Overexpression of arginine decarboxylase in transgenic plants

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The activity of arginine decarboxylase (ADC), a key enzyme in plant polyamine biosynthesis, was manipulated in two generations of transgenic tobacco plants. Second-generation transgenic plants overexpressing an oat ADC cDNA contained high levels of oat ADC transcript relative to tobacco ADC, possessed elevated ADC enzyme activity and accumulated 10–20-fold more agmatine, the direct product of ADC. In the presence of high levels of the precursor agmatine, no increase in the levels of the polyamines putrescine, spermidine and spermine was detected in the transgenic plants. Similarly, the activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase were unchanged. No diversion of polyamine metabolism into the hydroxycinnamic acid–polyamine conjugate pool or into the tobacco alkaloid nicotine was detected. Activity of the catabolic enzyme diamine oxidase was the same in transgenic and control plants. The elevated ADC activity and agmatine production were subjected to a metabolic/physical block preventing increased, i.e. deregulated, polyamine accumulation. Overaccumulation of agmatine in the transgenic plants did not affect morphological development.

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INTRODUCTION

Polyamines are small polycations found in most organisms and cells and are essential for cellular proliferation and normal cellular function [1–5]. The diamine putrescine is the precursor of the triamine spermidine and the tetra-amine spermine. In animals and fungi, putrescine is synthesized directly from ornithine by ornithine decarboxylase (ODC; EC 4.1.1.17). In plants and some bacteria, an additional indirect route to putrescine via the activity of arginine decarboxylase (ADC; EC 4.1.1.19) is present [6]. The direct product of ADC is agmatine, which is converted into putrescine via an N-carbamoylputrescine intermediate [7]. Spermidine is synthesized from putrescine by the addition of an aminopropyl group donated by decarboxylated S-adenosylmethionine. The enzymes involved are spermidine synthase and S-adenosylmethionine decarboxylase (SAMDC). Spermine is synthesized from spermidine by the addition of an aminopropyl group. The enzyme involved, spermine synthase, is distinct from spermidine synthase [8].

Plant polyamine metabolism is unusual in that the polyamines can be conjugated via an amide bond to hydroxycinnamic acids [9,10]. In tobacco, the conjugated polyamine pool can be larger than that of the free polyamines, depending on the tissue involved [11]. The function of the conjugated forms of polyamines is not known. However, the conjugated forms of agmatine found in barley have been shown to exert an anti-fungal effect [12]. In addition, many plants form toxic alkaloids from polyamines [13], and the principal alkaloid of tobacco is nicotine.

We have recently isolated the first plant ODC cDNA [14] and have shown that the critical amino acid residues required for the active site in mouse ODC are conserved in both plant ODC and ADC, which have related sequences. Although plant ADC cDNA has been cloned from oat [15], tomato [16], pea [17], *Arabidopsis* [18], soya bean (accession no. U35367) and *Datura stramonium* (A. Michael, unpublished work), relatively little is known about its regulation and physiological function. Both ODC and ADC belong to the group IV of pyridoxal 5'-phosphate-dependent decarboxylases [19]. Use of substrate-based specific enzyme-activated suicide inhibitors of ODC and ADC indicates that

ODC and ADC have distinct and different roles in plant growth and physiology [20,21].

To test the feasibility of modifying ADC activity and altering the control of the plant polyamine metabolic pathway, we overexpressed an ADC cDNA from oat in tobacco and analysed two generations of transgenic plants for the activities of key polyamine-biosynthetic enzymes, accumulation of free and conjugated polyamines and the level of the tobacco alkaloid nicotine.

EXPERIMENTAL

Recombinant constructs

Oat ADC cDNA was a gift from Dr. Russel Malmberg (University of Georgia, Athens, GA, U.S.A.). A 2124 bp *EcoRI* fragment containing oat ADC cDNA [15] was ligated into the *EcoRI* site of pJIT60 [22]. The pJIT60 plasmid contains a cauliflower mosaic virus (CaMV) 35S RNA promoter with duplicated enhancer sequences and the termination region of CaMV. The orientation of the oat ADC cDNA within the 35S promoter cassette was verified by restriction analysis, and the sense construct was digested with *KpnI*–*XhoI* to excise the 35S–ADC–terminator as a 3.6 kbp fragment. The *KpnI*–*XhoI* fragment was ligated into the *KpnI*–*Sall* sites of pBIN19 [23]. Triparental mating with the helper strain RK2013 and the LBA4404 *Agrobacterium tumefaciens* [23] strain mobilized the pBIN19 construct from XL1-Blue to LBA4404. The structural integrity of the pBIN19 construct in *Agrobacterium* was verified by miniprep preparation of plasmid and restriction analysis.

