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Polyamine Homeostasis in Transgenic Plants Overexpressing Ornithine Decarboxylase Includes Ornithine Limitation

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It was reported recently that overexpression of human ornithine decarboxylase (ODC) cDNA in transgenic rice plants resulted in increased steady-state concentration of polyamines, i.e., enough biosynthetic control is invested at this step to enable adjustment of polyamine levels. To investigate critically whether constitutive overexpression of ODC is sufficient to control steady-state polyamine levels, we expressed an ODC cDNA from *Datura stramonium* in transgenic tobacco plants. Transgenic progeny of self-fertilised primary transformants exhibited increases in ODC activity of 25-fold in leaves and 5-fold in flower buds. However, the increase in putrescine levels was only 1.5- to 2.1-fold in leaves and 1.1- to 1.3-fold in flower buds. Emphatically, no changes to spermidine or spermine steady-state levels or to soluble or insoluble hydroxycinnamic acid-conjugated polyamines were observed. Ornithine feeding to cell suspension cultures derived from the transgenic plants indicated that putrescine accumulation was limited in part by ornithine availability. These results demonstrate that a large increase in the capacity of the tobacco plants to decarboxylate ornithine does not result in a comparable increase in the level of free or conjugated polyamines. Plant polyamine homeostatic mechanisms efficiently accommodate increased ODC activity, suggesting that polyamine biosynthetic control is invested at multiple interdependent steps.

Key words: arginine decarboxylase, hydroxycinnamic amide, ornithine decarboxylase, polyamine, putrescine.

Polyamines are evolutionarily ancient and ubiquitous small polycations that are important for a range of cellular processes, notably cell growth and division, stabilisation of nucleic acids and membranes, protein synthesis and chromatin function (*1*, *2*). The biosynthetic pathway of polyamines is regulated at transcriptional, post-transcriptional, translational and post-translational levels. (*3–5*). The complexity of this regulation indicates the importance of maintaining polyamine homeostasis in the cell, while still allowing rapid response of the pathway to mitogenic signals or external stress. In animals and fungi, the diamine putrescine is synthesized from ornithine by ornithine decarboxylase (ODC). Plants and some bacteria have an additional route to putrescine, from arginine via the activity of arginine decarboxylase (ADC). ADC produces agmatine, which in some bacteria is converted directly to putrescine by the activity of agmatine ureohydrolase and in plants is converted first to *N*-carbamoylputrescine by agmatine iminohydrolase and then to putrescine by *N*-carbamoylputrescine amidohydrolase (*1*, *6*, *7*). Relative contributions of ADC and ODC to the polyamine pathway vary between plant species, developmental stages and in response to stress (*6–8*). The higher polyamines spermidine and spermine are formed from putrescine by the successive additions of aminopropyl groups obtained from decarboxylated *S*-adenosylmethionine, mediated by the aminopropyl transferases spermidine synthase and spermine synthase (*1*, *3*). *S*-adenosylmethionine decarboxylase (AdoMetDC), produces the decarboxylated *S*-adenosylmethionine for these steps and embodies another important control point for polyamine homeostasis and response (*4*). Plant polyamines can also be conjugated to hydroxycinnamic acids to form a range of acid-soluble or acid-insoluble amides (*6*, *9*).

Our objective was to investigate the role of ODC in plant polyamine biosynthesis and to determine whether overexpression of ODC can alter the steady-state levels of polyamines. A recent paper reported that constitutive overexpression of human ODC in transgenic rice plants is sufficient to increase the steady-state levels of putrescine, spermidine and spermine (*10*). However, only primary transformants were analysed and no demonstration of the segregation of biochemical phenotypes with transgenic genotype was provided. The levels of polyamines in plant cells are responsive to stress and growth rates (*1*, *7*). Genetic transformation of plants usually involves a tissue culture stage, and this can give rise to somaclonal cytological perturbations. Such somaclonal variation is often associated with epigenetic activation of transposons and modification of DNA methylation resulting in cytologically abnormal plants (*11*, *12*). To exclude metabolic variation due to somaclonal effects in plants coming out directly from tissue culture, it is essential to analyse segregating progeny in the second generation, thereby allowing direct assessment of the effects of the transgene on metabolism. We have produced and analysed two generations of tobacco plants over-expressing an ODC cDNA from *D. stramonium*. This is the first

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report of the over-expression of a plant ODC in transgenic plants, and we observed that overexpression produced a large increase in ODC activity but only a small increase in putrescine content and no changes to spermidine or spermine steady-state content. The putrescine level was increased in transgenic ODC overexpressing cell suspension cultures by feeding ornithine, indicating that ornithine was limiting in part for putrescine accumulation.

MATERIALS AND METHODS

Plasmid Construction—DNA manipulation was performed by standard protocols (*13*) or according to manufacturers' instructions. A 1,576 bp *D. stramonium* ODC cDNA (*14*) was excised from pBluescript SK+ with PstI and *Xho*I. The cDNA was subcloned into the *Pst*I and *Sal*I sites of pJIT60, placing the sequence in a sense orientation between a Cauliflower Mosaic Virus (CaMV) 35S RNA promoter with duplicated enhancer sequences and the CaMV termination sequence (*15*). The same cDNA was also excised from pBluescript with *Eco*RI and *Xho*I and subcloned into the *Eco*RI and *Sal*I sites of pJIT60 to produce an antisense construct. Both constructs were then digested with *Kpn*I and *Xho*I and ligated into the *Kpn*I and *Sal*I sites of the pBin19 *Agrobacterium tumefaciens* binary transformation vector (*16*). The sense and antisense constructs and an empty pBin19 vector were transferred into *A. tumefaciens* strain LBA4404 by triparental mating as described by Bevan (*16*).

