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Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation

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Abstract

Transformed root cultures of *Nicotiana rustica* have been generated in which the gene from the yeast *Saccharomyces cerevisiae* coding for ornithine decarboxylase has been integrated. The gene, driven by the powerful CaMV35S promoter with an upstream duplicated enhancer sequence, shows constitutive expression throughout the growth cycle of some lines, as demonstrated by the analysis of mRNA and enzyme activity. The presence of the yeast gene and enhanced ornithine decarboxylase activity is associated with an enhanced capacity of cultures to accumulate both putrescine and the putrescine-derived alkaloid, nicotine. Even, however, with the very powerful promoter used in this work the magnitude of the changes seen is typically only in the order of 2-fold, suggesting that regulatory factors exist which limit the potential increase in metabolic flux caused by these manipulations. Nevertheless, it is demonstrated that flux through a pathway to a plant secondary product can be elevated by means of genetic manipulation.

Introduction

The value of root cultures, transformed by *Agrobacterium rhizogenes*, for the study of secondary product formation by tissues grown *in vitro* [8, 9] has become widely recognised. Such cultures display secondary product synthesis with similar characteristics to that of the parent plant species, show chromosomal stability over long periods of growth *in vitro* [1] and are amenable to genetic manipulation [10, 11, 30]. These properties enable the long-term effects of introducing and over-expressing genes coding for enzymes which are important in pathways leading to secondary product synthesis to be assessed.

An important early intermediate in the synthesis of nicotine and polyamines in *Nicotiana* species is putrescine, which may be derived directly from ornithine by decarboxylation or from arginine via agmatine and carbamoyl putrescine (Fig. 1). Previous results have shown that feeding low levels of putrescine (1 to 5 mM) can have a stimulatory effect on the production of nicotine by transformed root cultures of *N. rustica* [32]. In this paper we report on the effect of introducing an ornithine decarboxylase (ODC) gene into

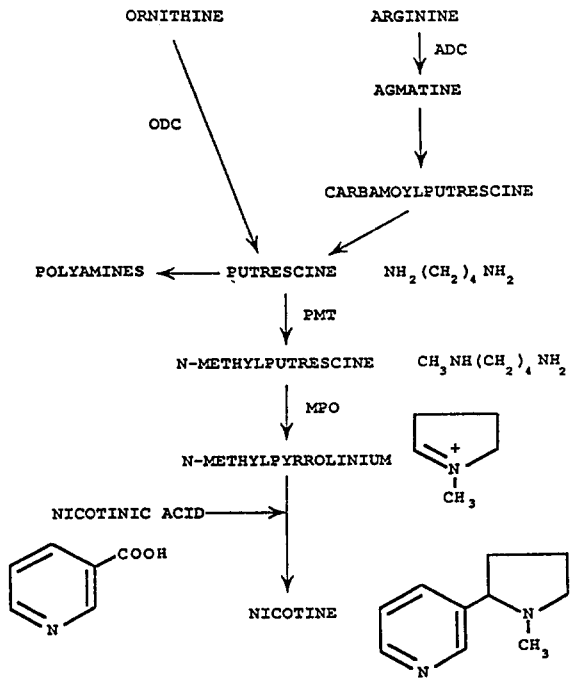

Fig. 1. Patway showing the formation of nicotine from ornithine or arginine and nicotinic acid.

transformed root cultures under a constitutive promoter in order to enhance the potential supply of endogenous putrescine.

The complete coding sequence for ornithine decarboxylase (EC 4.1.1.17) is known from several sources, including the yeast *Saccharomyces cerevisiae* [5]. The gene from this source (hereafter referred to as the yODC gene) contains no introns, making it a suitable candidate for overexpression in transformed root cultures of *Nicotiana* in order to assess the effects this may have on nicotine biosynthesis in transgenic tissue. The constitutive promoters CaMV35S [22] and CaMV35S with a duplicated enhancer sequence (designated CaMV35S^{++}) [17] were chosen to facilitate constitutive expression of the gene in transformed roots after introduction using a binary vector/*A. rhizogenes* delivery system [10]. Previous results have shown these promoters to be active in transformed roots of *Nicotiana* and to allow the constitutive expression of reporter genes in transformed roots of *N. rustica* while the cultures remain in the growth phase [25].

Materials and methods

Vector construction

DNA manipulations were carried out generally as described by Maniatis *et al.* [20].

Foreign genes were introduced into transformed roots by co-transformation with the pBin19-based vectors pF1E20 and pF1H10 described previously [11]. These both contain the CaMV35S promoter [22], the latter with the addition of an upstream duplicated enhancer sequence [17]. Both vectors contain the CaMV35S polyadenylation sequences.

A plasmid containing the yeast coding sequence for the ODC structural gene (pM13MP10) was obtained from Fonzi and Sypherd [5]. A *Hin*d III : *Bsm* I fragment was isolated, comprising the coding sequence but excluding the 3′ polyadenylation and the 5′ promoter sequences. This fragment was cloned into the *Hin*d III : *Sph* I site of pMTL24, allowing the coding sequence of the yODC gene to be isolated as a *Xba* I fragment of about 1.65 kb. This fragment was inserted into the *Xba* I sites of the expression cassettes. Control tissues contained a chloramphenicol 3-*O*-acetyltransferase (CAT) gene, inserted into pFIH10, instead of the yODC gene.