Plant transformation

Plants of *Nicotiana tabacum* cv Xanthi XHFD8 were used for leaf disc transformation [24]. Shoots were regenerated from leaf discs on Murashige Skoog medium containing 2 mg/l 6-benzylaminopurine, 0.1 mg/l NAA, 100 mg/l kanamycin sulphate and 500 mg/l cefotaxim. Plantlets were then transferred to hormone-free medium containing 100 mg/l kanamycin sulphate. Rooted plants were transferred to soil in a greenhouse.

Abbreviations used: ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SAMDC, S-adenosylmethionine decarboxylase; CaMV, cauliflower mosaic virus; AIH, agmatine iminohydrolase; NCPAH, N-carbamoylputrescine amidohydrolase; DAO, diamine oxidase; PHT, putrescine hydroxycinnamoyltransferase.

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Molecular analysis of plants

The same frozen powder obtained from grinding young leaves in liquid nitrogen was used for all molecular and biochemical analyses. Genomic DNA was isolated at the same time as total RNA using a scaled-up version of the method of Verwoerd et al. [25]. Southern- and Northern-blot analysis was carried out using standard procedures [26]. The full-length oat ADC cDNA and an 800 bp PCR-derived tobacco ADC cDNA fragment (A. J. Michael, unpublished work) were used as hybridization probes. The gel-purified oat and tobacco ADC fragments were isotopically labelled using a PRIME-IT II kit (Stratagene). All membranes were washed at high stringency ($0.1 \times \text{SSC}/0.1\%$ SDS at 65°C , where $1 \times \text{SSC}$ is $0.15\text{ M NaCl}/0.015\text{ M}$ sodium citrate, pH 7.0).

Polyamine analysis

Polyamines were extracted twice from duplicate frozen leaf powder samples with 5 vol. of 5% (w/v) cold trichloroacetic acid for 4 h on ice. The homogenates were centrifuged, supernatants pooled and the pellets resuspended in trichloroacetic acid. Part of the supernatant and the resuspended pellets were hydrolysed with 12 M HCl (v/v) for 18 h at 110°C in sealed tubes. The hydrolysed samples were dried and resuspended in trichloroacetic acid. Free polyamines and polyamines liberated after hydrolysis were quantified after derivatization with dansyl chloride as described by Smith and Davis [27]. Dansylated polyamines were separated by HPLC on a C_{18} ($5\text{ }\mu\text{m}$) column ($250\text{ mm} \times 4.6\text{ mm}$) using a gradient from 60 to 100% acetonitrile in water in 23 min, and detected fluorimetrically.

As derivatization of agmatine by dansyl chloride is not quantitative, it was separated on a Novapak C_{18} ($5\text{ }\mu\text{m}$) column ($15\text{ mm} \times 3.9\text{ mm}$) by reverse-phase ion-pair chromatography. Agmatine was detected fluorimetrically after post-column reaction with *o*-phthalaldehyde as described previously [28]. Separation was achieved using a gradient from 5% solvent B [0.2 M sodium acetate/acetonitrile/methanol ($11.6:4:1$, by vol.), 10 mM heptanesulphonic acid, pH 4.5] to 60% solvent B in solvent A (0.1 M sodium acetate, 10 mM heptanesulphonic acid, pH 4.5) in 25 min [29]. The trichloroacetic extract was injected after filtration through a $0.22\text{ }\mu\text{m}$ Millipore-GS filter.

