformed plant cells. Previously, it has been shown that gene *2* codes for an amidohydrolase able to convert IAM into IAA. We have isolated *Nicotiana tabacum* regenerates transformed with either gene *1* or genes *6a* and *6b* of the T-DNA. The tobacco plants transformed with gene *1* contain 500–1000-times more IAM as compared to plants transformed with genes *6a* and *6b*, and as compared to untransformed control plants. No drastic differences in endogenous IAA concentrations were observed between the three plant types analyzed.

Indole-3-acetamide *Indole-3-acetic acid* Agrobacterium tumefaciens *T-DNA gene* Nicotiana tabacum

1. INTRODUCTION

Agrobacterium tumefaciens induces crown gall tumors upon infection of most dicotyledonous plants [1]. All oncogenic *A. tumefaciens* strains harbor large tumor-inducing (Ti) plasmids [2]. Crown gall tumor formation is the result of the transfer, stable integration and expression of a well-defined Ti plasmid segment, the T-DNA, into the plant nuclear DNA (for a recent review, see [3]).

At least 3 T-DNA genes are directly involved in tumor induction. Mutants in gene *4* or in gene *1* and/or *2*, respectively, produce tumors that sprout many roots or many shoots on some plant hosts [4–7]. In analogy to what is known about plant growth regulators, the effect of gene *4* can be thought of as 'cytokinin-like', and the combined effects of genes *1* and *2* can be thought of as 'auxin-like' [4,7]. Recently, it has been demonstrated that gene *2* codes for an enzyme which is able to hydrolyse IAM into IAA [8,9]. Furthermore, there is accumulating evidence that gene *1* is directly or indirectly responsible for the synthesis of a substrate (most likely IAM) for the enzyme encoded by gene *2* [10]. The data presented here demonstrate that gene *1* is involved in the accumulation of IAM in transformed cells.

Abbreviations: BHT, 2,6-di(*tert*-butyl)-4-methylphenol; IAA, indole-3-acetic acid; IAM, indole-3-acetamide; RT, retention time

2. MATERIALS AND METHODS

2.1. *Isolation of* N. tabacum *plants transformed with either gene* 1 *or genes 6a and 6b*

The *Nicotiana tabacum* var. W38 regenerates, rG1 and rG6a/6b, transformed with gene *1* and genes *6a* and *6b*, respectively, were isolated as in [11] and by using *A. tumefaciens* strains containing respectively pGV3850::pG1 or pGV3850::pG6a-6b [10].

Plants were grown as sterile shoot cultures on hormone-free Linsmaier and Skoog agar medium [12] at 25°C and 16 h light. Subcultures were made every 5–6 weeks.

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2.2. *Extraction and analysis of IAA and IAM*

Upon harvesting (2 weeks after subculture) the tissues (about 10 g fresh wt) were homogenized (Virtis '45', 2 min, full speed) in methanol (9 ml/g fresh wt, 0.1 mg BHT/ml) and kept overnight at −20°C. For recovery measurements, 3-indolyl-[1-^{14}C]acetic acid (4000 dpm, 2.18 GBq/mmol) and 3-[5(n)-^{3}H]IAM (10000 dpm, 788 GBq/mmol) were added to the extract.

After centrifugation (28000 × g, 15 min) the extract was evaporated in vacuo and solubilized in 10 ml of 50% methanol. This sample was passed through two consecutive Sep-pak C-18 cartridges (Waters Ass.). After further washing with 10 ml methanol/water (50:50, v/v), the combined eluate was evaporated in vacuo to 1/4 of its volume, acidified with KH_2PO_4/H_3PO_4 (0.5 M, pH 2.5) and passed through a Sep-pak C18 cartridges, prewashed with the acid buffer. Both IAA and IAM were then recovered from the cartridge by eluting with 5 ml of diethyl ether (the diethyl ether was washed 3 times with water, passed through a cotton plug, and 0.1 mg BHT/ml was added), which was then removed by an N_2 stream. Subsequently, the extract was subjected to gel permeation chromatography. A Sephadex LH-20 column (30 × 1 cm) was eluted with methanol in darkness at 4°C. Both IAA and IAM were recovered quantitatively at 1.4 column volumes (45 min, 0.7 ml/min).

At this stage the samples were subjected to HPLC (Perkin Elmer series 2, 5 μm Rosil C18 HL, 25 × 0.46 cm I.D.) and measured on line with a Shimadzu RF530 fluorescence detector (excitation at 285 nm and emission at 360 nm). Retention times of IAA and IAM for each system used are given in table 1. Taking into account the final extraction yield of each individual sample and the specific fluorescence of IAA and IAM, the original IAA and IAM concentrations were calculated and expressed in pmol/g fresh wt.