Tissue culture

Transformed roots of *Nicotiana rustica* var. V12 (a highly inbred line) were induced using *A. rhizogenes* strains carrying binary vectors and selected as described previously [8, 10]. A number of lines, each derived from a single root tip from individual wound spots, were established and analysed separately for each promoter type in order to assess the range of biochemical phenotypes seen after transformation. Care was taken to ensure that all root lines were subcultured at the same time, and that all lines were cultured under precisely the same conditions to ensure that environmental factors did not interfere in the comparison between lines.

Southern and northern hybridizations

Extraction of DNA from hairy roots and Southern blotting were as described previously [12]. RNA extraction was carried out by grinding 10 g of blotted dry root tissue in liquid N_2 and transferring to 20 ml of a buffer composed of 400 mM NaCl, 50 mM Tris pH 9, 1% w/v SDS, 1 mM aurintricarboxylic acid (Sigma), 50 mM EDTA pH 8.0 and 10 mM dithiothreitol for 5 min at 37 °C followed by two phenol/$CHCl_3$ and two $CHCl_3$ extractions. 25 ml of supernatant was added to 15 ml of 8 M LiCl and left overnight at 0 °C to allow RNA to precipitate. The RNA was pelleted by centrifugation at 12000 *g* for 30 min, washed twice in 10 ml of 3 M sodium acetate pH 6.0 and finally once in 70% (v/v) ethanol. The pellets were partially dried and dissolved in 1 ml Analar diethylpyrocarbonate-treated H_2O. This method, derived from Jepson [14], routinely gave RNA preparations from hairy roots (100–200 μg RNA/g tissue) showing very little polysaccharide contamination or degradation of rRNA. This contrasts with previously reported procedures (e.g. [19]), in which polysaccharide contamination was a persistent problem. The isolation of poly(A)$^+$ RNA was carried out using oligo dT cellulose (Pharmacia) columns, as described by Maniatis *et al.* [20].

Northern blotting was carried out using 5 μg poly(A)$^+$ RNA in formaldehyde denaturing gels as described by Fourney *et al.* [6]. A BRL DNA ladder of 0.16–1.77 kb was used for the estimation of transcript sizes. Slot blots were carried out in an apparatus (BRL, Bethesda) using 3 μg poly(A)$^+$ RNA (as measured by OD_{260}, OD_{230} and OD_{280} values [20]) treated at 70 °C for 10 min and absorbed onto nitrocellulose filters in 3 × SSC (1 × SSC = 150 mM NaCl + 15 mM Na citrate, pH 7.5) and baked at 80 °C for 1 h. 100–150 ng DNA was ^{32}P-labelled by the method of Feinberg and Vogelstein [4] and hybridized to northern blots for 16 h at 42 °C in buffer composed of 50% deionized formamide, 6 × SSPE (1 × SSPE = 150 mM NaCl + 10 mM NaH_2PO_4 + 1 mM EDTA, pH 7.5), 5 × Denhardt's solution, 0.5% SDS and 0.2% denatured salmon sperm DNA. Hybridisation was followed by 2 × 30 min washes in 2 × SSPE, 0.2% SDS and 1 × 30 min wash in 0.2 × SSPE, 0.02% SDS at 42 °C. Filters were exposed to preflashed Fuji RX film for 2–5 days at −70 °C with the use of intensifying screens. Poly(A)$^+$ RNA slot blots were also hybridized to ^{32}P-labelled DNA coding for ribosomal RNA [7] to check whether the level of rRNA in each preparation of poly(A)$^+$ mRNA was the same, which was confirmed to be the case.

Enzyme assays

Ornithine decarboxylase, arginine decarboxylase (ADC; EC 4.1.1.19), putrescine N-methyltransferase (PMT) and N-methylputrescine oxidase (MPO) were extracted and assayed as described by Robins *et al.* [27].

Protein determination

Protein was determined by the dye-binding method [2] using the Bio-Rad Laboratories dye concentrate as described in their literature and BSA fraction V (Sigma) as the protein standard.

Polyamine analysis

Polyamines were extracted and prepared for analysis essentially as described by Hiatt and Malmberg [13]. Quantitation of the free or acid-liberated polyamines was based upon HPLC of their dansyl derivatives. Good results were obtained at room temperature by using a Spherisorb ODS2 3 μm column (Pharmacia, Milton Keynes, UK) eluted with a water: acetonitrile gradient (Solvent A = 30% acetonitrile, solvent B = 90% acetonitrile; solvent profile 15% B to 100% B over 12 min, then 100% B for a further 8 min; flow 0.75 ml/min). A fluorescence detector was routinely employed (excitation 335 nm, emission 510 nm).

N-Methylpyrrolinium assay

The N-methylpyrrolinium ion was estimated as its cyanide adduct [21]. Roots were homogenized in 20 mM H_2SO_4 (5 ml per 1 g), the homogenate extracted with ether, and the aqueous phase neutralized and split into 2 portions. To one was added excess KCN. Both portions were then made alkaline (ca pH 11) and nicotine alkaloids and the newly formed 1-methyl-2-cyanopyrrolidine were extracted into ether. Following removal of the ether under reduced pressure, the residues were dissolved in 0.1 M $(NH_4)H_2PO_4$ containing 1% (v/v) acetonitrile. HPLC analysis was carried out using a Nucleosil 10SA (Jones Chromatography) and a μBondapak C_{18} (Waters) column connected in series, eluted with 0.1 M $(NH_4)H_2PO_4$ + 1% (v/v) acetonitrile at 1.2 ml/min and monitored at 200 nm. Although the major alkaloids and 1-methyl-2-cyanopyrrolidine were well separated, minor components sometimes interfered, and in these cases N-methylpyrrolinium was estimated by the difference between the KCN-treated and control extracts. Standard N-methylpyrrolinium was obtained using pea diamine oxidase to oxidize a solution of N-methylputrescine to completion in a manner analogous to that described for tobacco N-methylputrescine oxidase by Mizusaki *et al.* [21].