Assay of enzyme activities

ADC, ODC and SAMDC

Frozen leaf powder was homogenized in 0.1 M Hepes buffer containing 10 mM dithiothreitol, 10 mM EDTA, 0.1 mM pyridoxal $5'$ -phosphate and 0.5% (w/v) ascorbic acid. Insoluble poly(vinyl pyrrolidone) (100 mg/g of powder) was added before homogenization. The homogenates were centrifuged for 25 min at 17000 g and the supernatants loaded on to a PD10 column (Pharmacia) and eluted with the same buffer minus the ascorbic acid. The eluates were used for analysis of enzyme activity. Enzyme reaction mixtures contained $100\text{ }\mu\text{l}$ of extract and $20\text{ }\mu\text{l}$ of radiolabelled substrate. The ODC assay mixture contained $0.1\text{ }\mu\text{Ci}$ of L-[$1\text{-}^{14}\text{C}$]ornithine (57 mCi/mmol) and unlabelled ornithine (2.5 mM). L-[$U\text{-}^{14}\text{C}$]arginine ($0.1\text{ }\mu\text{Ci}$, 307 mCi/mmol) and unlabelled arginine (1.25 mM final concentration) were added for the analysis of ADC activity. For SAMDC activity, $20\text{ }\mu\text{l}$ of $5\text{ }\mu\text{Ci/ml}$ *S*-adenosyl-L-carboxy[^{14}C]methionine diluted in 4.5 mM unlabelled SAM was used. Each reaction was performed in duplicate at 37°C for 45 min. The ADC, ODC and SAMDC activities were determined by measurement of $^{14}\text{CO}_2$ released from substrates [30]. Protein concentration was de-

termined by the method of Bradford [31] using the Bio-Rad dye reagent.

Agmatine iminohydrolase (AIH) and *N*-carbamoylputrescine amidohydrolase (NCPAH)

Frozen leaf powder was homogenized as described above. After centrifugation the clear supernatants were dialysed for 24 h against 0.01 M Hepes buffer containing 1 mM dithiothreitol and 1 mM EDTA. The dialysed extracts were centrifuged and used for the assays. Assays were performed in a total volume of $80\text{ }\mu\text{l}$ containing $50\text{ }\mu\text{l}$ of extract and 12.5 mM substrate (agmatine or *N*-carbamoylputrescine). After 1 h at 37°C the reactions were stopped by addition of $10\text{ }\mu\text{l}$ of 50% trichloroacetic acid. The controls consisted of extracts without substrate and substrate without enzyme. *N*-Carbamoylputrescine and putrescine, the products of AIH and NCPAH respectively, were quantified after HPLC separation and post-column derivatization as described above.

Diamine oxidase (DAO)

DAO activity was determined as described previously [32]. The pyrrolidine was extracted in toluene after addition of NaHCO_3 and counted using a Philips liquid-scintillation counter.

Putrescine hydroxycinnamoyltransferase (PHT)

PHT activity was determined by the radiometric method of Negrel et al. [33] using putrescine and caffeoyl-CoA as substrates. Labelled caffeoylputrescine was separated in the presence of authentic standard by HPLC on a Novapak column and detected at 310 nm . Elution was achieved using a gradient from 87% solvent A (0.5% trichloroacetic acid, 10 mM heptanesulphonic acid) in acetonitrile to 50% solvent A. The gradient was optimized using both detection at 310 nm and post-column derivatization with *o*-phthalaldehyde to avoid co-elution of putrescine and caffeoylputrescine.

RESULTS

Generation of transgenic tobacco plants overexpressing oat ADC cDNA

Tobacco was chosen as the transgenic system because of its ease of transformation and regeneration. We chose to use oat ADC cDNA to avoid co-suppression [34] problems in tobacco and because the cDNA has been well characterized. The oat ADC cDNA contained 19 bp of untranslated leader sequence [15], and the entire 2.1 kbp cDNA was excised as an *EcoRI* fragment and subcloned into the *EcoRI* site of pJIT60 [22], which contains a CaMV 35S promoter with duplicated enhancer sequences and a CaMV transcriptional termination region. The 35S promoter-ADC-CaMV terminator cassette was subcloned into the *Agrobacterium* binary vector pBin19 [23], and, after triparental mating, the *Agrobacterium tumefaciens* LBA4404 strain [35] was used for the inoculation of tobacco leaf discs. Kanamycin-resistant plantlets were regenerated, rooted in kanamycin-containing medium and transferred to soil. DNA from young leaves was used for Southern-blot analysis to determine if the kanamycin-resistant plants contained the oat ADC construct. A 2.1 kbp *EcoRI* band corresponding to the oat ADC cDNA was present in all of the kanamycin-resistant putative transformants but not in the two untransformed control plants (results not shown). To determine if the oat ADC was expressed in tobacco, RNA gel-blot analysis of the transformed plants was performed. A PCR-derived tobacco ADC probe was isolated from tobacco root cultures and

Figure 1 Overexpression of oat ADC cDNA in young leaves of regenerated transformed tobacco plants

Each track was loaded with 10 μ g of total RNA from young leaves. Each panel represents an RNA gel-blot from identical formaldehyde-agarose gels run in parallel and probed with either the entire oat ADC cDNA or an 800 bp PCR-derived tobacco ADC cDNA fragment.

