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Reduction in the endogenous *arginine decarboxylase* transcript levels in rice leads to depletion of the putrescine and spermidine pools with no concomitant changes in the expression of downstream genes in the polyamine biosynthetic pathway

Abstract We investigated whether down-regulation of arginine decarboxylase (ADC) activity and concomitant changes in polyamine levels result in changes in the expression of downstream genes in the polyamine pathway. We generated transgenic rice (*Oryza sativa* L.) plants in which the rice *adc* gene was down-regulated by expression of its antisense oat (*Avena sativa* L.) ortholog. Plants expressed the oat mRNA *adc* transcript at different levels. The endogenous transcript was down-regulated in five out of eight plant lineages we studied in detail. Reduction in the steady-state rice *adc* mRNA levels resulted in a concomitant decrease in ADC activity. The putrescine and spermidine pool was significantly reduced in plants with lower ADC activity. Expression of the rice *ornithine decarboxylase* (*odc*), *S-adenosylmethionine decarboxylase* (*samdc*) and *spermidine synthase* (*spd syn*) transcripts was not affected. We demonstrate that even though levels of the key metabolites in the pathway were compromised, this did not influence steady-state transcription levels of the other genes involved in the pathway. Our results provide an insight into the different regulatory mechanisms that control gene expression in the polyamine biosynthetic pathway in plants by demonstrating that the endogenous pathway is uncoupled from manipulations that modulate polyamine levels by expression of orthologous transgenes.

Keywords Antisense · Arginine decarboxylase · *Oryza* · Polyamine

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Abbreviations ADC (*adc*): arginine decarboxylase protein (gene) · DIG: digoxigenin · ODC (*odc*): ornithine decarboxylase protein (gene) · SAMDC (*samdc*): adenosylmethionine decarboxylase protein (gene) · SPD SYN (*spd syn*): spermidine synthase protein (gene) · RT–PCR: reverse transcription–polymerase chain reaction

Introduction

Manipulation of metabolic pathways in plants through molecular genetic approaches is now possible as a result of a significant increase in our knowledge base in terms of how such, often complex, networks are controlled and regulated. An important step forward in our ability to understand and modulate plant biosynthetic pathways is the availability of cloned genes encoding key enzymes involved in the pathway. This, together with the identification of useful mutant phenotypes and advances in gene transfer technology make it possible to pose important biochemical questions that need to be addressed before we embark on useful strategies to

engineer complex pathways in plants. Sophisticated genomic tools, availability of transcription factors that influence metabolism in a holistic manner and a better understanding of how the biochemistry of a given pathway may be controlled spatially and/or temporally provide additional means of manipulating plant metabolism.

Transgenic manipulation of polyamine metabolism has become a valuable tool for studying their physiological roles in plants (for review, see Kumar and Minocha 1998; Bhatnagar et al. 2002). Cellular content of polyamines has been modulated by over-expression or down-regulation of *arginine decarboxylase* (*adc*), *ornithine decarboxylase* (*odc*) and *S-adenosylmethionine decarboxylase* (*samdc*; for review, see Kumar and Minocha 1998; Bhatnagar et al. 2002). Over-expression of heterologous *adc* or *odc* cDNA generally causes the production of high levels of putrescine (DeScenzo and Minocha 1993; Bastola and Minocha 1995; Burtin and Michael 1997; Capell et al. 1998). In most cases, a relatively small increase in spermidine and spermine has been observed despite significantly large increases in putrescine levels in transgenic cells (Bassie et al. 2000; Lepri et al. 2001; Sivamani et al. 2001). This observation, combined with the fact that under stress conditions mostly putrescine levels appear to fluctuate without major changes in spermidine or spermine levels, suggests that the levels of spermidine and spermine are under a tight homeostatic regulation (Bhatnagar et al. 2002).