Nicotine assay

Procedures for the extraction and assay of nicotine by HPLC were as described previously [23, 26].

Statistical analysis

Control root lines were assumed to be normally distributed with respect to the observed values for each of the biochemical attributes investigated. Individual manipulated lines or groups of lines were compared with the controls, the null hypothesis being that manipulation was without measurable effect. In the case of individual manipulated lines this involved testing the significance of the observed deviation from the mean of control values, while a t-test for the significance of the difference between two means was employed when comparing manipulated and control groups.

Results and discussion

Integration of the yODC gene into the genome of N. rustica *and its effect on total ODC activity*

The coding sequence for the yODC gene was inserted into each expression cassette on a *Xba* I fragment and, as described in Materials and methods, was introduced into transformed roots of *N. rustica*. The presence of an integrated yODC gene in the genome of root cultures derived by this method was confirmed by Southern blotting. In the case of the yODC gene under the control of the CaMV35S^{++} promoter, copy number was shown to range from 3 to 7 inserts per genome (Fig. 2). A number of lines in which the ODC gene was placed under the control of the CaMV35S or nopaline synthase promoters were also obtained and were each shown to contain several copies of the intact yODC gene (data not shown). The presence of the CAT gene in control tissue was similarly demonstrated (data not shown).

A number of lines containing yODC under the CaMV35S^{++} promoter were assessed for the level of activity of ODC and the metabolically related enzymes ADC, PMT and MPO (see Fig. 1). Activity in these and control lines, determined at 7 or 8 days after subculture, when endogenous activity of all four enzymes is high (R.J. Robins, unpublished results) and at 21 to 26 days, when endogenous activity is very low, is shown in Fig. 3. Although some lines containing CaMV35S^{++}-yODC showed little difference from controls, line numbers 8 and 15 contained levels of ODC activity about 3-fold greater than that found in controls at the period of maximum expression of the endogenous ODC (difference significant at $p < 0.05$). When examined at a late