Table 1 ADC activity and accumulation of agmatine, putrescine, spermidine and spermine in young leaves of transformed tobacco plants overexpressing the oat ADC cDNA

C1 and C2 are untransformed control plants. The same frozen leaf powder used for the RNA gel-blot analysis was used for the enzyme assay and the agmatine and polyamine quantification. The ADC activity is expressed as nmol of CO_2/h per mg of protein. Agmatine and polyamines were measured from the same trichloroacetate extract and expressed as nmol/g fresh weight.

Plant	ADC activity	Agmatine	Putrescine	Spermidine	Spermine
C1	14	5	289	374	54
C2	13	2	325	490	71
A12	183	113	219	259	41
A13	190	202	154	292	37
A14	281	142	233	404	53
B12	155	134	229	349	42
B13	14	4	181	293	36
B15	175	117	279	370	52
B16	123	228	196	380	40
B17	163	76	338	418	55

exhibited 84.6% identity with the tomato ADC cDNA over approx. 800 bp. Figure 1 shows that the CaMV 35S promoter efficiently transcribed the oat ADC cDNA in all the transformants except the B13 plant, which, although transformed, did not accumulate the oat ADC transcript.

ADC activity, agmatine and polyamine content in regenerated transgenic plants

Polyamine biosynthesis in animals and fungi is regulated at several levels including translational control [4]. Even though the oat ADC cDNA does not contain a long untranslated leader region, which in the case of the mammalian SAMDC leader is involved in cell-specific translational down-regulation [36], it was possible that the increased ADC transcript levels would not result in increased enzyme activity. In addition, the oat ADC cDNA encodes a 66 kDa proenzyme [15] that is activated by enzymic clipping into two polypeptides [37,38]. To address directly whether the oat ADC mRNA was translated and the resulting protein processed and thereby active, we assayed extracts from young leaves of transgenic and control tobacco plants for the presence of increased ADC activity. All the plants with a high level of oat ADC transcript possessed an elevated ADC activity that was 10–20-fold greater than that of the untransformed control plants (Table 1). The higher ADC ac-

Figure 2 Southern-blot analysis of the segregating progeny of the A12 and A14 lines

Plant genomic DNA was digested with *EcoRI* and size-fractionated on a 0.8% agarose gel. Each track was loaded with 30 μ g of *EcoRI*-cut DNA. The oat ADC band is indicated by an arrow. Size markers are also as indicated.

tivities were also operational *in planta*, as shown by the increased level of agmatine (Table 1). The same frozen leaf powder that was used for the DNA and RNA blot analyses was also used for the ADC, agmatine and polyamine analyses. Transformed plants accumulated at least 20–65-fold more agmatine compared with the mean level in control plants. The B13 plant, which although transformed did not accumulate the oat ADC transcript, also did not possess increased ADC activity or agmatine accumulation.

ADC is thought to be a key enzyme in plant polyamine biosynthesis and in order to determine whether increased agmatine biosynthesis would affect polyamine metabolism, we analysed the transgenic plants for the effect of increased agmatine levels on the accumulation of putrescine, spermidine and spermine. Even though the agmatine accumulation in the transformed plants was greatly increased, no real change in the levels of putrescine, spermidine or spermine could be detected (Table 1). None of the transformed plants exhibited altered morphology and all set seed normally upon self-fertilization.

Molecular and biochemical analysis of the progeny of two transgenic lines with elevated ADC activities

To investigate further the regulation of polyamine biosynthesis in tobacco, we examined the effect of the increased agmatine levels on the size of the other polyamine pools. Physiological analysis of the first-generation regenerated plants was limited by the fact that the individual transformants were not at the same stages of development. To analyse a more physiologically homogeneous population of plants we decided to analyse second-generation offspring. The progeny resulting from the self-fertilization of two lines carrying the oat ADC construct (A12 and A14) were employed for analysis at the molecular and biochemical levels. These plants segregated in a Mendelian manner into siblings with the oat ADC transgene and those without it. Analysis of these segregants ensured that the syngenic control and transgenic siblings were at exactly the same developmental stage at the time of sampling and were identical except for the presence of the transgene. Seeds from the A12 and A14 plants were sown directly in soil, and young leaves from plants of each

Figure 3 Overexpression of the oat ADC cDNA in young leaves of the segregating progeny of the A12 and A14 lines

Each panel represents a 1.2% formaldehyde gel run in parallel. Each track was loaded with 10 μ g of total RNA from young leaves. The RNA gel blots were probed with either the entire oat ADC cDNA or an 800 bp PCR-derived cDNA probe of the tobacco ADC.