Among the regulatory mechanisms controlling ADC, ODC and SAMDC activities, feedback control by polyamines has been described in different systems. In osmotically stressed oat leaves, spermidine inhibits post-translational processing of the ADC precursor with a subsequent decrease in mature ADC (Borrell et al. 1995). Exogenously added polyamines suppressed ADC activity in tobacco cell cultures, also suggesting the existence of a feedback regulatory mechanism for ADC (Hiatt et al. 1986). With regard to the feedback control of plant ODC by polyamines, the sensitivity of ODC activity to these molecules suggests that polyamine synthesis via ODC may be regulated, at least in part, by simple end-product accumulation (Slocum and Richardson 1991). However, the data obtained by Hiatt et al. (1986) suggest that this may not be a general mechanism since exogenously added polyamines did not suppress ODC activity in tobacco cell cultures. In contrast to the mammalian or yeast enzymes, the plant SAMDC enzyme is not stimulated by putrescine, and it appears that the accumulation of cellular polyamines inhibits SAMDC activity (Hiatt et al. 1986). In tobacco cell cultures, treatment with 1 mM spermidine resulted in a rapid decrease in SAMDC activity by blocking the synthesis of the enzyme (Hiatt et al. 1986). This suggested that there might be different mechanisms involved in regulating ADC, ODC and SAMDC activities (Tiburcio et al. 1997).

For the past several years we have been investigating molecular and biochemical aspects of the polyamine biosynthetic pathway in plants, using rice as a model. In the course of these studies we generated transgenic plants expressing different polyamine biosynthetic genes, including *adc*, *odc* and *samdc* (Capell et al. 1998; Noury et al. 2000; Lepri et al. 2001; Thu-Hang et al. 2002). We have demonstrated that by over-expressing the *Datura samdc* cDNA, rice leaf tissue can accumulate 2-fold higher putrescine and 2.5-fold higher spermidine levels when compared to wild type, with a concomitant increase in ADC and ODC activity. No changes in *samdc* and *spermidine synthase* (*spd syn*) transcripts were observed (Thu-Hang et al. 2002). We subsequently wished to investigate whether down-regulation of the rice *adc* gene would result in depletion of the polyamine pool and whether this would influence expression of other genes in the pathway. We used transgenic rice plants in which the rice *adc* gene was down-regulated by expression of its antisense oat ortholog. Transcript accumulation of the rice *odc*, *samdc* and *spd syn* was not affected. Reduction in the steady-state rice *adc* mRNA levels resulted in a concomitant decrease in ADC activity. We demonstrated that even though levels of the key metabolites in the pathway were compromised, this did not influence steady-state transcript levels of the other genes involved in the pathway. Our results indicate that even though the endogenous polyamine pool in these plants is altered substantially, this does not have any effect on the steady-state mRNA of the other downstream genes in the polyamine pathway.

Materials and methods

Plasmids, transformation and plant regeneration

The 2.124-kb oat *adc* cDNA (Bell and Malberg 1990) was excised as an *Eco*RI fragment from pAMC2 (Burtin and Michael 1997) and subcloned into the *Eco*RI site of pJIT60 (Gurineau et al. 1992) which contains a 35S CaMV promoter with duplicated enhancer sequences and a *nos* termination region. Using the restriction enzyme *Sal*I, we confirmed the antisense orientation of the oat *adc* cDNA. This plasmid was subsequently referred to as *35S:adca*. Bombardment, selection and regeneration of transgenic material were as described previously (Sudhakar et al. 1998; Capell et al. 2000).

Polymerase chain reaction (PCR) and reverse transcription–polymerase chain reaction (RT–PCR)

Genomic DNA was extracted from leaf tissue according to the method of Edwards et al. (1991). Genomic PCR amplifications were carried out in a total volume of 50 μl, comprising 100 ng genomic DNA, 1× Roche PCR buffer [50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl$_2$, 0.1% Triton X-100], 400 μM each deoxynucleoside triphosphate, 100 nM of each primer and 2.5 units of *Taq* DNA polymerase (Roche). The accession number for the oat *adc* sequence, primer sequences and the predicted amplification product size are indicated in Table 1. For the oat *adc* cDNA we carried out 35 amplification cycles: denaturation (96 °C, 40 s), annealing (70 °C, 30 s) and extension (72 °C, 2 min 30 s). The product was visualized on a 1% Tris–borate buffer (TBE) agarose gel.

