

# *Agrobacterium* T-DNA gene 1 codes for tryptophan 2-monooxygenase activity in tobacco crown gall cells

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Cloned tobacco crown gall tissue induced by the *Agrobacterium tumefaciens* C58 T-DNA mutant pGV3132, defective for the T-DNA-encoded amidehydrolase (*iaaH*), accumulates about 1000-times more indole-3-acetamide (IAM) when compared to untransformed tobacco callus and crown gall tissue induced by a T-DNA mutant defective for gene 1. In vitro experiments demonstrated that this IAM accumulation is correlated with the active conversion of Trp into IAM. The results presented in this report provide biochemical evidence that the T-DNA gene 1 encodes a tryptophan 2-monooxygenase (*iaaM*) activity in transformed plant cells. This gene cooperates with the gene 2-encoded amidehydrolase (*iaaH*) in the T-DNA-controlled indole-3-acetic acid (IAA) biosynthesis pathway in crown gall cells.

<i>Indole-3-acetamide</i>	<i>Indole-3-acetic acid</i>	<i>Tryptophan 2-monooxygenase</i>	<i>T-DNA gene</i>
(Nicotiana tabacum, Agrobacterium tumefaciens)			

## 1. INTRODUCTION

Transformation of plant cells with *Agrobacterium tumefaciens* causes the formation of tumors which grow in in vitro culture in the absence of auxin and cytokinin. This specific property of crown gall tissue is the result of the transfer, stable integration and expression of the T-DNA into the plant nuclear DNA (review [1]). T-DNA mutants in gene 1 (*iaaM*) and/or gene 2 (*iaaH*) produce tumors that sprout shoots indicating that it is the combined activity of both genes which is responsible for the 'auxin effect' of the T-DNA [2–5].

It has been shown that gene 2 codes for an amidehydrolase which converts IAM into IAA [6–8]. The natural occurrence of IAM in plants is doubtful [9]. However, this compound has been shown to be an intermediate in IAA biosynthesis by the plant-pathogenic *Pseudomonas savastanoi* [10–13]. Much less is known about the function of gene 1 in crown gall cells. It has been demonstrated

that both genes 1 and 2 are needed to produce auxin effects, and that the *iaaM* gene of *P. savastanoi* is able to complement the T-DNA gene 1 deficiency in vivo [14,15]. Furthermore, it has been shown [16] that *Nicotiana tabacum* regenerates transformed with gene 1 contain high amounts of IAM.

This paper reports on the T-DNA gene 1-controlled IAM biosynthesis in tobacco crown gall tissues and on the in vitro tryptophan 2-monooxygenase activity which catalyses the conversion of Trp into IAM. The results are discussed in relation to the T-DNA-controlled IAA biosynthesis in tobacco crown gall cells.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains, plant material and tissue culture

The T-DNA mutants pGV3132 and pGV3845 are described in [5]. Transformed cell clones were

obtained by cocultivation of mesophyll protoplasts of *N. tabacum* cv. petit Havana SR1 [17,18] with agrobacteria as described [19]. The examined SR1 lines are described in table 1. The SR1 transformed clones were cultured on Linsmaier and Skoog mineral salts and vitamins [20], 3% sucrose, 0.8% Difco agar, without hormones. The SR1 callus line was cultured on the same medium supplemented with 2 mg/l NAA and 0.3 mg/l BAP.

## 2.2. Extraction and analysis of IAM

The extraction, purification and quantitative analyses of IAM are described in [16].

## 2.3. Enzyme sources and assay

Frozen tissues (10 days after subculture, 6 g fresh wt) were homogenized in 20 ml cooled buffer (0.1 M Tris-HCl, pH 8, 10% sucrose, 5 mM EDTA, 10 mM MgCl<sub>2</sub> and 10 mM 2-mercaptoethanol). After centrifugation (20000 × g, 15 min, 4°C) the supernatant fluids were concentrated by precipitation with ammonium sulfate (90% saturation). Precipitates were dissolved in 3.0 ml assay buffer (0.1 M Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 40 mM KCl, and 10 mM 2-mercaptoethanol) and dialysed at 4°C against 4 × 1 l of this buffer.