Plant Material and Transformation—Leaf discs of tobacco (*Nicotiana tabacum* cv Xanthi XHFD8) were transformed on three separate occasions with *A. tumefaciens* (*17*). Discs were incubated for 2 d on MS medium containing 1× MS basal salts (*18*, Sigma), 2% (w/v) sucrose, 0.05% (w/v) MES, and 0.3% (w/v) Phytagel (Sigma), then transferred to a selective medium (MS medium supplemented with 300 mg/liter carbenicillin disodium (Melford Laboratories, Ipswich, UK), 100 mg/liter kanamycin sulphate, 10 mg/liter dimethylaminopurine, and 1 mg/liter indole acetic acid). After 3–5 weeks, plantlets were transferred to rooting medium (MS medium incorporating 300 mg/liter carbenicillin and 50 mg/liter kanamycin). Rooted plants were transferred to pots in a greenhouse with a day temperature of 20°C, night temperature 18°C, 16 h day from September to April. Plants were fed weekly with Solufeed high potash fertiliser (Kings Horticulture, Colchester, Essex, UK). Primary transformants were allowed to self-fertilise and the resulting seeds germinated on soil.

Generation and Maintenance of Cell Suspension Cultures—Callus and cell suspension cultures were produced by the method of May and Leaver (*19*). Seeds from line 405 (ODC over-expressing) and a pBin19 control line were sterilised in 10% (v/v) bleach containing 1–2 drops of detergent for 10–15 min. After 5 washes with distilled water, seeds were plated onto MS medium with 0.8% (w/v) Phytagel supplemented with 100 mg/liter kanamycin and left for 7–10 d to germinate. Seedlings were transferred to Magenta vessels (Sigma) containing the same medium and grown for 6–8 weeks. Healthy seedlings were dissected, and cut surfaces of leaves, petioles and stems were pushed into callus-inducing medium (*19*) and left in the dark for several weeks to allow the formation and proliferation of callus. Callus was subcultured every 2–3 weeks. A suspension cell culture was produced from young, friable callus, which was crushed then shaken in 50 ml of MS cell culture medium [1× MS basal salts, 3% (w/v) sucrose, 100 mg/liter myo-inositol, 200 mg/liter KH_2PO_4, 1 mg/liter thiamine-HCl] supplemented with 0.2 mg/liter 2,4-dichlorophenoxyacetic acid and 50 mg/liter kanamycin, in the dark for 10 d. The cells were harvested, crushed, and transferred to 100 ml of the same cell culture medium. This process was repeated until a fairly dense solution was obtained; thereafter 10-ml aliquots of cultures were subcultured into 90 ml of fresh medium every 10 d. The pattern of growth was assessed by inoculation of replicate flasks, with material being harvested, dried and weighed every 2 days to determine when the stationary phase occurred.

For ornithine feeding experiments, nine flasks were set up from the same parent flask, with three flasks containing 10 mM ornithine in the cell suspension medium and six containing no added ornithine. After 11 days of growth, 10 mM ornithine was added to three of the untreated flasks. Seven hours later all of the cultures were harvested onto paper towels, blotted dry and frozen in liquid nitrogen.

Molecular Analysis of Plants—For RNA, DNA, and biochemical analyses young leaves were taken from plants before flowering. Total RNA and genomic DNA were extracted together as described previously (*14*). Southern and Northern blot analyses were performed as described previously (*13*) using Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech UK, Bucks., UK). RNA size markers were obtained from Promega. The *D. stramonium* ODC 1,576 bp cDNA excised from pBluescript (*14*) was used as a probe to detect expression of the transgene on RNA gel blots. As the *D. stramonium* ODC sequence might cross-hybridise with the tobacco ODC, the CaMV 35S RNA promoter fragment was excised from pJIT60 with *Kpn*I and *Sma*I to produce a 730 bp probe for the detection of 35S-ODC constructs integrated into genomic DNA. The pBin19 transformation vector was digested with *Pst*I, and the 1,955 bp fragment encompassing the T-DNA region was used as a probe to detect pBin19 DNA in plants transformed with the empty vector. An approximately 500 bp tobacco ubiquitin sequence (*20*) was used to confirm equal loading on gels. DNA probes were gel-purified (Qiaex II, Qiagen, Crawley, W. Sussex, UK) and radiolabelled to a high specific activity by random priming with High Prime (Roche Diagnostics, E. Sussex, UK) using ^{32}P-dCTP (Amersham Pharmacia). Filters were hybridised overnight in QuikHyb (Stratagene) at 65°C, rinsed twice in 2× SSC (sodium chloride/sodium citrate, *13*) at room temperature, then washed twice for 30 min in 0.1× SSC, 0.1% (w/v) SDS at 65°C. Radioactivity was detected on X-ray film (RX, Fuji, Tokyo) or using a Fuji BAS 1500 phosphorimager. The same ground frozen leaf and flower bud powder used for RNA analysis was used to examine polyamine biosynthetic enzyme activity and polyamine content.

Assay of ODC, ADC, AdoMetDC, and LDC Enzyme Activity—ODC, ADC, and AdoMetDC enzyme activities were assayed by quantification of the release of $^{14}CO_2$ from substrates incubated with cell-free extracts, as

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described previously (*21*). The protein concentration of the cell-free extracts was determined by the method of Bradford (*22*) with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK), using BSA (Sigma) to produce a standard curve. LDC enzyme activity was measured by the same procedure using an assay mixture containing 2.5 mM unlabelled lysine and 0.1 mCi L-[U-^{14}C]lysine (323 mCi/mmol, Amersham Pharmacia).