Total RNA was extracted from 2-month-old leaves and tillers of transgenic plants and wild type using the RNeasy Plant Mini Kit

Table 1 Primers used in PCR, RT–PCR analysis and probes. PCR product sizes: pRadc-3, pRadc-4 = 400 bp; pOatadc-1, pOatadc-5 = 1.5 kb; pRodc-1, pRodc-2 = 700 bp; pRsamdc-1, pRsamdc-2 = 700 bp; pRspdsyn-1, pRspdsyn-2 = 900 bp; pAct-1, pAct-2 = 600 bp. *EST* Expressed sequence tag, *ORF* open reading frame

[a]Forward primer
[b]Reverse primer

Accession number	Primer name	Nucleotide position in the EST (E) or ORF (O)	Sequence
C99671 (rice *adc*)	pRadc-3[a] pRadc-4[b]	1 (E)	5′-AGCGCGCTGGTGTGCGCACCA-3′ 5′-TGTCGCAGGTGAGGTCGGAG-3′
X56820 (oat *adc*)	pOatadc-1[a] pOatadc-5[b]	1 (O)	5′-CGGCGATGTGTACCATGTCGAGGG-3′ 5′-GCGGGTGCAGCGGCATCGTCTCGG-3′
BE040058 (rice *odc*)	pRodc-3[a] pRodc-4[b]	60 (E)	5′-GCGTTTTATGCGATTTGCGAACGG-3′ 5′-CCCAGTCTAAACAAGCCGGAACCG-3′
Y07766 (rice *samdc*)	pRsamdc-1[a] pRsamdc-2[b]	1023 (O)	5′-GGAGATCCAGCCAAAGCCTGGCC-3′ 5′-CCCAGGGGAGAAGATTGCCCAG-3′
AJ251298 (rice *spd syn*)	pRspdsyn-1[a] pRspdsyn-2[b]	196 (O)	5′-GGATGGTTCTCCGAGATTAG-3′ 5′-GATCTAGTTGGCCTTGGATC-3′
X16280 (rice *actin-1*)	pAct-1[a] pAct-2[b]	1 (O)	5′-ATGGCTGACGCCGAGGATAT-3′ 5′-AGGAGTGGTGACTGAGTAAC-3′

(Qiagen). A single tiller from each plant was separated and grown hydroponically in Yoshida's solution (Yoshida et al. 1972) to induce root growth. Roots were harvested 2 weeks later and RNA analyses were carried out using the same kit as before. Aliquots of 200 ng total RNA were used in each RT–PCR reaction. Reverse transcription was performed using the Access RT–PCR System (Promega). The resulting oat *adc* cDNA was amplified as described above, using the same primers and cycling conditions. As a negative control, particular RNAs without RT were also subjected to RT–PCR. Products from 25 cycles after separation in a 1% TBE agarose gel were capillary-blotted on positively charged nitrocellulose membranes (Roche). Blots were hybridized with the oat *adc* probe (Table 1) under the same conditions used for DNA gel blot analysis (as described subsequently). Exposure time was 10 min. For each plant, rice *actin-1* transcripts also were amplified as constitutive expression controls as described in Fu et al. (2001). Re-hybridization of the blot with the rice *actin-1* probe (Table 1) was carried out as described in Hloch et al. (2001). Exposure time was 20 min.

DNA and RNA gel blot analysis

Rice (*Oryza sativa* L.) DNA, RNA and oat (*Avena sativa* L.) DNA was isolated from leaf and root tissue according to the procedure of Creissen and Mullineaux (1995). Tissue from plants grown exactly under the same conditions as described for PCR analysis was used. Following *Hind*III or *Eco*RI digestion and electrophoresis on a 1% TBE agarose gel (Sambrook et al. 1989), DNA (15 µg) was transferred to a positively charged nylon membrane (Roche). Nucleic acids were fixed by baking at 80 °C for 2 h. Filters were washed in 2×SSC for 30 min and then pre-hybridized at 42 °C for 2 h using the digoxigenin (DIG)-easy hybridization solution (Roche). The primer sequences used to make the oat *adc* probe and the predicted amplification product size are indicated in Table 1. The probe was labeled using the PCR DIG probe synthesis kit (Roche). Alkali-labile DIG-11-dUTP was incorporated into the probe in a final volume of 50 µl comprising 4 µM dATP, 4 µM dCTP, 4 µM dGTP, 3.2 µM dTTP, 0.8 µM DIG-11-dUTP, 1× Roche PCR buffer [50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100], 2.5 units of *Taq* DNA polymerase (Roche), 0.1 mM each of the forward and reverse sequence primers and 200 pg of the plasmid. We carried out 35 amplification cycles: denaturation (96 °C, 10 s), annealing (70 °C, 10 s), and extension (72 °C, 1 min 30 s). Labeled oat *adc* probe was purified using the QIAquick Gel Extraction Kit (Qiagen) and denatured at 68 °C for 10 min prior to use. Hybridization was performed at 42 °C overnight. The membranes were washed twice for 5 min in 2×SSC, 0.1% SDS at room temperature, and then twice (15 min) in 0.5×SSC, 0.1% SDS at 68 °C. Genomic *Eco*RI-digested rice and oat DNA (5 µg) from wild-type plants was probed with the oat *adc* DIG-labeled probe and washed twice (15 min) in 2×SSC, 0.1% SDS at 68 °C. Chemiluminescence detection was carried out according to the manufacturer's instructions using the DIG Luminescence