Standard enzyme assays were carried out in 500 µl enzyme extract containing 160 nmol Trp and 86000 dpm L-[G-<sup>3</sup>H]Trp (2.46 TBq/mmol, Amersham) which corresponded to a final specific activity of 540 dpm/nmol [<sup>3</sup>H]Trp in the reaction mixture. At defined time intervals at 30°C, 50 µl of the reaction mixture was added to 40 µl MeOH (+ 0.1 M HCl), immediately frozen in liquid nitrogen and kept at -20°C until analysis by HPLC. The protein content of the enzyme extract

was assayed by the Coomassie brilliant blue method [21].

## 3. RESULTS

Table 2 shows that tobacco SR1 crown gall tissues transformed with *A. tumefaciens* C58 T-DNA mutant pGV3132 and therefore containing an active gene 1, but no gene 2 activity, accumulated large amounts of IAM. An endogenous level of 17160 pmol IAM/g fresh wt was found 12 days after subculture. In comparison, untransformed SR1 callus and the SR1 3845 line, defective for gene 1 but with the amidohydrolase activity encoded by gene 2, contained 31.0 and 10.9 pmol IAM/g fresh wt, respectively.

Table 2  
Endogenous IAM concentrations

Cell line	IAM (pmol/g fresh wt)
SR1 callus	31
SR1 3132	17160
SR1 3845	11

Table 1  
SR1 cell lines

Cell line	Origin of T-DNA	Affected T-DNA genes	Morphology
SR1 callus	—	—	undifferentiated
SR1 3132	pGV3132	gene 2 ( <i>iaaH</i> )	teratoma
SR1 3845	pGV3845	gene 1 ( <i>iaaM</i> )	teratoma

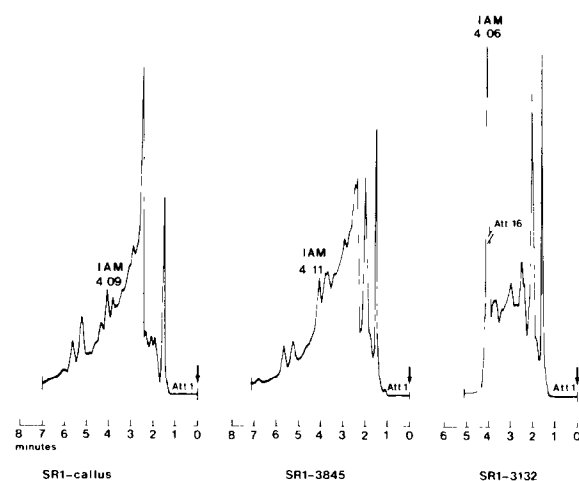


Fig.1. IS-HPLC spectrofluorimetric elution profiles of the tryptophan 2-monooxygenase reaction mixtures of SR1 callus, SR1 3132 and SR1 3845 extracts. [<sup>3</sup>H]Trp elutes at 19.5 min (not shown). IS-HPLC, 5 µm Rosil (18 HL, 25 × 0.47 cm IS, MeOH-H<sub>2</sub>O-HAc (40:60:0.05, v/v), 1.5 ml/min. Detection: on-line spectrofluorimeter (Shimadzu 530) λ<sub>ex</sub> 285 nm, λ<sub>em</sub> 360 nm.

The *in vitro* conversion of Trp into IAM was tested in cell-free extracts from SR1 3132 and compared to extracts from SR1 3845 and to SR1 callus. Fig.1 shows that in extracts from the gene 2-deficient SR1 3132 tumor line, Trp was actively converted into IAM. The identity of the IAM peak obtained by IS-HPLC was further qualitatively and quantitatively confirmed by two HPLC analyses using subsequently neutral reversed-phase (RP) and ion-pairing reversed-phase (IP) HPLC (fig.2). Furthermore, the specific activity of the [ $^3$ H]IAM fraction obtained after the final IP-HPLC was 590 dpm/nmol. This value corresponds to the specific activity of [ $^3$ H]Trp