Table 2 Agmatine and free polyamine content in young leaves of the A12 and A14 lines overexpressing the oat ADC cDNA

The A12 and A14 progeny were 80 and 87 days old respectively at the time of sampling. Control values represent the mean $\pm$ S.D. between independent plants ($n = 5$). Agmatine and polyamines were measured using the same trichloroacetate extract and are expressed as nmol/g fresh weight.

Line	Plant	Agmatine	Putrescine	Spermidine	Spermine
A12	Controls	8 $\pm$ 3	276 $\pm$ 26	454 $\pm$ 35	68 $\pm$ 8
	3	94	355	479	64
	4	128	348	436	59
	5	163	363	492	71
	7	112	264	394	55
	10	171	283	407	53
A14	Controls	8 $\pm$ 2	387 $\pm$ 44	497 $\pm$ 95	70 $\pm$ 9
	2	143	377	514	76
	3	153	256	407	53
	7	93	365	449	65
	8	102	376	484	69
	10	145	400	420	58

line sampled at 80 days for A12, and 87 days for A14 plants after sowing. Five plants of each line segregating without the transgene were chosen as controls and five plants from each line segregating with the transgene were chosen as the transgenotes. We were not able to differentiate between homozygous and heterozygous transgenics. Figure 2 is a Southern-blot analysis of the segregating progeny of the A12 and A14 lines demonstrating that A12-3,4,5,7,10 and A14-2,3,7,8,10 retained the oat ADC cDNA. To ensure that each transformant expressed the oat ADC cDNA, the accumulation of both the oat and tobacco ADC transcripts was analysed by RNA gel-blot hybridization. Figure 3 shows that, as with the primary transformants, in the segregating transgenic progeny, oat ADC cDNA was highly expressed, whereas the tobacco ADC transcript accumulation was similar in both transgenic and syngenic control plants. The results from the Southern- and Northern-blot analyses confirmed that the oat ADC cDNA was stably integrated and transcribed in the A12 and A14 transgenic offspring.

The level of agmatine in the A12 and A14 non-transgenic control plants was low (8 nmol/g fresh weight), as with the first-

Table 3 ADC, ODC and SAMDC activities in young leaves of the A12 and A14 progeny overexpressing the oat ADC cDNA

The A12 and A14 progeny were 80 and 87 days old respectively at the time of sampling. Control values represent the mean $\pm$ S.D. between independent plants ($n = 5$). Enzyme activities are expressed as nmol of CO₂/h per mg of protein.

Line	Plant	ADC	ODC	SAMDC
A12	Controls	16.9 $\pm$ 1.5	15.1 $\pm$ 2.9	0.59 $\pm$ 0.10
	3	214	11.4	0.50
	4	311	12.3	0.50
	5	305	18.3	0.52
	7	258	11.8	0.48
	10	246	17.8	0.75
A14	Controls	27.2 $\pm$ 3.9	13.4 $\pm$ 3.4	1.82 $\pm$ 0.40
	2	221	14.8	2.20
	3	165	10.1	2.05
	7	131	12.6	1.98
	8	106	14.5	1.74
	10	165	9.5	1.32

generation control plants, whereas for the transformed plants the level ranged between 93 and 171 nmol/g fresh weight (Table 2). On average there was 16-fold more agmatine in both the A12 and A14 transgenic plants. Accumulation of putrescine, spermidine and spermine was unaffected by the elevated levels of agmatine, being quantitatively unchanged between control and transformed plants. In young leaves of the non-transgenic control plants, the proportions agmatine/putrescine/spermidine/spermine were 0.024:1.00:1.43:0.21 whereas in the transgenic plants the ratio between agmatine and putrescine had risen to 0.40:1.00.