Detection Kit. After washing, the membranes were incubated with CSPD(R) Chemiluminescent Substrate (Roche) and subsequently exposed to X-ray film (Fuji Photofilm Co., Kanawa, Japan) for 30 min at 37 °C.

Denatured RNA (30 µg) from leaf tissue and roots was subjected to electrophoresis on a 1.2% agarose–formaldehyde gel using 1×Mops buffer (Sambrook et al. 1989). Hybridization of the RNA gel blots from leaf tissue and roots with the oat *adc* probe was as described above for DNA procedures. Membranes were exposed to X-ray film for 10 min at 37 °C. Hybridization of the RNA gel blot with the rice probes was carried out as described subsequently. Accession numbers, primer sequences used to make the rice *adc*, rice *odc*, rice *samdc* and rice *spd syn* probes and the predicted amplification product sizes are indicated in Table 1. Probes were labeled using the PCR DIG probe synthesis kit (Roche). We carried out 35 amplification cycles: denaturation (96 °C, 10 s), annealing [64 °C (rice *adc*), 65 °C (rice *odc*), 65 °C (rice *samdc*), 60 °C (rice *spd syn*), 10 s], and extension (72 °C, 1 min). Labeled probes were purified using the QIAquick Gel Extraction Kit (Qiagen) and denatured at 68 °C for 10 min prior to use. Transfer and hybridization were carried out as described above for DNA procedures. Re-probing of the membranes was performed as described in Hloch et al. (2001). Membranes were exposed at 37 °C to X-ray film for 30 min for *adc*, 50 min for *odc*, 30 min for *samdc*, and 1 h for *spd syn*.

All RNA experiments were repeated at least twice from independent RNA isolations. Oat and rice *adc* steady-state mRNA hybridization signals were quantified using Quantity One (Quantification Software; Bio-Rad) and the resulting values were normalized using values obtained from RNA loading levels.

Determination of ADC activity

Leaf tissue from 2 month-old plants at the same stage as described for molecular analysis were used for ADC activity measurements. Tissue was extracted in buffer (0.1 M Tris, pH 7.6, and 2 mM DTT) at a ratio of 300 mg ml^{-1} buffer. Polyvinylpyrrolidone (100 mg) was added during grinding. Following centrifugation at 12,000 g for 20 min, the supernatant was used directly in enzyme activity assays. Tissue was always processed immediately after harvest and all assays were performed using fresh extracts. Enzyme assays were carried out in 1.5-ml Eppendorf tubes. A 6-mm-diameter filter paper disc impregnated with 50 µl of 2 N KOH and transfixed with a 3-cm needle was used to trap the $^{14}CO_2$ liberated during the reaction. The reaction mixture for ADC activity contained 20 µl of extraction buffer (pH 7.6), 160 µl of crude enzyme and 20 µl of the substrate mix [20 µl of L-[U-^{14}C]-arginine (specific activity 11 GBq mmol^{-1}, radioactive concentration 1,850 kBq ml^{-1}; Amersham International) diluted with 20 µl non-radioactive arginine (500 mM) and 60 µl of distilled water] to give a final concentration of 10 mM arginine. Two hundred microliters of 10% (v/v) perchloric acid was added to stop the reaction. After further

incubation for 45 min the filter paper was placed in scintillation minivials with 2 ml scintillation liquid (OptiPhase Hisafe II; Fisons Chemicals) and radioactivity was measured in a Wallac 1219 Rackbeta liquid scintillation counter. Protein determination was carried out as described in Bradford (1976), using bovine serum albumin as a standard. One nkat of ADC activity was defined as the amount (μmol) of $^{14}CO_2$ released per min and per mg protein.