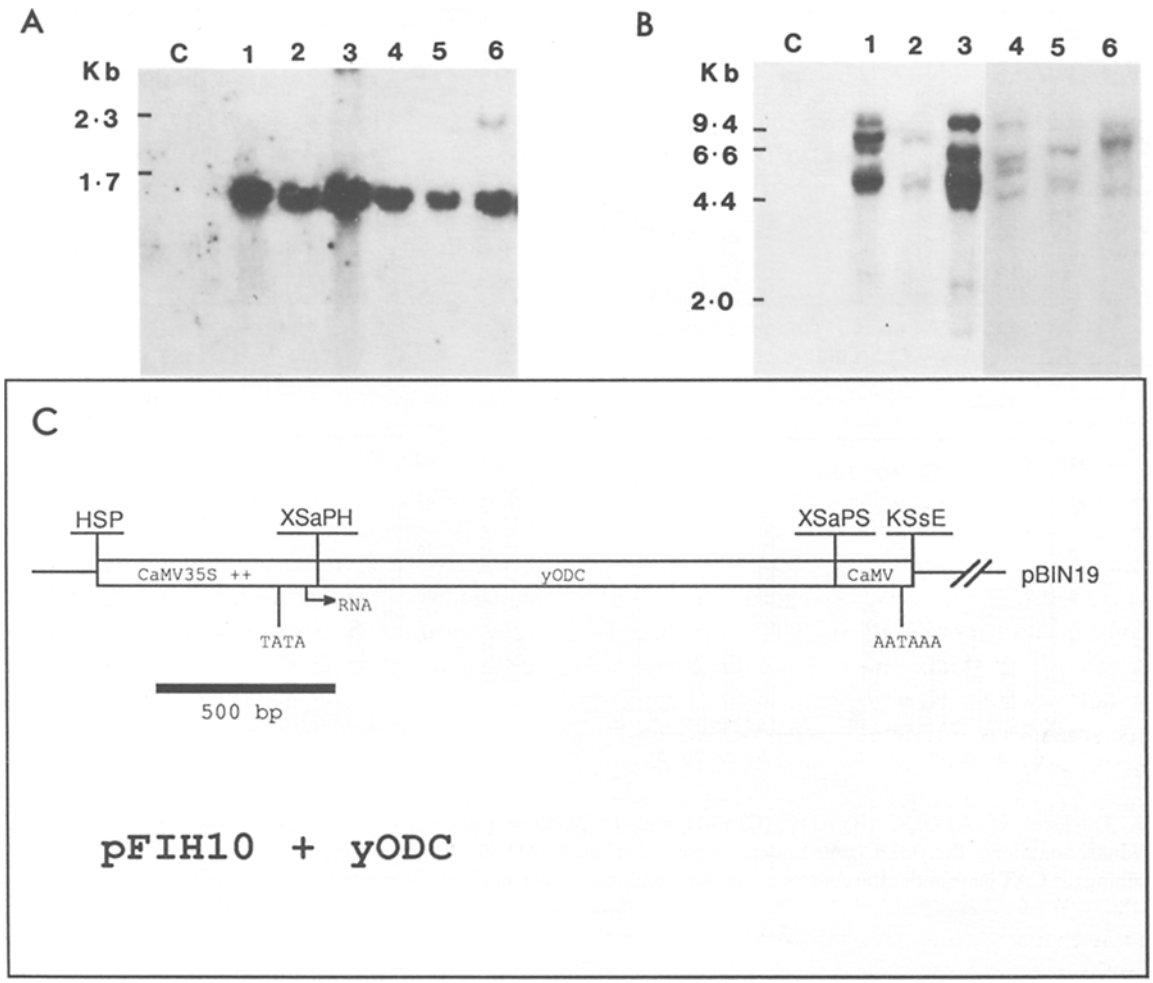

Fig. 2. Southern blot of transformed root lines of *Nicotiana rustica* showing presence of the coding sequence of the gene for ornithine decarboxylase from yeast.

A. DNA was digested with *Xba* I before electrophoresis. Lanes 1–6 (corresponding to roots lines 14, 15, 1, 3, 6 and 8, respectively) contained the yODC gene under the control of the CaMV35S $^{++}$ promoter, as shown diagrammatically in part C. Control tissue (C; line 24), contained the CAT gene under the control of the CaMV35S $^{++}$ promoter. The filter was probed with a ^{32}P-labelled coding sequence of the yODC gene. The presence of a 1.65 kb fragment in all transformed lines, but not the control, shows that the coding sequence for the yODC gene was structurally intact in all lines.

B. DNA from lines described above was digested with *Eco* R1 and probed with a ^{32}P-labelled copy of the yODC coding sequence. This digestion ensured that the probe hybridized to sequences across integration borders. Each band of different molecular weight represents a separate integration event.

C. Diagrammatic representation of the yeast ODC gene driven by the CaMV35S $^{++}$ promoter in pFIH10. H = *Hin*d III, S = *Sph* I, P = *Pst* I, X = *Xba* I, Sa = *Sal* I, K = *Kpn* I, Ss = *Sst* I, E = *Eco* RI.

stage in the growth cycle, these lines continued to show a high level of activity, which at this stage was 10 to 20 times in excess of the mean of that found in control tissues of similar age. Line 6 similarly showed greatly enhanced ODC activity late in the growth cycle (significantly different from controls at $p < 0.01$), as well as a high peak ODC activity (Fig. 3A). At least 3 of the 6 lines examined, therefore, show evidence of the yODC gene having affected measurable ODC activity. Other lines may have been similarly affected, but to an extent insufficient for their