The increased ADC activity in young leaves of the transgenic progeny compared with control progeny can be seen clearly in Table 3. ADC activity in the A12 transgenic plants was on average 16-fold higher than its activity in the non-transgenic control plants and, in A14 transgenic plants, ADC activity was on average 6-fold higher than in the controls. The ODC and SAMDC activities did not show any difference between the transgenic and control plants, but SAMDC activity was more than 2-fold higher in the A14 than A12 plants (Table 3).

Conjugated polyamine, bound polyamine and alkaloid levels in A12 and A14 segregating progeny

As the increased agmatine production resulting from overexpression of oat ADC did not result in increased putrescine levels, we decided to investigate other possible destinations of the agmatine, including conjugated and bound polyamines and the tobacco alkaloid nicotine. In most plant species, polyamines can be found free, as amides of hydroxycinnamic acids or bound to macromolecules [6]. In tobacco, conjugated polyamines represent the main component of the polyamine pool, especially in flowers and roots [39].

We extracted polyamines with trichloroacetic acid, and separated free and conjugated polyamines from the bound forms by centrifugation. Conjugated and bound forms were quantified by dansylation of the released polyamines after acid hydrolysis. Table 4 shows that the levels of conjugated putrescine and spermidine were unchanged in transgenic compared with control plants in the progeny of A12 and A14 lines. An apparent reduction in the levels of conjugated spermidine in the transgenic progeny of A12 was not significant compared with the levels in the control plants. Similarly there was no change in the levels of the bound polyamines. No conjugated or bound spermine was

Table 4 Conjugated polyamines, bound polyamines and nicotine content in young leaves of the A12 and A14 progeny overexpressing the oat ADC cDNA

The A12 and A14 progeny were 80 and 87 days old respectively at the time of sampling. Control values represent the mean $\pm$ S.D. between independent plants ($n = 5$). Polyamines and nicotine are expressed as nmol/g fresh weight and μ mol/g fresh weight respectively.

Line	Plant	Conjugated polyamines		Bound polyamines		Nicotine
		Putrescine	Spermidine	Putrescine	Spermidine	
A12	Controls	843 $\pm$ 244	453 $\pm$ 128	24 $\pm$ 5	73 $\pm$ 21	2.06 $\pm$ 0.58
	3	640	273	16	50	1.27
	4	574	324	13	66	1.58
	5	571	250	12	97	1.72
	7	617	306	16	46	2.28
	10	560	280	20	57	2.10
A14	Controls	987 $\pm$ 268	507 $\pm$ 140	27 $\pm$ 8	154 $\pm$ 8	2.37 $\pm$ 1.22
	2	1801	881	25	168	2.50
	3	644	366	37	131	1.87
	7	1113	702	36	170	1.30
	8	828	343	40	118	1.33
	10	688	250	20	130	1.76

detectable in young leaves of the transgenic or control plants. Overall, in both transgenic and control plants, the levels of conjugated putrescine and spermidine were higher in young leaves of A14 transgenotes than in the progeny of A12, reflecting the 7-day difference in sowing times between the A12 and A14 lines. This developmental difference resulted in the A14 plants being closer to the transition from vegetative to floral development [40], and correlated with increased polyamine metabolism in newly initiated leaves.

Putrescine can enter the alkaloid pathway in tobacco upon methylation by putrescine *N*-methyltransferase [41]. Nicotine is synthesized in the roots and transported to the leaves. The level of nicotine in young leaves of the segregating progeny of the A12 and A14 lines was analysed and the results presented in Table 4. No difference in nicotine levels were found between the control and transgenic lines of either line. Thus the elevated agmatine accumulation did not result in increased free putrescine, spermidine, spermine, conjugated putrescine, conjugated spermidine, bound putrescine or spermidine, or nicotine levels.

Other related enzyme activities in the A14 progeny

Although agmatine is the precursor of putrescine, two other enzyme activities are required to produce putrescine from agmatine. AIH hydrolyses agmatine to *N*-carbamoylputrescine [42] and NCPAH then hydrolyses *N*-carbamoylputrescine to putrescine [6]. These two steps are not thought to be rate-limiting. AIH and NCPAH were active in tobacco (Table 5); this is the first demonstration of their joint activities in tobacco. No difference in AIH or NCPAH activity was detected between the transgenic and normal progeny (Table 5).