Polyamine analysis

Crude extracts from leaves from 2-month-old plants and seeds were dansylated and separated by TLC (thin-layer chromatography) as described earlier (Bassie et al. 2000). The dansyl-polyamine bands were identified on the basis of their Rf values after visualisation under UV light (312 nm) and comparison to dansylated polyamine standards. The image of the chromatogram was captured and analysed by Quantity One (Quantification Software; Bio-Rad). The relative amount of dansyl-polyamines in each sample was determined by calculating the integrated optical density of the bands compared to the integrated optical density of the appropriate dilution of the dansylated control samples. Results were expressed as nmol g^{-1} fresh weight (fw).

Statistical analysis

As control values for biochemical analyses (enzyme activity and polyamine content) we used *hpt*-transformed plants in addition to wild-type controls (average of three samples each from six independent lines; $n = 36$). Hygromycin-resistant transformants and wild-type control values were not significantly different ($P > 0.05$) in terms of enzyme activity and polyamine levels (Lepri et al. 2002). For biochemical analyses of transgenic material (enzyme activity and polyamine content) we used the average value of three samples from each sibling ($n = 3$) and each measurement was repeated twice. The data were analyzed by two-way analysis of variance followed by a *t*-test using the Residual Mean Square in the ANOVA as the estimate of variability.

Results

The 1.5-kb oat *adc* probe, detects the rice *adc* gene

To confirm that the antisense effect was due to the homology between sequences, we digested rice and oat genomic DNA with *Eco*RI and hybridised at low stringency to the 1.5-kb DIG-labelled region of the oat *adc* cDNA. *Eco*RI digests of rice and oat DNA yielded one fragment at ≈12 kb that hybridised strongly to the *adc* probe in both species. In oat, a second genomic fragment of ≈7 kb was also detected (Fig. 1).

Molecular characterization of the transgenic population

The transformation vector containing the oat antisense *adc* cDNA was constructed as described in Materials and methods. Gene transfer and recovery of primary transformants were carried out as described in Capell et al. (2000). We analyzed 12 independent transgenic rice plant lineages and we confirmed integration of the
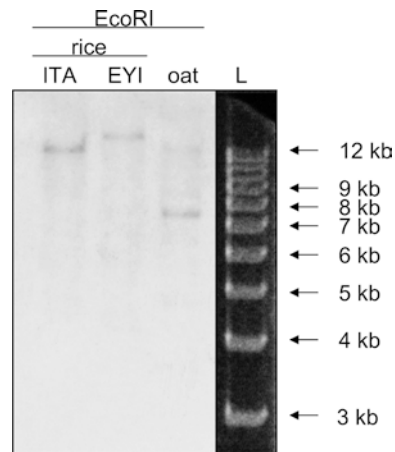

Fig. 1 Gel blot analysis of *Eco*RI-digested genomic rice (*Oryza sativa*, genotypes ITA and EYI) and oat (*Avena sativa*) DNA. The blot was probed with the 1.5-kb DIG-labelled PCR product from *35S:adca* at low stringency. Exposure time 30 min. *L* Molecular size marker (1-kb DNA ladder; Invitrogen)