HPLC Analysis of Polyamines—Polyamines were extracted once from frozen, powdered plant material in 5% (w/v) trichloroacetic acid (TCA) containing 1×10^{-4} M 1,7-diaminoheptane as an internal standard as described previously (*21*). Soluble and insoluble conjugated polyamines were released by hydrolysis of the TCA extract or the washed, resuspended pellet from the extraction. Polyamines were dansylated overnight in duplicate by an adaptation of the method of Smith and Davis (*23*). Sample aliquots of 200 μl were incubated with 100 μl of saturated Na_2CO_3 and 600 μl of dansyl chloride (10 mg/ml in acetone) for 16 h in the dark in open tubes to allow gradual evaporation of the acetone. Excess dansyl chloride was removed by 30-min incubation with 150 μl of proline (300 mg/ml). The reaction was then extracted with 1 ml toluene, centrifuged for 5 min at 13,000 ×g and 800 μl of the upper phase was dried with nitrogen and resuspended in 500 μl of acetonitrile. Samples were filtered through Acrodisc CR PTFE filters (Gelman Sciences, MI, USA). Dansylated polyamines were separated by HPLC using a Sphereclone 5μ C18 ODS(2) column (250 × 4.6 mm; Phenomenex, Macclesfield, Cheshire, UK) with fluorescence detection (excitation wavelength 340 nm, emission wavelength 510 nm). Solvent A was HPLC-grade water, solvent B was acetonitrile and the gradient was run for 50 min at a flow rate of 1.2 ml/min with the following concentrations: t = 0 min: 40% A, 60% B; t = 25 min: 0% A, 100% B; t = 40 min: 40% A, 60% B; t = 50 min: 40% A, 60% B.

RESULTS

Production and Molecular Analysis of Primary Transformed Plants—The *D. stramonium* ODC cDNA encodes a protein of 431 amino acids (*14*) exhibiting 90% identity with the ODC of tobacco (accession no. BAA83427), and it was inserted in both sense and antisense orientations under the control of the constitutive plant viral CaMV 35S RNA promoter. After *Agrobacterium*-mediated transformation of tobacco leaf discs and *in vitro* regeneration of shoots and roots, the transformed plants were transferred to soil in a greenhouse. The presence of the transgene was assessed by Southern analysis of genomic DNA from leaves. Digestion of the genomic DNA with *Eco*RI excised the 0.8 kbp CaMV 35S promoter in sense transformants and both promoter and ODC cDNA sequence (2.4 kbp) in the antisense transformants (Fig. 1). As it was formally possible that the hybridising sequences, which were probed with CaMV 35S RNA promoter sequence, could have arisen from persisting *Agrobacterium* cells on the leaves, we also demonstrated integration of the transgenes into the tobacco genomic DNA by digestion with *Hin*dIII. The control plants contained only the pBin19 binary vector and did not show any hybridisation to the CaMV 35S RNA promoter sequence. Eight plants with the sense construct and three with the antisense construct were examined. RNA gel blot analysis of leaf material after hybridisation to the *D. stramonium* ODC cDNA probe revealed that all transformants expressed a message of approximately 2.7 kb at varying levels (Fig. 2). Accumulation of the antisense message was low in all three antisense transformants.

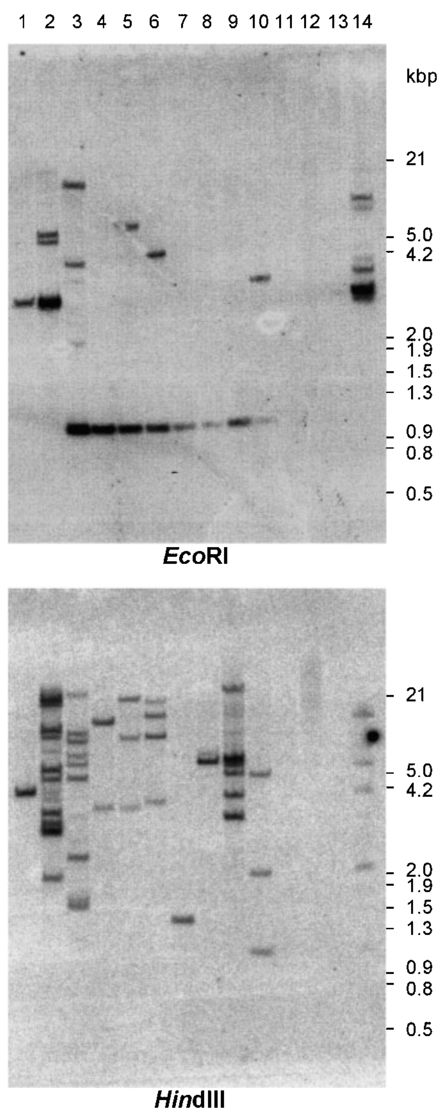

Fig. 1. **Southern blot analysis of tobacco ODC sense and antisense primary transformants.** Southern blot analysis of genomic DNA. 20 μg genomic DNA was digested with *Eco*RI or *Hin*dIII. Lanes 1, 2, 14: ODC antisense plants 310, 312, and 302; lanes 3–10: ODC sense plants 403, 404, 405, 406, 409, 411, 413, 416; lanes 11, 12: pBin19 empty vector transformants; lane 13: no sample loaded.