If the increased agmatine pool was not being channelled into putrescine, spermidine, spermine, their conjugates, bound polyamines or nicotine, it was possible that agmatine itself was being conjugated to cinnamic acids. Agmatine conjugates of this type have been found in barley leaves [6]. Negrel et al. [33] have shown that PHT, which catalyses the conjugation of putrescine to an activated hydroxycinnamic acid, can also use agmatine as a substrate. We compared the activity of this enzyme in the leaf extracts from A14 transgenic and control plants and found no overall difference (Table 5). Moreover, in all the transformed and

Table 5 AIH, NCPAH, DAO and PHT activities in young leaves of 87-day-old A14 progeny

Control values represent the mean $\pm$ S.D. between independent plants ($n = 5$). Enzyme activities are expressed as nmol of CO₂/h per mg of protein.

Plant	AIH	NCPAH	DAO	PHT
Controls	61.7 $\pm$ 7	35 $\pm$ 11	8 $\pm$ 3.5	1.57 $\pm$ 0.35
2	67	34	16.9	1.30
3	70	26	5.4	1.32
7	62	31	4.5	1.11
8	59	25	9.9	1.02
10	57	23	14.1	1.22

control plants from the two lines, no conjugated or bound agmatine was detected after hydrolysis. To confirm these results, the conjugated polyamines were extracted and purified by cation-exchange chromatography [40] and analysed by HPLC as described in the Experimental section. No extra compound was detected in extracts of transgenic plants compared with the controls (results not shown).

In bacteria, agmatine can be metabolized to γ -guanidinobutyraldehyde by either agmatine oxidase [43] or agmatine dehydrogenase [44]. Our attempts to find this metabolism in tobacco were unsuccessful. DAO can also catabolize polyamines in plants and can use agmatine as a substrate [45]. We measured the activity of DAO in the progeny of A14 but no difference in DAO activity between the control and transgenic plants was detected (Table 5).

In conclusion, the only effect we could detect in the tobacco plants overexpressing the oat ADC cDNA was increased ADC activity and an elevated level of agmatine. Polyamine biosynthesis and related pathways were not affected. All transgenic progeny of the A12 and A14 lines were morphologically normal.

DISCUSSION

Malmberg and colleagues [15,37,38] demonstrated that the oat ADC protein is made as a 66 kDa proenzyme and is processed to 42 and 24 kDa polypeptide fragments by proteolytic cleavage. Processing is necessary for enzyme activation, and the processing enzyme is apparently distinct from ADC. It is evident that leaf extracts from the regenerated transgenic tobacco plants that we have produced, overexpressing the oat ADC cDNA, exhibited increased ADC activity *in vitro*. The 20–65-fold increased accumulation of agmatine in the first-generation regenerated plants *in vivo* confirms that the measured increase in the ADC activity is representative of an elevated activity *in vivo*. It is therefore reasonable to assume that the monocot oat ADC must be correctly processed in the dicot tobacco.

Biochemical analyses of the first-generation transgenics were carried out on young leaves because the oat ADC was originally isolated from leaves, and free and conjugated polyamines as well as nicotine could be analysed in the same organ. Increased agmatine levels correlated with accumulation of the oat ADC transcript in the transformed plants. However, the increased agmatine levels did not result in elevated putrescine accumulation, and the levels of spermidine and spermine were unchanged in the transgenic plants. The enzymes AIH and NCPAH are necessary for the formation of putrescine from agmatine via *N*-carbamoylputrescine [6,42]. It has not been previously reported that the AIH and NCPAH are rate-limiting so it was possible that flux may have been channelled into other polyamine-derived

metabolites. To examine in more detail the fate of the accumulated agmatine in the transgenic plants, it was necessary to analyse the segregating progeny derived from the self-fertilization of the first-generation plants. This was essential because the regenerated transgenic plants were at different stages of development and, in addition, like all plants regenerated from tissue culture, may have been subjected to somaclonal variation. By choosing to analyse segregating progeny from two different transgenic lines, we were able to eliminate possible artifacts caused by insertion mutagenesis due to the transferred DNA.