35S:adca by genomic DNA gel blot analyses (Capell et al. 2000). Twenty fertile phenotypically normal plants (R1) from each of eight randomly selected primary transformants (R0) were analysed for the presence of the transgene. Segregation analysis was performed by PCR using the set of primers pOatadc-1 and pOatadc-5 (Table 1). We observed that in all lines the *35S:adca* transgene segregated in a ratio of approximately 3:1 (results not shown) and confirm that single or multiple copies of the transgene was/were integrated in a single genetic locus. These results are consistent with previous reports describing the genomic organisation of multiple integrated transgenes in rice (Kohli et al. 1998; Fu et al. 2000). Genomic DNA gel blot analysis of representative samples from each lineage (up to four siblings) is shown in Fig. 2 (*adc* DIG-labelled probe). Digests were carried out using either *Hind*III, which cuts once within the transgene (Fig. 2a) or with *Eco*RI, which releases a 2.1-kb diagnostic fragment comprising the entire coding sequence of the oat *adc* cDNA (Fig. 2b, c). In the *Hind*III digest, each line showed a unique integration pattern, confirming that plants originated from independent transformation events (Fig. 2a). R1 progeny had rather simple integration patterns and these were identical with those of the corresponding primary transformant(s). The *Eco*RI digest demonstrated that six out of the eight lines contained an intact 2.1-kb fragment corresponding to the oat *adc* cDNA coding sequence (lines N82, N84, N92, N93, N96 and N97; Fig. 2b, c). In several lineages, additional fragments were seen, indicating the existence of multiple rearranged copies of the *35S:adca* and also lower molecular weight species representing integration of truncated copies of the transgene. Lineages N88 and N95 showed hybridization signals higher than the expected 2.1 kb (Fig. 2c), most likely resulting from the loss of one *Eco*RI site during the integration process.
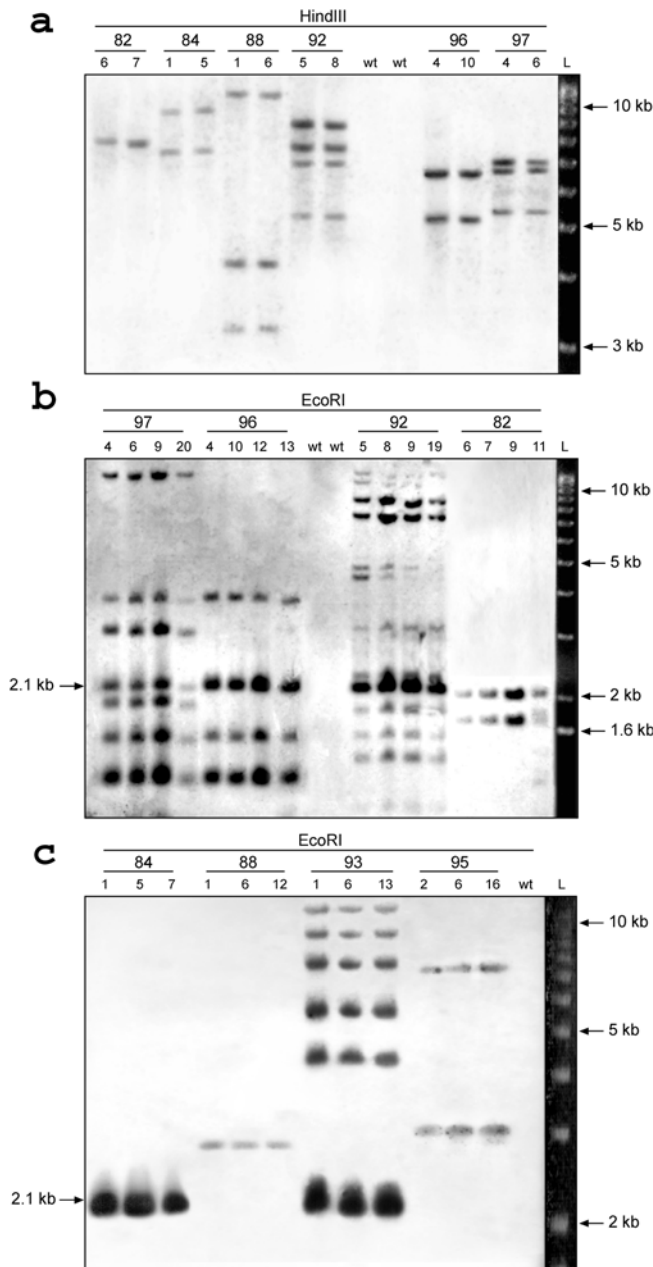
Fig. 2a–c DNA gel blot analysis of R1 progeny from wild-type (*wt*) rice and plants harbouring *35S:adca*. Fifteen micrograms of genomic DNA was digested with *Hind*III (**a**) or *Eco*RI (**b, c**) and blots were probed with the 1.5-kb DIG-labelled PCR product from *35S:adca* at high stringency. Exposure time 30 min. Expected size of cDNA fragment, ≈2.1 kb is shown on the left-hand side of the panel (*arrow* in **b** and **c**). *L* Molecular size marker (1-kb DNA ladder; Invitrogen). Numbers above gels represent siblings (*lower number*) from the same parental line (*upper number*)

The oat *adc* transcript is expressed in all primary transgenic plants and progeny