ODC, ADC, and AdoMetDC Activities in Primary Transformants—Three sense and three antisense independently transformed plants were selected for further study and compared with three independently transformed plants containing the empty pBin19 transformation vector. Analysis of young leaves and unopened flower buds revealed a large increase in ODC activity in the ODC sense plants compared to the Bin19 control plants (Table

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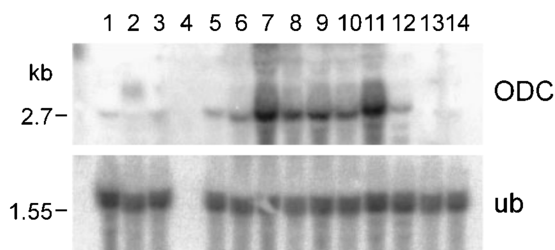

Fig. 2. **RNA gel blot analysis of tobacco ODC sense and antisense primary transformants.** RNA gel blot analysis. The *D. stramonium* ODC cDNA was hybridised to 15 μg total RNA. Lanes 1–3: ODC antisense plants 302, 310, 312; lane 4: no sample; lanes 5–12: ODC sense plants 403, 404, 405, 406, 409, 411, 413, and 416; lanes 13, 14: pBin19 control plants. The blot was hybridised to tobacco ubiquitin (ub) as a loading control.

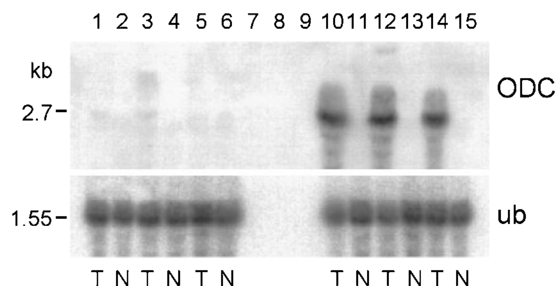

Fig. 3. **Expression of *D. stramonium* ODC in segregating progeny of primary transformants.** The *D. stramonium* ODC cDNA was hybridised to 15 μg total RNA extracted from ODC antisense (302, 310, 312) and sense (405, 411, 413) segregating progeny. Lanes 1, 2: line 302; lanes 3, 4: line 310; lanes 5, 6: line 312; lanes 7–9: no sample loaded; lanes 10, 11: line 405; lanes 12, 13: line 411; lanes 14, 15: line 413. T, transgenic; N, syngenic. The blot was hybridised to ubiquitin (ub) as a loading control.

1). There were increases of 10.7-, 4.3-, and 16.6-fold in leaves and 6.4-, 5.5-, and 7.7-fold in flower buds of ODC sense lines 405, 411, and 413 respectively, compared to the mean values of the control plants. In the control plants, ODC activity was 3.9-fold higher in flower buds than in leaves. There was no noticeable difference between the ODC activities of the control and ODC antisense plants, suggesting that the antisense construct had not affected ODC activity. The ODC sense plants did not exhibit any consistent change in ADC or AdoMetDC activities compared to the control plants.

Analysis of Second-Generation Segregating Progeny—Three ODC over-expressing plants were chosen for further analysis (405, 411, and 413) and self-fertilised. The segregating progeny were analysed by RNA gel blot hybridisation to detect transgenic progeny expressing the *D. stramonium* ODC and syngenic progeny that had segregated without the transgene (Fig. 3). Under the stringent hybridisation conditions employed, the *D. stramonium* ODC did not hybridise to the tobacco ODC. A low level of the *D. stramonium* ODC antisense message was detectable in the segregating transgenic progeny of antisense lines 302, 310, and 312.

Increased ODC activity in the second-generation plants segregated with the presence of the expressed *D. stramonium* ODC transgene (Table 2). In young leaves of transgenic plants ODC activities were 18.7-, 25.7-, and 23.1-fold higher than in syngenic plants of lines 405, 411, and 413 respectively. In transgenic flower buds the increase was 3.2-, 5.5-, and 4.8-fold higher in lines 405, 411, and 413 compared to the corresponding syngenic flower buds. However, the mean ODC activity of syngenic leaves was only 10% of that of syngenic flower buds, whereas the mean ODC activity of transgenic leaves was 66% of that of transgenic flower buds. AdoMetDC activity was not detectably changed in leaves (Table 2). Progeny

Table 1. ***In vitro* ODC, ADC, and AdoMetDC enzyme activities in young leaves and unopened flower buds from ODC sense and antisense primary transformants.** Mean enzyme activities in (A) young leaves and (B) unopened flower buds of transgenic ODC sense, ODC antisense and pBin19 primary transformants. Values are the means of duplicate assays ± the standard deviation. S, sense; AS, antisense; n.d., not determined; [a], single assay.

	Plant		Mean enzyme activity (nmol CO_2 mg^{-1} h^{-1})		
			ODC	ADC	AdoMetDC
A	405	S	272.54 ± 10.14	7.21 ± 1.29	0.96 ± 0.06
	411	S	109.09 ± 2.82	1.40 ± 0.04	0.52 ± 0.05
	413	S	424.11 ± 2.01	3.02 ± 0.05	0.36 ± 0.03
	302	AS	18.71 ± 2.79	15.40 ± 0.45	0.70 ± 0.25
	310	AS	12.02 ± 0.62	14.69 ± 1.76	0.75 ± 0.13
	312	AS	11.57 ± 1.23	8.17 ± 3.13	0.67 ± 0.05
	pBin19 1		27.86 ± 7.09	5.73[a]	n.d.
	pBin19 2		16.87 ± 0.82	7.96 ± 1.84	n.d.
	pBin19 3		31.68 ± 0.26	7.28 ± 0.07	n.d.
B	405	S	638.46[a]	14.38 ± 1.95	5.54 ± 0.01
	411	S	546.46 ± 8.88	10.74 ± 0.51	4.33 ± 0.08
	413	S	796.06 ± 46.29	15.41 ± 0.48	7.27 ± 0.49
	302	AS	43.94 ± 2.70	9.96 ± 0.23	2.88 ± 0.07
	310	AS	86.98 ± 10.13	12.51 ± 0.84	5.01 ± 0.18
	312	AS	154.02 ± 2.42	21.84 ± 7.34	7.24 ± 0.82
	pBin19 1		77.35 ± 0.07	9.81[a]	n.d.
	pBin19 2		93.98 ± 0.46	10.13 ± 0.77	n.d.
	pBin19 4		126.20 ± 3.78	6.31 ± 0.06	n.d.