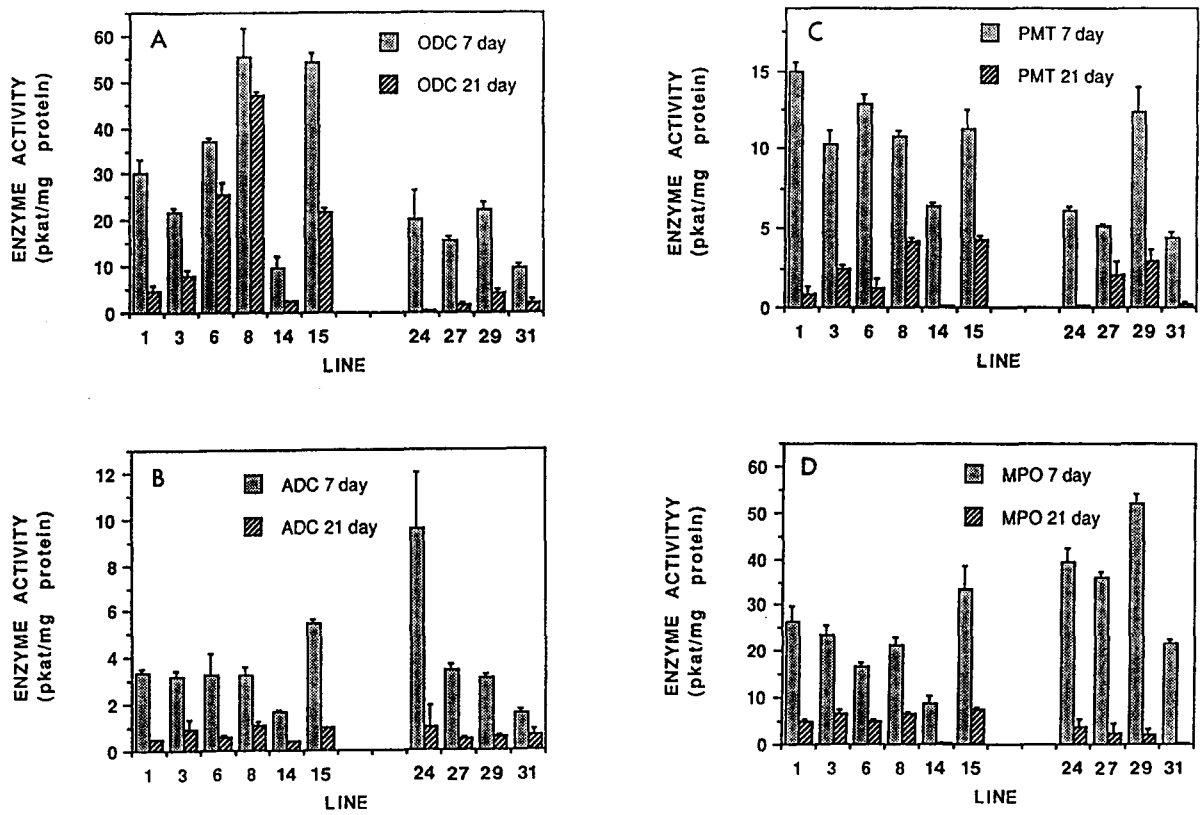

Fig. 3. The levels of (A) ODC, (B) ADC, (C) PMT and (D) MPO in a number of lines of *N. rustica*. Lines 1 to 15 represent individuals containing the yODC gene under the control of the CaMV35S$^{+\,+}$ promoter, while lines 24 to 31 are control lines containing the CAT gene under the control of the same promoter construct. Values shown represent duplicate assays on duplicate flasks ± s.e.

detection amidst the background variation which exists in control lines. In contrast to these observations, fluctuations in the levels of ADC, PMT and MPO in lines containing the yODC gene fell within the range determined for the controls at either time perod (Fig. 3B, C, D), though there is a suggestion that the mean PMT activity early in the growth cycle might have been slightly increased.

Several lines containing the yODC under the control of the CaMV35S promoter alone were also studied and did not show a level of ODC activity which was significantly elevated over that of the controls at either stage in the growth cycle (data not shown).

Detailed analysis of clone 8

Clone 8 was selected for more detailed examination on the basis of having high ODC levels at both time points studied. Figure 4 shows a time course of expression of ODC in this line and in control line 24. As is clearly seen, the level of ODC activity was about 2.5-fold higher than in the control at day 7 and remained high until day 24 when the culture had entered stationary phase. The control, in contrast, showed a decline in activity after day 7 to a very low value at day 24, in keeping with that observed in a number of other wild-type *Nicotiana* (R.J. Robins, W.R. McLauchlan and E. Bent, unpublished data) and *Datura* [24, 27] transformed root lines. The temporal activity profile for ODC in line 8 was similar, however, to that observed for CAT activity

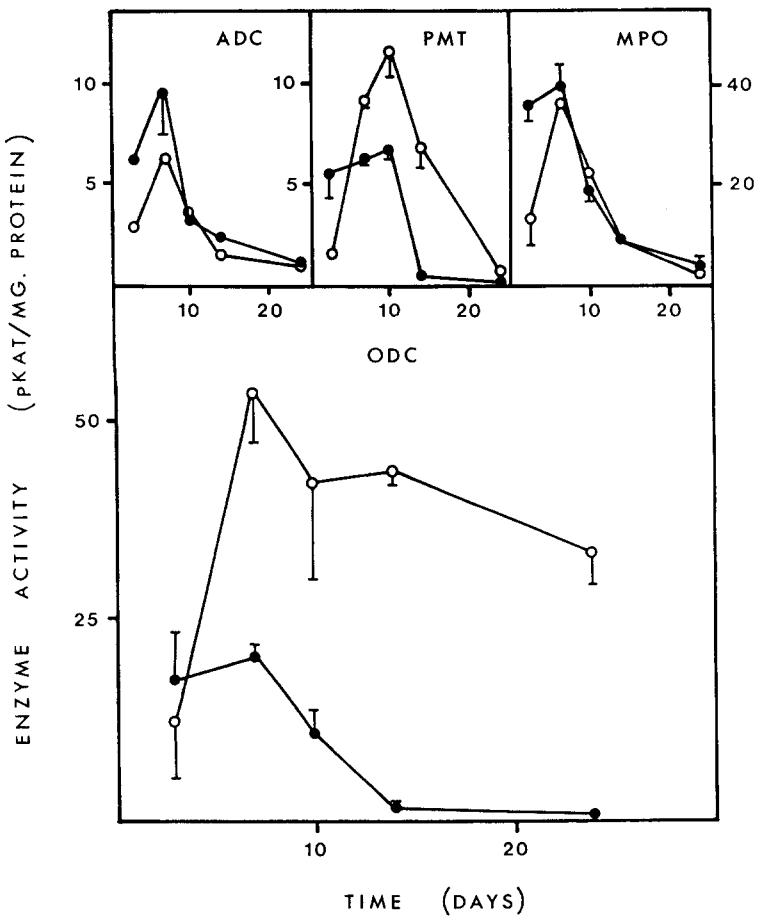

Fig. 4. The patterns of activity for ODC, ADC, PMT and MPO in line 8 (o) containing the yODC gene and line 24 (•) containing the CAT gene, both under the control of the CaMV35S^{++} promoter. Values represent duplicate assays on duplicate flasks ± s.e.

under control of the same promoter in mid and late stages of culture [25] in showing a slow and steady decline from the peak of activity as the cultures entered stationary phase. Unlike the situation with ODC, ADC levels in line 8 resembled those of the control, as did the MPO levels. PMT activity profiles apparently differed slightly between the two lines, but the precise significance of this effect is at present unclear.

Northern blotting of RNA from line 8 showed the presence of a 2.1 kb transcript homologous to the yODC gene, suggesting a poly(A)$^+$ tail of about 100 nucleotides (Fig. 5A). The presence of this transcript was readily detected in tissue extracted at early, mid and late stages in the growth cycle (Fig. 5B). Interestingly no signal was detected in the case of the control line, implying that the plant ODC differs significantly from yeast ODC at the nucleic acid level. At the present time it is not possible to examine the expression of the endogenous ODC gene in transformed roots as this gene has yet to be cloned, and antibodies are not available.