We extracted total RNA from leaves, tillers and roots from all 32 plants representing the 8 lineages and we analysed oat *adc* antisense expression by RT–PCR (Fig. 3a, b and c, respectively). Out of the eight lines analysed, six showed mRNA expression in all four

siblings in leaves (Fig. 3a), tillers (Fig. 3b) and roots (Fig. 3c; N84-1, 5, 7, 8; N88-1, 6, 12, 13; N92-5, 8, 9, 19; and N96-4, 10, 12, 13). When RNA samples were subjected to RT-PCR analysis in the absence of reverse transcriptase, no amplification products were obtained. Thirty micrograms of total RNA from leaves and roots was used for RNA gel blot analysis. Leaves and roots from lines N82-7, N88-1 and N96-4 accumulated the oat *adc* transcript, with roots showing substantially higher levels of expression compared to leaves (Fig. 4b, d). The remaining two lineages (N93-1 and N95-16) which appeared not to express the transcript were subsequently shown to contain low but detectable levels of the oat *adc* mRNA following blotting and probing the RT–PCR product from leaf tissue with the oat *adc* DIG-labelled probe (Fig. 4e). When RT was not included in the reaction mixture no amplification bands were obtained (data not shown). All RT–PCR experiments were repeated at least twice from independent RNA isolations and produced the same results.

This three-stage analysis confirmed constitutive expression of the antisense oat *adc* transcript in all lineages, albeit at varying levels. Two lineages (N82 and N97) expressed a second transcript with a lower molecular weight, most likely resulting from expression of an additional truncated copy of the integrated transgene (Fig. 3a–c).

Expression of the oat antisense *adc* transcript only affects expression of its rice ortholog

A rice *adc* 400-bp sequence (Table 1) was used as a probe to investigate expression of the rice *adc* in plants shown to express the introduced *35S:adca*. Levels of rice *adc* steady-state transcript were reduced in five of the eight lines analysed. Representative siblings from each line (N88-1, N92-9, N95-16, N96-4 and N97-20) are shown (Fig. 5b). Line N95 hardly exhibited any expression of the rice *adc* (Fig. 5b). Levels of the endogenous rice *adc* gene remained unaffected in three lines (representative siblings, e.g. N82-7, N84-8 and N93-1 are shown in Fig. 5b). The membranes were re-probed sequentially with a 700-bp DIG-labelled probe from the rice *odc* sequence, a 700-bp probe from the rice *samdc* sequence and a 900-bp probe from the rice *spd syn* sequence (Table 1). Rice *odc*, *samdc* and *spd syn* steady-state transcript accumulation remained unchanged compared to wild type (Fig. 5c, d and e, respectively).

Transgenic rice plants with reduced levels of the *adc* transcript also show reduction in ADC activity

Multiple tissue segments (3–4 cm in length) from the central section of leaves from R1 progeny were harvested from greenhouse-grown plants at the same developmental stage. ADC activity was measured in

Fig. 3a–c RT–PCR analysis of *35S:adca* transcript levels from R1 progeny of rice. Samples were prepared from total RNA extracted from leaf (**a**), tillers (**b**) and roots (**c**). Specific primers amplifying a 1.5-kb fragment from the oat *adc* cDNA were used as described in Materials and methods and Table 1. *wt* Wild type, *L* molecular size marker (1-kb DNA ladder; Invitrogen). In **b** and **c**, '–' indicates the negative control (water). Numbers above gels represent siblings (*lower number*) from the same parental line (*upper number*)
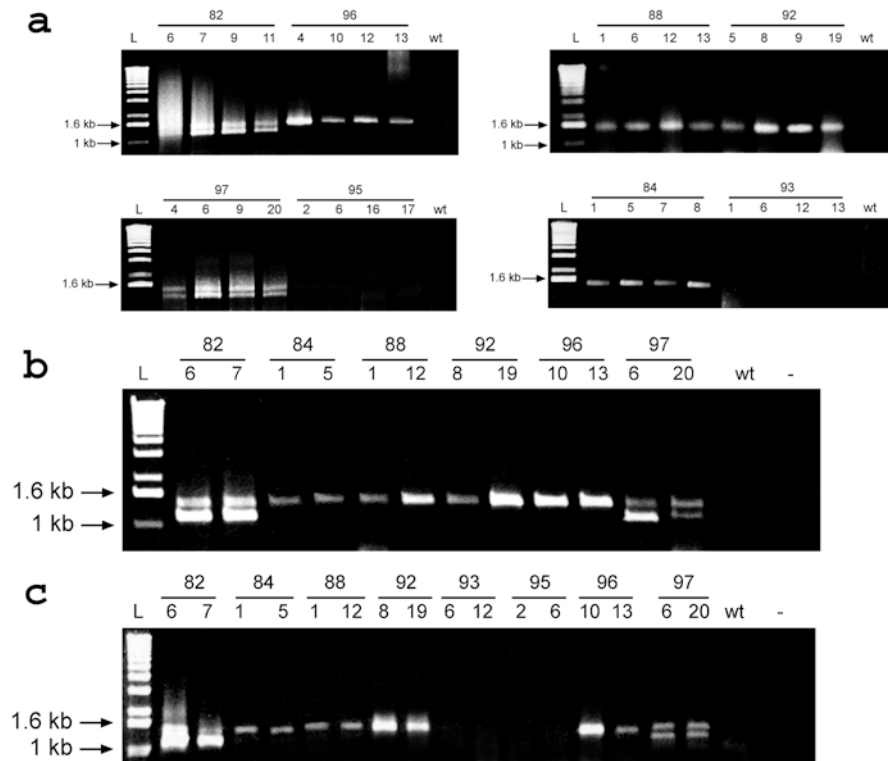
randomly selected siblings from all eight lineages. We also included samples from negative segregants, wild type and *hpt* controls. We measured significant reductions in ADC activity in leaves in three of the eight lineages (N92, N95 and N97). A 60% reduction in ADC activity was measured in leaves from these plants (1.11 ± 0.32 nkat mg^{-1} protein; $P < 0.01$) while the remaining plants exhibited no significant variation in ADC activity (2.82 ± 0.5 nkat mg^{-1} protein, $P > 0.05$) when compared to the control population (3.5 ± 0.72 nkat mg^{-1} protein; Fig. 6a).