Table 2. ***In vitro* ODC, ADC and AdoMetDC enzyme activities in young leaves and unopened buds from ODC-overexpressing segregating progeny.** Mean enzyme activities in (A) young leaves and (B) unopened buds of ODC-overexpressing segregating progeny. Values are the means of duplicate assays on 1–3 independent plants ± the standard deviation. T, transgenic; N, syngenic; n, number of plants in sample; [a], single assay; [b], two plants used for flower bud analysis.

	Line		*n*	Mean enzyme activity (nmol CO_2 mg^{-1} h^{-1})		
				ODC	ADC	AdoMetDC
A	405	T	3	304.11 ± 49.81	1.41 ± 0.21	0.82 ± 0.10
	405	N	3	16.25 ± 7.15	2.28 ± 0.98	0.75 ± 0.31
	411	T	3	525.17 ± 138.47	1.89 ± 0.23	1.06 ± 0.18
	411	N	3	20.45 ± 7.72	3.12 ± 0.27	0.88 ± 0.12
	413	T	3	194.22 ± 38.76	0.64 ± 0.10	0.33 ± 0.03
	413	N	1	8.44 ± 1.07	1.18 ± 0.09	0.34[a]
B	405	T	3	374.65 ± 129.87	7.16 ± 1.91	7.18 ± 0.20
	405	N	3	115.68 ± 56.29	10.96 ± 2.65	6.52 ± 1.37[b]
	411	T	3	601.78 ± 318.09	7.21 ± 0.82	n.d.
	411	N	1	116.12 ± 8.67	6.36[a]	n.d.
	413	T	3	572.82 ± 79.04	7.81 ± 0.56	n.d.
	413	N	1	210.15 ± 1.93	14.21 ± 0.27	n.d.

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Table 3. **Free polyamine content of young leaves and unopened flower buds from ODC-overexpressing segregating progeny.** (A) free polyamines in young leaves, (B) free polyamines in unopened buds, (C) insoluble conjugated polyamines in young leaves, (D) insoluble conjugated polyamines in unopened flower buds. Measurements are the means of duplicate dansylation reactions from single plants ± the standard deviation. T, transgenic; N, syngenic.

	Line		Mean polyamine content (nmol g^{-1} fresh weight)		
			Putrescine	Spermidine	Spermine
A	405E	T	892.17 ± 8.58	553.68 ± 5.94	100.32 ± 10.39
	405J	N	561.61 ± 8.70	598.19 ± 3.14	87.49 ± 5.15
	411J	T	755.20 ± 0.10	537.64 ± 0.62	80.62 ± 0.55
	411G	N	565.10 ± 0.10	620.79 ± 33.08	83.42 ± 1.03
	413B	T	798.01 ± 2.61	532.14 ± 1.00	97.60 ± 1.06
	413I	N	413.84 ± 2.21	493.30 ± 6.82	67.04 ± 2.58
B	405A	T	855.57 ± 13.92	407.63 ± 1.34	98.08 ± 0.42
	405I	N	711.58 ± 9.41	457.50 ± 37.47	117.88 ± 16.79
	411J	T	764.87 ± 0.70	523.70 ± 0.59	145.48 ± 3.64
	411C	N	586.87 ± 0.13	498.18 ± 15.09	112.35 ± 6.14
	413C	T	791.67 ± 4.00	414.28 ± 14.65	83.81 ± 4.95
	413I	N	580.62 ± 9.73	376.87 ± 11.13	94.80 ± 6.55
C	405E	T	1.162 ± 0.006	14.278 ± 0.409	0.035 ± 0.004
	405J	N	0.913 ± 0.0025	14.350 ± 0.537	0.056 ± 0.003
	411J	T	1.236 ± 0.005	15.138 ± 0.093	0.069 ± 0.009
	411G	N	2.416 ± 0.022	17.316 ± 0.583	0.090 ± 0.016
	413B	T	0.916 ± 0.250	13.933 ± 3.120	0.079 ± 0.017
	413I	N	1.786 ± 0.158	16.076 ± 0.513	0.074 ± 0.017
D	405A	T	10.173 ± 0.093	127.313 ± 2.706	2.944 ± 0.027
	405I	N	3.609 ± 0.055	93.874 ± 17.106	1.299 ± 0.078
	411J	T	5.186 ± 1.363	86.178 ± 3.690	2.091 ± 0.551
	411C	N	3.939 ± 0.672	102.405 ± 29.350	1.826 ± 0.351
	413C	T	4.483 ± 0.680	74.604 ± 0.524	1.370 ± 0.203
	413I	N	4.671 ± 0.385	115.653 ± 51.602	2.733 ± 0.392

of the three antisense lines 302, 310, and 312 were also analysed, but although transgenic plants expressed the *D. stramonium* antisense cDNA, there were no differences in enzyme activities between transgenic and syngenic progeny (results not shown).

Polyamine Content in Second-Generation Plants—In marked contrast to the large increases in ODC activity in the transgenic plants, the increase in putrescine accumulation was relatively low. Increases in free putrescine in transgenic leaves were only 1.6-, 1.5-, and 2.1-fold higher, and in flower buds they were 1.1-, 1.3-, and 1.3-fold higher than in syngenic plants of lines 405, 411, and 413 respectively (Table 3). There was no consistent change in free spermidine or spermine levels (Table 3) or in the levels of acid-soluble polyamine conjugates (results not shown). Line 405 did show a 2.8-fold increase in insoluble conjugated putrescine (Table 3), but the absolute levels of this conjugate were very low in all plants. Plants from the antisense lines showed no notable changes in polyamine content in leaves or buds (results not shown).