Two main effects of insertion of the yODC gene under the control of the CaMV35S^{++} promoter are seen to have occurred in line 8 and are apparent to varying degrees in other lines (Fig. 3), the variation possibly reflecting differences in the expression of the yODC gene relative to endogenous ODC in different lines. Firstly, the level of ODC activity at the peak of expression has been stimulated. Secondly, the time-frame of expression has been altered. The maximum level of stimulation, about 3-fold at the peak of endo-

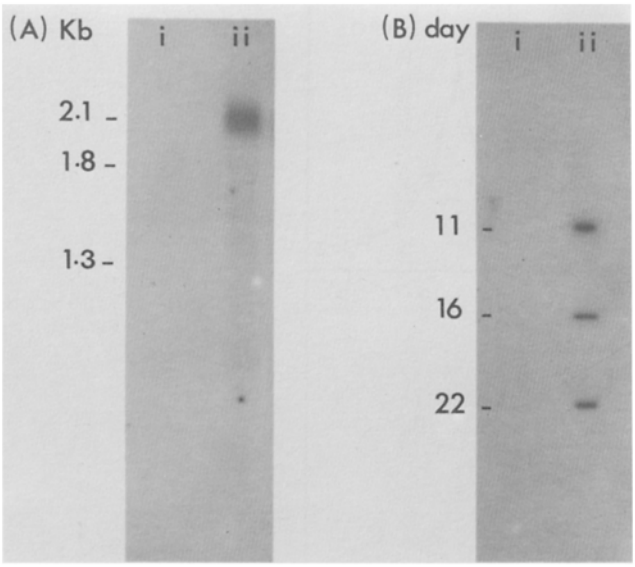

Fig. 5. Expression of the yODC gene in transformed line 8. A. poly(A) mRNA was extracted (i) from control line 24 containing the CAT gene and (ii) from line 8 containing the yODC gene, both under the control of the CaMV35S^{++} promoter. The blot was hybridized with ^{32}P-labelled yODC DNA. The presence of a transcript in line (ii) of ~2.1 kb suggests a poly(A)$^{+}$ tail of about 100 nucleotides.
B. Poly(A)$^{+}$ mRNA was isolated from line 8 at day 11 (early-mid growth phase), day 16 (mid-late growth phase) and day 22 (late growth into early stationary phase) after subculture. mRNA was extracted from control line 24 containing the CAT gene under the CaMV35S^{++} promoter at the same time points of its growth cycle. A slot blot of mRNA hybridized with the ^{32}P-labelled yODC gene showed no hybridization to mRNA from the control line (i), but positive hybridization to mRNA from the yODC line (ii). The yeast ODC message is present in tissue throughout the growth cycle.

genous activity, is less than that seen for certain other metabolically important enzymes being expressed under the control of the CaMV35S promoter. For example levels of EPSP synthase were increased 40-fold [29] and glutamine synthase 35-fold [3], even without the use of constructs containing a duplicated enhancer sequence. The relatively modest stimulation of peak ODC activity observed may be related to the fact that ODC is a tightly regulated gene, the complexity of this regulation reflecting its important role in the synthesis of polyamines. In particular there is considerable post-transcriptional [16] and post-translational regulation [18], and the yODC gene construct will presumably not be independent of such regulation which may act to buffer any biochemical effects of the genetic manipulation.

Due to the stimulation of ODC activity in some lines, however, it was nevertheless pertinent to examine the levels of putrescine, polyamines and alkaloids in manipulated and control tissues to assess the effects the observed additional enzyme activity might have had on metabolite pool sizes.

Effects of yODC over-expression on intermediary metabolite pools in transformed root lines

Amine levels in roots were assayed at day 7–10, near the peak of ODC activity, and later on in the growth cycle at day 18–23. Free putrescine, the immediate product of the ODC reaction, was found to be present at higher levels in several CaMV35S^{++}-yODC lines than in control lines (Fig. 6A). Line 6 showed a level about twice that of the mean of controls both early on and late in the growth cycle (significantly different from controls at $p < 0.05$ late in the growth cycle), while line 8 also showed an elevated free putrescine level late in the growth cycle (significant at $p < 0.05$). Stimulation of putrescine might also have occurred in some of the other lines, but the effects were not significant when individual lines were compared to the control population. In contrast to these results for free putrescine, no significant differences were observed in the levels of the polyamines spermidine plus spermine (Fig. 6D). This confirms the observation of Hiatt and Malmberg [13] that the formation of these compounds from putrescine is strongly regulated, so that a constant level is maintained irrespective of putrescine supply. A similar result was obtained for acid-labile putrescine conjugates (Fig. 6B). Although line 6 showed a somewhat elevated level late in the growth cycle, the observed value still did not exceed that which might reasonably be expected for a control line ($p > 0.05$).