The ADC overexpression and agmatine overaccumulation effects were essentially the same in the transgenic segregating progeny of the second-generation as in the first-generation plants. The levels of agmatine were rather lower but no effect on putrescine, spermidine or spermine levels could be detected, and the ODC and SAMDC activities were the same as in the controls. It was possible that the increased agmatine was being channelled into the hydroxycinnamic acid-conjugated polyamine pool via putrescine and spermidine, or that the putrescine-conjugating enzyme PHT was using agmatine as a substrate [33] and conjugating it directly. However, no change in the conjugated putrescine and spermidine pools could be found and no spermine or agmatine conjugates could be detected in the leaf extracts. Thus the increased ADC activity and agmatine levels did not result in changes in the free and conjugated polyamine pools. Another possibility was that the increased ADC activity and agmatine level might result in an increase in alkaloid production via putrescine. Nicotine is synthesized in the roots and transported to the leaves [41]; the CaMV 35S promoter is active in roots [46]. Hamill et al. [47] reported that overexpression of yeast ODC in hairy root cultures of *Nicotiana glauca* resulted in a slight increase (less than 2-fold) in nicotine accumulation. The putrescine required for nicotine biosynthesis in tobacco is generated primarily through the ADC, rather than the ODC, pathway [48,49]. However, in the plants overexpressing ADC, we detected no change in nicotine levels between the transgenic and control plants. AIH and NCPAH activities did not seem to be down-regulated in the transgenic plants. Likewise, the activity of the conjugating enzyme PHT was unchanged.

The question arises as to whether the tight regulation of putrescine biosynthesis from agmatine was due to the pathway compensating in some undetected manner for the increased agmatine level or whether the agmatine is somehow inaccessible to the rest of the pathway because of rapid degradation or sequestration by compartmentalization. DAO activity was not affected by the increased agmatine levels, but DAO could be operating below its catalytic capacity in normal leaves and thus be capable of passively responding to an increase in agmatine accumulation by rapidly catabolizing increased putrescine. Agmatine itself can also act as a substrate for DAO. Overproduction of agmatine could result in sequestration to the vacuole before further metabolism; polyamines have been detected in the vacuole [50]. One possibility is that if ADC is localized to the chloroplast despite lacking an obvious transit peptide [51], the targeting of the monocot oat ADC may not be as effective in the dicot tobacco plant.

The deregulated expression of ADC and overproduction of agmatine did not result in any change in polyamine levels. It has been suggested that accumulation of polyamines is toxic because of the effect of their breakdown products [52]. The results from the ADC overexpression are in keeping with results from the overexpression of human ODC in mouse [53], yeast ODC in hairy root cultures of *N. glauca* [47] and mouse ODC in tobacco [54]. None of the ODC overexpressing systems produced a change in spermidine and spermine accumulation. Increased

ODC activity in transgenic mice overexpressing human ODC did not result in increased putrescine levels except in testes and brain [53,55]. Similarly overexpression of yeast ODC in *N. glauca* did not significantly increase putrescine levels [47], and overexpression of mouse ODC in tobacco increased putrescine accumulation 2.5-fold in the most extreme case [54]. Recently, the human ODC gene has been expressed in transgenic mice under the control of the mouse metallothionein I promoter [56]. Even though there was a nearly 150-fold increase in hepatic putrescine, there was only a transient increase in spermidine and spermine before they returned to normal basal levels. It is probable that to increase putrescine accumulation from arginine, there must be a co-ordinated increase in the activities of ADC, AIH and NCPAH. In this respect, it is interesting to note that, when barley seedlings were fed with HCl, causing elevated putrescine accumulation, NCPAH activity increased 2-fold, as did that of ADC [57], suggesting that NCPAH activity must be co-ordinately up-regulated with ADC to increase putrescine levels. Previous studies on the role of ADC in putrescine biosynthesis have not investigated the activities of AIH and NCPAH in parallel with ADC. Our results with ADC overexpression reflect a more general phenomenon in plant metabolism, which has led to a re-evaluation of how metabolic pathways are regulated [58]. Employing the distinction made by Stitt and Sonnewald [58] between the regulatability and regulatory capacity of an enzyme, it is obvious from previous studies that ADC is a highly regulated enzyme during stress responses [59], but the capacity of ADC alone to influence polyamine biosynthesis appears to be limited. Plant polyamine metabolism is not only plastic, with two pathways via ODC and ADC to putrescine, but, in addition, flux control in the ADC pathway may be shared by several components. We are now trying to determine the nature of the metabolic/physiological block preventing the accumulation of putrescine in 35S ADC transgenic plants.

We thank Cathie Martin and Julie Hofer for constructive criticism of the manuscript, Russel Malmberg for the gift of oat ADC cDNA clone, Jonathan Negrel for a sample of caffeoyl-CoA and Lionel Perkins for maintaining the plants. D.B. was the recipient of an E.C. Human Capital and Mobility fellowship (no. ERBBI02CT25110).

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