Lysine Decarboxylase Activity—Mammalian ODC is capable of decarboxylating lysine with low efficiency. We reasoned that the increased ODC activity in the transgenic plants might also result in increased lysine decarboxylation. Lysine decarboxylation was assayed in young leaves and flower buds of segregating progeny of ODC-overexpressing lines (Table 4). There was very little lysine decarboxylase (LDC) activity detectable in either

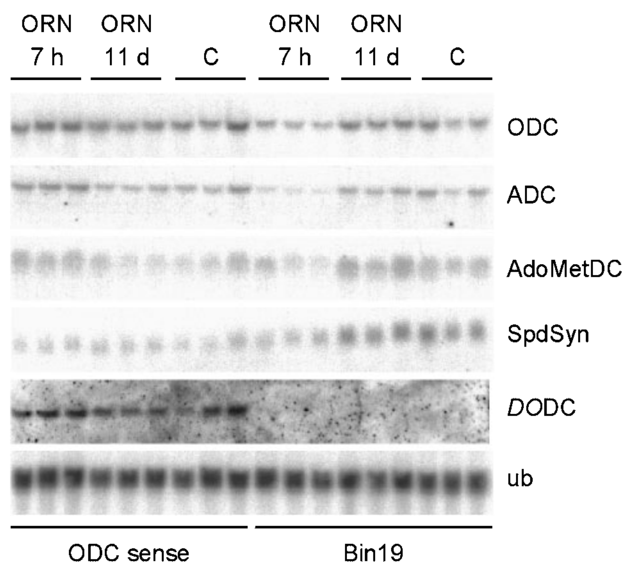

Fig. 4. **Effect of ornithine on steady-state mRNA levels in ODC-overexpressing and pBin19 control suspension cultures.** Four replicate blots containing 15 μg total RNA in each lane were produced and hybridised to sequences for tobacco ODC, ADC, AdoMetDC, and spermidine synthase (SpdSyn) and *D. stramonium* ODC (*D*ODC). Lanes 1–9: 405 ODC over-expressing cell cultures fed with ornithine for 7 h (lanes 1–3), 11 d (lanes 4–6), or untreated (lanes 7–9); lanes 10–18: pBin19 cell cultures fed with ornithine for 7 h (lanes 10–12), 11 d (lanes 13–15), or untreated (lanes 16–18). Blots were hybridised to ubiquitin (ub) as a loading control.

transgenic or syngenic plants. The low LDC activity in tobacco was noted previously (*24*).

Ornithine Feeding to Transgenic Cell Suspension Cultures—One possible explanation for the relatively low accumulation of putrescine compared to the increase in ODC activity in ODC-overexpressing plants is the availability of ornithine. We produced cell suspension cultures from the ODC-overexpressing line 405 and as a control we used a transgenic suspension culture generated from a plant containing the empty pBin19 *Agrobacterium* transformation vector. Expression of the *D. stramonium* ODC transgene in line 405 cells was confirmed by RNA gel blot analysis (Fig. 4). Growth curves showed that the cultures reach stationary phase 10 d after subculturing (results not shown). Triplicate cultures were fed with either 10 mM ornithine for 11 d from subculturing, or for 7 h after 11 d growth, or harvested at 11 d after no treatment. It is difficult to compare absolute levels of message, enzyme activity and polyamine content in two different cell lines due to variations in growth and density; however, we found interesting differences in the pattern of response to feeding between the ODC-overexpressing cells and the control cell cultures. In the control, feeding for 7 h with ornithine resulted in decreased steady-state mRNA levels for tobacco ODC, ADC, AdoMetDC, and spermidine synthase, while the message levels after 11 d incubation with ornithine were equivalent to those in untreated 11 d cultures (Fig. 4). In contrast, ODC-overexpressing cells did not show a consistent change in mRNA levels upon ornithine feeding for 7 h.

The pBin19 control cells exhibited slight increases in the mean ODC and ADC enzyme activities after 11 d

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Table 4. ***In vitro* LDC enzyme activity in tissue from ODC-overexpressing progeny.** T, transgenic; N, syngenic. Values are the mean of duplicate assays; [a], single assay.

Tissue			Mean enzyme activity (nmol CO_2 mg^{-1} h^{-1})
405A	leaf	T	0.257 ± 0.02
405I	leaf	N	0.349 ± 0.07
411B	leaf	T	0.417 ± 0.15
411G	leaf	N	0.257 ± 0.04
413E	leaf	T	0.463 ± 0.09
413I	leaf	N	0.668[a]
411J	bud	T	0.716 ± 0.10
411C	bud	N	0.631 ± 0.13

Table 5. ***In vitro* ODC, ADC and AdoMetDC enzyme activities in ODC sense and Bin19 suspension cell cultures fed with ornithine for 7 h or 11 d.** Mean enzyme activities in ODC-overexpressing (405) and pBin19 suspension cell cultures grown for 11 d without treatment (C), grown for 11 d then fed with ornithine for 7 h, or cultivated with ornithine for 11 d. Measurements are the means of duplicate assays on 3 flasks ± the standard deviation.