The effect of overexpressing the yODC gene on putrescine-derived intermediary metabolites involved in nicotine biosynthesis (see Fig. 1) was

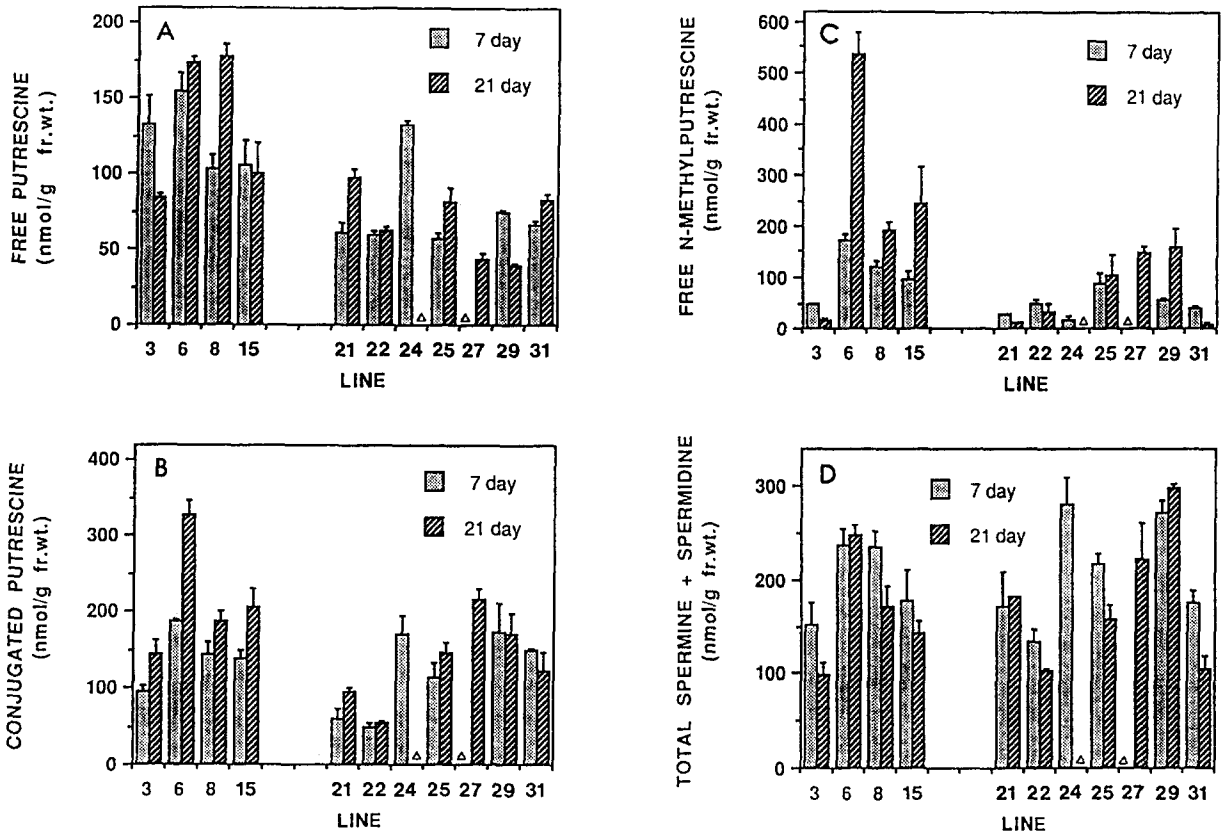

Fig. 6. The levels of various pools of ornithine-derived amines in manipulated and control transformed roots of *N. rustica*. (A), Free putrescine; (B), Conjugated putrescine; (C), Free N-methylputrescine; (D), Total spermidine plus spermine. Values represent duplicate analyses of duplicate samples ± s.e. △ = Not determined.
Lines 3 to 15 contain the yODC gene under the control of the CaMV35S^{++} promoter, and lines 21 to 31 are control lines containing the CAT gene under this promoter.

made somewhat difficult to determine by the substantial level of variation seen between individual control lines in relation to the expected magnitude of any effects arising from genetic manipulation. Nevertheless, in the case of N-methylputrescine, line 6 did show significantly enhanced steady-state levels both early on and later in the growth cycle (significant at $p < 0.05$ and $p < 0.01$ respectively) (Fig. 6C). Analysis of the levels of N-methylpyrrolinium, however, showed no significant differences between control lines (62–193 nmol/g fr.wt; N = 3) and manipulated lines (85 nmol and 134 nmol/g fr. wt., for lines 6 and 8 respectively), at least in mid growth cycle (day 15–18).

Thus, while the effects of over-expressing the yODC gene upon levels of putrescine and derivatives are not dramatic, some lines have elevated putrescine and N-methylputrescine levels, particularly in the later stages of growth when the enzymes in the metabolic pathway leading to N-methypyrrolinium are poorly expressed in control tissues. In other lines, and with other metabolites, no clear increases are apparent however. It would appear that while the levels of certain metabolites can show a degree of variation in control tissues, there must be internal factors which tend to buffer the effects of expression of the yODC gene and prevent large fluctuations from occurring in the levels of metabolic intermediates in the root tissue. Nevertheless the levels of putrescine and N-methylputrescine can be

stimulated by such manipulation, confirming that the yODC gene is biochemically active and raising the possibility that the flux towards nicotine can also be affected.