After a final purification and removal of all salts by reversed phase (water) HPLC the IAM-containing fraction was analyzed by mass spectrometry (63°C, 75 eV, Finigan type 3200, Data System 6000) and compared with reference IAM.

2.3. *Preparation of 3-[5(n)-^{3}H]IAM*

1.85 MBq 3-[5(n)-^{3}H]IAA (788 GBq/mmol, Amersham) was solubilized in 300 μl methanol and methylated ([^{3}H]Me-IAA) as in [13]. This reaction mixture was then treated with gaseous ammonia at room temperature for about 5 h. [^{3}H]IAM (RT 4.40 min) was separated quantitatively from [^{3}H]IAA (RT 7.40 min) and [^{3}H]Me-IAA (RT 9.00 min) by preparative reversed phase ion suppression HPLC (see table 1).

3. RESULTS

N. tabacum plants transformed with either gene *1* (rG1) or genes *6*a and *6*b (rG6a/6b) and untransformed control plants were extracted as described in section 2.

First, the extracts were analyzed by ion suppression reversed phase HPLC (fig.1A). The [^{3}H]IAA-containing fractions were collected, reduced under vacuo and subjected to ion-pairing reversed phase HPLC. Similar results were obtained by quantitation of the IAA peaks in both chromatographic systems (table 2).

The [^{3}H]IAM-containing fractions were successively analyzed by reversed phase (pH 6.7) HPLC and ion-pairing reversed phase HPLC (fig.1B,C). Quantitation of the [^{3}H]IAM containing peaks of rG1 gave in all chromatographic systems endogenous IAM concentrations of about 1500 pmol/g fresh wt, whereas in the untransformed tobacco control plant only 1.0 pmol/g fresh wt was found. Ion suppression

Table 1

Retention time of IAA and IAM

System		Retention time (min)	
		IAA	IAM
(A)	Ion suppression	7.40	4.40
(B)	Reversed phase (pH 6.7)	2.05	4.40
(C)	Ion-pairing	6.11	5.25
(D)	Reversed phase (H_2O)	–	4.47

Column: 5 μm Rosil C18 HL, 25 × 0.46 cm I.D. (A) MeOH-H_2O-HAc (40:60:0.05, v/v), 1.5 ml/min; (B) MeOH-0.01 M phosphate buffer, pH 6.7 (40:60, v/v), 1.5 ml/min; (C) MeOH-0.01 M tetrabutylammonium hydroxide in 0.001 M phosphate buffer (pH 6.6) (45:55, v/v), 1 ml/min; (D) MeOH-H_2O (40:60, v/v), 1.5 ml/min

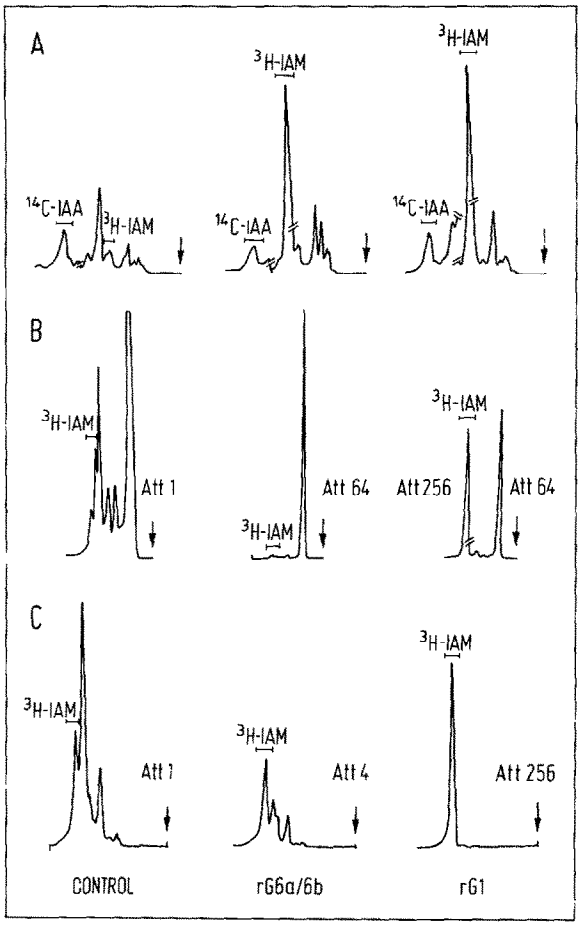

Fig.1. HPLC spectrofluorimetric elution profiles of an untransformed (control) and two transformed (rG6a/6b and rG1) plant extracts. The chromatographic systems are described in table 1. The [^{3}H]IAM containing peak in system A was analyzed in system B. Subsequently, the [^{3}H]IAM peak of system B was analyzed in system C. ↓, injection; ——, collected fraction; //, attenuation change. Attenuation in system A: IAA, att 16; IAM (control), att 32; IAM (rG6a/6b), att 64; IAM (rG1), att 256. The attenuations in systems B and C are indicated.