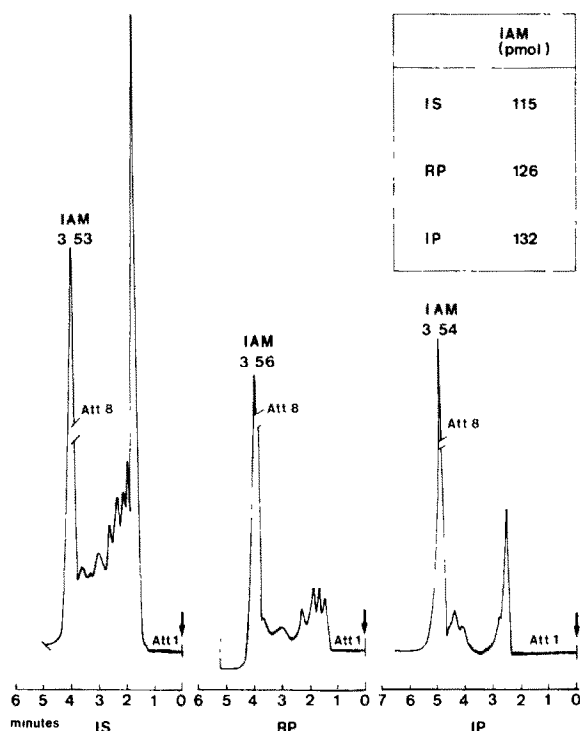


Fig.2. Identification of the IAM produced in an SR1 3132 enzyme extract by 3 subsequent analytical HPLC runs. The [ $^3$ H]IAM-containing peak in IS was analysed by RP and subsequently the [ $^3$ H]IAM peak of RP was analysed by IP. Column and detection, see fig.1. IS-HPLC, ion suppression, MeOH-H<sub>2</sub>O-HAc (40:60:0.05, v/v), 1.5 ml/min; RP-HPLC, neutral reversed-phase, MeOH-0.01 M phosphate buffer, pH 6.7 (40:60, v/v), 1.5 ml/min; IP-HPLC, ion-pairing, MeOH-0.01 M tetrabutylammonium hydroxide, pH 6.6 (45:55, v/v), 1 ml/min.

Table 3

Specific tryptophan 2-monooxygenase activity

Cell line	U <sup>a</sup> /mg protein
SR1 callus	17
SR1 3132	790
SR1 3845	7

<sup>a</sup> 1 unit = 1 pmol IAM/h

(540 dpm/nmol) in the reaction mixture, providing good evidence that all of the measured IAM originated from the Trp added to the reaction mixture. In extracts of SR1 callus and SR1 3845 lines a small, but significant, conversion of Trp into IAM was observed (fig.1). Taking into account the reaction time and the protein content of each reaction mixture, the specific tryptophan 2-monooxygenase activity was calculated and is presented in table 3. One unit of activity corresponds to the production of 1 pmol IAM/h. The SR1 3132 extract showed a specific enzyme activity of 790 U/mg protein whereas in extracts of SR1 callus and SR1 3845, respectively, 0.9 and 1.4 U/mg protein were found.

#### 4. DISCUSSION

The T-DNA genes 1 and 2 of the Ti plasmid of *A. tumefaciens* are involved in the biosynthesis of IAA in transformed plant cells. It has been shown that gene 2 (*iaaH*) codes for an amidohydrolase which catalyses the conversion of IAM into IAA [6-8].

Recently, it has been shown [15] that the *iaaM* gene of *P. savastanoi* complemented T-DNA gene 1 deficiency *in vivo*. Significant sequence homology of the T-DNA gene 1 with the *iaaM* gene of *P. savastanoi* [22] and the accumulation of IAM in *N. tabacum* plants transformed with gene 1 [16] have indicated that the T-DNA gene 1 codes for a tryptophan 2-monooxygenase.

The results presented here show that an accumulation of IAM occurs in the gene 2-deficient SR1 3132 tobacco crown gall line. Our data provide biochemical evidence that the T-DNA gene 1 codes for tryptophan 2-monooxygenase activity in transformed plant cells. Indeed, cell-free extracts of the SR1 3132 (2<sup>-</sup>) line were shown to convert ac-

tively Trp into IAM, the identity of the reaction product being confirmed by three subsequent analytical HPLC runs and by the specific activity of the [ $^3\text{H}$ ]IAM formed. The monooxygenase activity measured in SR1 3132 extracts taken 10 days after subculture corresponded to an IAM production of about 1800 pmol/h per g fresh wt. This activity is certainly sufficient to account for the endogenous level of IAM which accumulates in the *iaaH*-deficient SR1 3132 tumor line. The very low Trp  $\rightarrow$  IAM conversion observed in the SR1 callus and SR1 3845 (1 $^-$ ) might be attributed to an aspecific peroxidase activity as was also observed in *P. savastanoi* [10].

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