Effects upon nicotine biosynthesis

Introduction of the yODC gene under the control of the CaMV35S^{++} promoter was found to be capable of influencing the nicotine contents of root lines. Thus, whereas the mean nicotine content at day 14 of control lines containing the CAT gene under the control of the CaMV35S^{++} promoter was 2.28 ± 0.22 μmol/g fr.wt, that for 6 lines containing the yODC gene with this promoter was 4.04 ± 0.48 μmol/g fr.wt. This finding indicated that, on average, over-expression of the yODC gene by the CaMV35S^{++} promoter had significantly ($p < 0.05$; t-test) enhanced nicotine accumulation. Examination of individual lines revealed that 3 out of the 6 lines containing yODC showed nicotine levels approximately twice the mean of controls (Fig. 7). Expressing the yODC gene under the CaMV35S promoter (without the duplicated enhancer sequence) on the other hand had little influence on nicotine levels in the population, the mean of 8 lines being 2.65 ± 0.40 μmol/g fr.wt ($p > 0.4$; t-test). This indicates a requirement for a very strong promoter before the yODC gene is able significantly to affect nicotine metabolism, in line with our observations on the effects of promoter strength on the expression of ODC activity (see above).

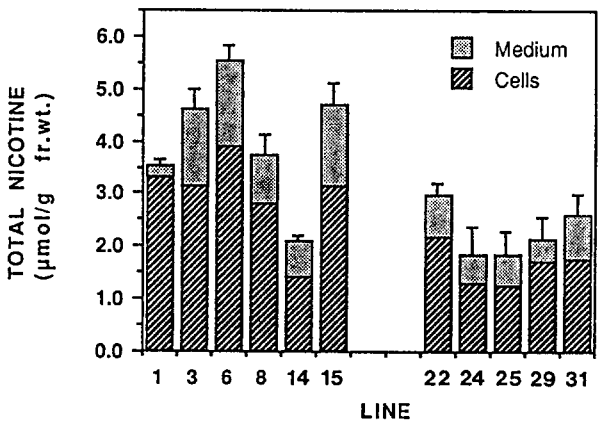

Fig. 7. Nicotine concentrations in manipulated transformed lines of *Nicotiana rustica* 14 days after sub-culture. Bars show the total nicotine in the system per g fresh weight, the hatched area representing the extra-cellular portion. Results are the means of analyses on triplicate flasks, with the standard error for total nicotine content also being shown.
Lines 1 to 15 contain the yODC gene under the control of the CaMV35S^{++} promoter and lines 22 to 31 are control lines which contain the CAT gene under the control of this promoter.

Although the overall effect of expressing the yODC gene under the CaMC35S^{++} promoter is to increase both ODC activity and nicotine levels in the population of root clones, there is not a precise correlation between ODC levels and nicotine levels in individual lines, suggesting that additional factors affect nicotine production. For instance, CaMV35S^{++}-yODC line 8, which had strongly elevated ODC levels relative to controls (Fig. 3), produced only slightly more nicotine than did several control lines (Fig. 7). A detailed regression analysis of control and manipulated lines confirmed the relatively weak association between nicotine levels and ODC activity, but revealed that the correlation was in fact significant ($r = 0.66$, 8 d.f., $p < 0.05$ for correlation between nicotine and ODC early in the growth cycle). The fluctuations observed may be due to the slight differences in growth rate and root morphology which are seen between clones, leading to variation in other biochemical factors besides putrescine supply which may potentially influence nicotine accumulation, for instance the availability of nicotinic acid [26], or alkaloid turnover. It should also be borne in mind that ADC, rather than ODC, may be more important as the endogenous source of putrescine for nicotine formation [31] and the effects of the insertion of the yODC gene seen here could be due to 'leak-through' into an ADC-based pathway. The extent to which this occurs could also be potentially variable.

Due to the presence of variation at all levels, it was not possible to examine any one individual CaMV35S^{++}-yODC line and state which proportion of its nicotine was due to the presence of the yODC gene and which proportion was due to

other, endogenous, factors affecting the nicotine content of that particular line. It was for this reason that it was important to look at the overall effect of genetic manipulation upon the average nicotine content of a number of transgenic lines. Similar observations have been made by others regarding variation in levels of foreign gene expression in transgenic plants necessitating a mean value of several clones to be calculated before judging the effect of a promoter upon gene expression (for example [15, 28]).

Future prospects for the genetic manipulation of nicotine production

While little is known of the *in vivo* regulation of the nicotine biosynthetic pathway, it would appear that increased substrate availability, due to enhanced ODC activity arising from expression of the yODC gene, can result in a significant increase in flux down the entire pathway. Other factors besides the supply of putrescine must however also be involved in regulating the accumulation of nicotine. This is apparent from the rather weak correlation between ODC activity and nicotine levels. Additionally, the enhancement of nicotine accumulation observed in manipulated lines is at best only approximately 2-fold even though the yODC gene expressed under the control of the CaMV35S^{++} promoter can produce increased ODC activity over a prolonged time period. These observations suggest that either the levels of other enzymes shown in Fig. 1 are, to some degree, limiting alkaloid production or that there may be other regulatory factors which can limit the effects of perturbations in metabolism.

Given that there are indications of a potentially flux limiting step beyond ODC, it may well be possible to elevate nicotine accumulation still further by subsequent genetic manipulation of other steps in the biosynthetic pathway. Evidence has been presented [24] that PMT, the first committed step in the biosynthetic pathway leading to nicotine, may be particularly important in the control of nicotine production in *N. rustica*. This enzyme is found to diminish rapidly at the onset of conditions under which nicotine accumulation ceases. Over-expression and/or de-regulated expression of PMT in combination with the ODC gene may cause yet higher levels of nicotine to accumulate in hairy roots of *Nicotiana*. Similarly, the supply of nicotinic acid [26] may become increasingly important as a limiting factor in manipulated lines. These problems can be addressed experimentally and so provide valuable information upon the genetic and biochemical control of nicotine production in hairy root cultures.

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