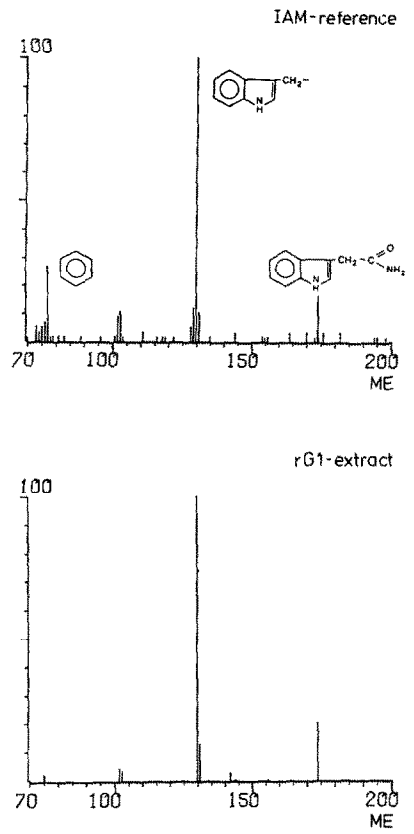

Fig.2. Mass spectra of synthetic IAM and a purified rG1 tobacco extract recorded at 63°C, 75 eV.

reversed phase HPLC analysis of rG6a/6b showed a [^{3}H]IAM containing peak corresponding with an apparent endogenous IAM concentration of 126 pmol/g fresh wt. Further purification of the tritiated IAM-containing peak showed the presence of a contaminating fluorescent compound which was resolved from IAM by reversed phase HPLC (pH 6.7) (fig.1B). This resulted in a

Table 2

Endogenous IAA and IAM concentration

Chromatographic system[a]	rG1		rG6a/6b		Control	
	IAA	IAM	IAA	IAM	IAA	IAM
Ion suppression	15	1410	11	126	12	–
Reversed phase (pH 6.7)	–	1473	–	4.6	–	1.1
Ion-pairing	18	1611	8.2	4.2	14.4	1.0

[a] cf. table 1

much lower endogenous IAM concentration (about 4 pmol/g fresh wt) consistent with two consecutive HPLC analyses (fig.1; table 2).

The purified IAM peam of a rG1 extract showed an identical mass spectrum as synthetic IAM (fig.2).

4. DISCUSSION

Here, we describe the isolation and analysis of *N. tabacum* regenerates transformed with either gene *1* (rG1) or genes *6a-6b* (rG6a/6b) as the only common T-DNA genes. Using different reversed phase HPLC systems, the endogenous IAA and IAM concentrations were analyzed in the two transformed (rG1 and rG6a/6b) tobacco plants and in control plants. No drastic differences in endogenous IAA concentrations were observed between the 3 plant types analyzed. However, the IAM content of rG1 was about 500–1000-times higher (about 1500 pmol/g fresh wt) compared to rG6a/6b and an untransformed control plant. The identity of IAM in rG1 plants was confirmed by its mass spectrum which was identical to the IAM reference spectrum. Confusion with indole-3-acetoldoxime was excluded since in the IAM spectra no significant fragmentation ion at *m/e* 157 [14] was found.

These data lead to the conclusion that the T-DNA gene *1* is responsible for the accumulation of IAM in transformed plants. Either gene *1* encodes an enzyme that synthesizes IAM or encodes a regulator protein that activates a pre-existing plant gene. Since gene *2* codes for an enzyme that converts IAM into IAA [8,9], we propose that the T-DNA pathway for auxin biosynthesis in crown gall cells is as follows: tryptophan ⟶ IAM ⟶ IAA. A similar auxin synthesis pathway has been described for the plant pathogenic bacterium, *Pseudomonas savastanoi* [15]. Based on this similarity, it seems likely that gene *1* encodes a tryptophan-2-monooxygenase.

The observation that *N. tabacum* (var.W38) plants transformed with an active gene *1* contain high amounts of IAM and normal amounts of IAA demonstrates that IAM cannot be converted at a significant level to IAA in tobacco plants. This is consistent with the observation that plants transformed with gene *1* grow and develop in a normal fashion.

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