Line	Treatment	Mean enzyme activity (nmol CO_2 mg^{-1} h^{-1})		
		ODC	ADC	AdoMetDC
405	7 h	42.81 ± 17.02	0.47 ± 0.07	0.14 ± 0.02
405	11 d	56.99 ± 14.47	0.45 ± 0.04	0.33 ± 0.02
405	C	78.75 ± 51.65	0.77 ± 0.10	0.29 ± 0.04
Bin 19	7 h	12.65 ± 3.72	0.57 ± 0.13	0.37 ± 0.05
Bin 19	11 d	16.67 ± 4.66	0.85 ± 0.11	0.68 ± 0.04
Bin 19	C	12.08 ± 3.32	0.67 ± 0.08	0.25 ± 0.03

feeding (Table 5) and clearer increases in AdoMetDC enzyme activity after 7 h and 11 d feeding. ODC-overexpressing cells had a significant level of internal variation between triplicate cell cultures samples, but on average these cultures showed a decrease in mean ODC and ADC activity after feeding with ornithine. There was also a clear 2-fold decrease in AdoMetDC activity after 7 h feeding, while the samples incubated with ornithine for 11 d had similar AdoMetDC activities to the untreated control.

Feeding with ornithine for 7 h caused no changes in the free polyamine content of control cells, but incubation for 11 d resulted in higher levels of free putrescine and spermidine and an increase in the mean spermine content (1.36-, 1.67-, and 1.4-fold increases respectively; Table 6). In contrast, ODC-overexpressing cells showed a more rapid response to feeding with an increase in free putrescine after 7 h incubation (1.36-fold) and a larger increase after 11 d (2.5-fold), but there were no changes in free spermidine or spermine. Polyamines levels in the medium were not analysed, as we reasoned that plant cells would more likely sequester polyamines to the vacuole rather than excrete them into the cell wall, where the hydrogen peroxide–producing polyamine oxidase resides.

Soluble conjugated polyamines and insoluble conjugated spermidine were only sporadically detectable in control and ODC-overexpressing cells, with the exception of soluble conjugated putrescine, which was detected in all ODC-overexpressing 405 cultures but showed no change upon feeding (results not shown). Insoluble conjugated putrescine levels were not affected in control cells after 7 h feeding but rose 3-fold in cultures fed for 11 d, and these cultures also showed a 1.44-fold increase in insoluble conjugated spermine (Table 6). The ODC sense

Table 6. **Polyamine content of ODC-overexpressing and pBin19 suspension cell cultures fed with ornithine for 7 h or 11 d.** Polyamine content of ODC-overexpressing (405) and pBin19 suspension cell cultures grown for 11 d without treatment (C), grown for 11 d then fed with ornithine for 7 h or cultivated with ornithine for 11 d. (A) free polyamines, (B) insoluble conjugated polyamines. Values are the means of duplicate assays on 3 flasks ± the standard deviation. n.d., not detectable.

	Line	Treat-ment	Mean polyamine content (nmol g^{-1} fresh weight)		
			Putrescine	Spermidine	Spermine
A	405	7 h	378.11 ± 51.80	156.39 ± 12.81	8.64 ± 0.52
	405	11 d	698.75 ± 62.18	130.76 ± 2.21	8.42 ± 0.63
	405	C	277.25 ± 69.14	138.91 ± 9.80	9.31 ± 0.62
	Bin 19	7 h	631.47 ± 95.85	106.36 ± 9.45	5.62 ± 0.53
	Bin 19	11 d	970.71 ± 82.68	193.82 ± 34.23	10.50 ± 1.64
	Bin 19	C	715.78 ± 44.94	115.82 ± 28.92	7.42 ± 2.22
B	405	7 h	105.64 ± 26.85	n.d.	0.78 ± 0.39
	405	11 d	102.39 ± 12.65	n.d.	0.76 ± 0.41
	405	C	63.80 ± 11.86	n.d.	0.93 ± 0.33
	Bin 19	7 h	8.47 ± 4.62	n.d.	0.94 ± 0.13
	Bin 19	11 d	27.49 ± 7.17	n.d.	1.71 ± 0.18
	Bin 19	C	9.27 ± 2.86	n.d.	1.18 ± 0.36

cells showed a small but definite increase in insoluble conjugated putrescine after 7 h (1.65-fold), and the 11 d incubation produced a similar increase (1.6-fold), but there was no change in the insoluble conjugated spermine content.

DISCUSSION

To assess the feasibility of increasing putrescine and polyamine levels in transgenic tobacco plants, the *D. stramonium* ODC cDNA was constitutively overexpressed. Previously we had shown that constitutive overexpression of the *D. stramonium* ADC cDNA in transgenic tobacco led to a 65-fold increase in the level of agmatine, the direct product of ADC (*21*). However, with ADC overexpression, there was no detectable increase in steady-state putrescine levels in second-generation transgenic progeny. As putrescine is the direct product of ODC, it seemed reasonable to suppose that overexpression of ODC might be more effective in increasing putrescine levels. This supposition was proved wrong and at most the increase in putrescine levels achieved was found to be 2.1-fold from a 25-fold increase in ODC activity and spermidine and spermine levels remained unchanged. Putrescine was not diverted to hydroxycinnamic acid conjugates in any significant quantity.

Expression of yeast ODC in transgenic hairy root cultures of *Nicotiana rustica* led to a 1.5- to 2.5-fold increase in ODC activity and up to 2-fold increase in putrescine and nicotine content (*25*). Expression of a C-terminally deleted mouse ODC cDNA in first-generation transgenic tobacco plants led to 2- to 3-fold increase in putrescine compared to untransformed control plants, and some plants exhibited an altered morphological phenotype consisting of stunted growth, wrinkled leaves and abnormal flowers (*26*). However, there were no changes in spermidine or spermine levels. When the mouse ODC was expressed in carrot suspension culture cells, a 10- to 20-fold increase in putrescine content was observed,

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whereas spermidine and spermine levels were essentially unchanged (*27*). Poplar suspension cells expressing the truncated mouse ODC also produced on a variable basis 2- to 10-fold higher levels of putrescine compared to untransformed cell lines. Spermidine content was the same in transgenic and untransformed controlled cell lines (*28*). Unfortunately, in each of these four heterologous systems described above, there is no demonstration that the biochemical phenotype segregates with the transformed genotype, which makes it difficult to fully interpret the significance of the findings. Recently, a paper appeared reporting that expression of the human ODC in transgenic rice can increase the steady-state levels of putrescine, spermidine and spermine in first generation vegetative tissues and seeds (*10*). Furthermore, this effect was cultivar-specific, suggesting that rice has evolved cultivar-specific polyamine homeostasis systems. The remarkable results in rice apart, the general trend in plants upon overexpression of ODC is for a relatively small increase in ODC activity (up to 25-fold), 2-fold increase in putrescine content and no change in spermidine and spermine levels. The plant polyamine biosynthetic pathway thus conforms to Le Chatelier's principle when ODC is overexpressed in transgenic plants, equilibrating to a steady-state that attenuates the effect of the ODC overexpression.

In contrast, ODC overexpression in mammalian systems has led to much higher levels of ODC activity and putrescine accumulation. Expression of the human ODC gene in transgenic mice resulted in 80-fold increased ODC activity in testes and 20-fold increased accumulation of putrescine (*29*, *30*). There was a 1.5-fold increase in spermidine but no change in spermine content. Subsequent treatment of transgenic mice overexpressing the human ODC gene with 5-fluoromethylornithine, a specific inhibitor of ornithine catabolism, resulted in a further increase in putrescine content by 2- to 3-fold, but spermidine and spermine levels were unaffected (*31*). A striking 8,000-fold increase in ODC activity in the liver of transgenic mice was obtained by overexpression of the human ODC gene transcribed from the mouse metallothionein I promoter (*32*). This increased ODC activity resulted in a 150-fold increase in putrescine content, whereas the spermidine and spermine levels remained at basal levels after a small transient increase upon induction of the metallothionein promoter. The tight regulation of the systemic polyamine steady-state levels appears to apply to both plants and mice.

The more modest increases in ODC activity upon overexpression in plants, compared to transgenic mice, is probably due to the highly regulated nature of the mammalian ODC and its low basal abundance. Plants do not contain a polyamine retroconversion pathway, and there is no evidence that the plant ODC is negatively regulated by polyamine levels, so the very tight homeostasis of the plant biosynthetic pathway requires explanation. Certainly one factor must be ornithine availability, as the ODC-overexpressing transgenic tobacco cell cultures have shown. However, ornithine feeding produced only 2.5-fold more putrescine in the ODC-overexpressing cells, while in the control cells ornithine feeding increased putrescine levels by up to 1.5-fold. It is evident that ornithine limitation is only one factor in maintaining putrescine homeostasis. There is no suggestion of ODC regulation by polyamines in plants and no identification of any antizyme-like gene. However, ODC binding proteins were identified in barley: a 16 kDa cytosolic protein and a 9 kDa chromatin-associated protein (*33*). These proteins are found associated with ODC in inactive complexes. It is possible that the ODC activity observed with *in vitro* assays may not reflect ODC activity *in vivo* if the ODC binding proteins are bound to ODC *in vivo* but disassemble *in vitro*. In a mammalian cell line expressing elevated levels of ODC activity that conferred resistance to α-difluoromethylornithine, the increased ODC activity was able to decarboxylate lysine, causing an accumulation of cadaverine (*34*). We did not detect any extra cadaverine in the ODC-overexpressing plants and, with the provision that we cannot be sure that the plant ODC can decarboxylate lysine (*35*), the absence of cadaverine accumulation could be interpreted as indicating low ODC activity *in vivo*. Another possible explanation for the small increases in putrescine accumulation is suggested by Minocha and colleagues, who found that when the mouse ODC was expressed in carrot cell suspensions, [^{14}C]putrescine feeding indicated higher rates of putrescine degradation in the transgenic cells (*36*). Overexpression of both ADC and ODC in tobacco in our laboratory has demonstrated the very tight control of the plant polyamine biosynthetic pathway. Our recent work leads us to propose that AdoMetDC is also a key regulatory point in plant polyamine homeostasis, as even a modest increase in AdoMetDC activity causes severe growth and developmental effects in transgenic tobacco plants (*37*). In contrast to expectations on the basis of the growth effects, the overexpression of AdoMetDC did not increase spermidine and spermine steady-state levels.

The plant ODC, ADC, and AdoMetDC activities are highly regulated but do not possess the ability to control the polyamine pathway when over-expressed individually. Conceptually this observation is not surprising and is a basic tenet of Metabolic Control Analysis (MCA). The essence of MCA is that control is shared by multiple steps in the metabolic pathway (*38–43*) and it is therefore unlikely that overexpression of a single step will result in an increase in flux and end-product accumulation. An additional complication of the polyamine pathway is that spermidine and spermine are physiologically active and excess polyamines disrupt normal cellular physiology, especially mRNA translation and chromatin function. This might explain why even ornithine provision could become limiting when ODC is overexpressed, so as to prevent build up of toxic levels of polyamines. Polyamine-mediated feedback inhibition can affect the transcriptional, post-transcriptional, translational and post-translational regulation of the polyamine biosynthetic enzymes (*1*). To understand the regulation of the polyamine pathway with a view to controlling it, it will be necessary to incorporate consideration of both the metabolic regulation and gene expression (hierarchical) regulation of the whole pathway and cellular pathways that interconnect with it.

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