A single tiller from each plant was separated and grown hydroponically in Yoshida's solution (Yoshida et al. 1972) to induce root growth after leaf samples were taken, as described earlier for molecular analyses. Roots were harvested 2 weeks later and ADC activity and polyamine content were determined. The three lines (N92, N95 and N97) that had shown significant reductions in activity in leaves (Fig. 6a) also showed significant reduction in ADC activity in roots (Fig. 6c). The minimum and maximum statistically significant reductions in activities were detected in plants N97-20 (2.5 ± 0.09 nkat mg^{-1} protein, $P < 0.05$) and N82-7 (1.46 ± 0.02 nkat mg^{-1} protein, $P < 0.01$) representing a 10 and 50% reduction, respectively compared to controls (2.77 ± 0.08 nkat mg^{-1} protein, Fig. 6c).

Putrescine and spermidine pools are reduced in transgenic plants in which the *adc* transcript and ADC activity are reduced

To determine whether down-regulation of ADC enzyme activity had a quantitative effect on the titers of putrescine, spermidine, and spermine, we measured free-polyamine concentrations in leaf tissue in progeny from the eight different lineages. Determination of free polyamines was carried out at the same time as ADC activity measurements for all tissues. Plants N92-9, N95-16 and N97-20 exhibited a maximum of 50% (N97-20, 217.50 ± 21 nmol g^{-1} fw, $P < 0.01$) and a minimum of 30% (N95-16, 284.22 ± 14 nmol g^{-1}fw, $P < 0.05$) reduction in putrescine concentration when compared to controls (426.74 ± 32 nmol g^{-1} fw; representative examples are shown in Fig. 6b). All the above plants also had a significant reduction in spermidine levels in leaves. Up to 60% reduction in spermidine content was measured in plant N92-9 (159.11 ± 28 nmol g^{-1} fw, $P < 0.001$) when compared to control levels (408.28 ± 42 nmol g^{-1} fw). No significant variation ($P > 0.05$) was observed in the levels of spermine in any of these lines in leaf tissues (representative examples are shown in Fig. 6b).

All eight lines had a significant reduction in putrescine levels in roots. A maximum 64% reduction in plant N95-16 (137.4 ± 13 nmol g^{-1} fw, $P < 0.01$) was measured compared to control levels (385.8 ± 36 nmol g^{-1} fw). Spermidine levels were reduced significantly in all lines that had exhibited significant reduction in putrescine levels. A maximum of 71% reduction in spermidine levels was measured in plant N95-16 (51.65 ± 9 nmol g^{-1} fw, $P < 0.001$) when compared to control levels (180.12 ± 8 nmol g^{-1} fw). Out of the eight lines that had a concomitant reduction in putrescine and spermidine levels in roots, only two lines had a significant reduction in spermine. Plant N95-16 had a 60% reduction (63.21 ± 19 nmol g^{-1} fw, $P < 0.01$) and
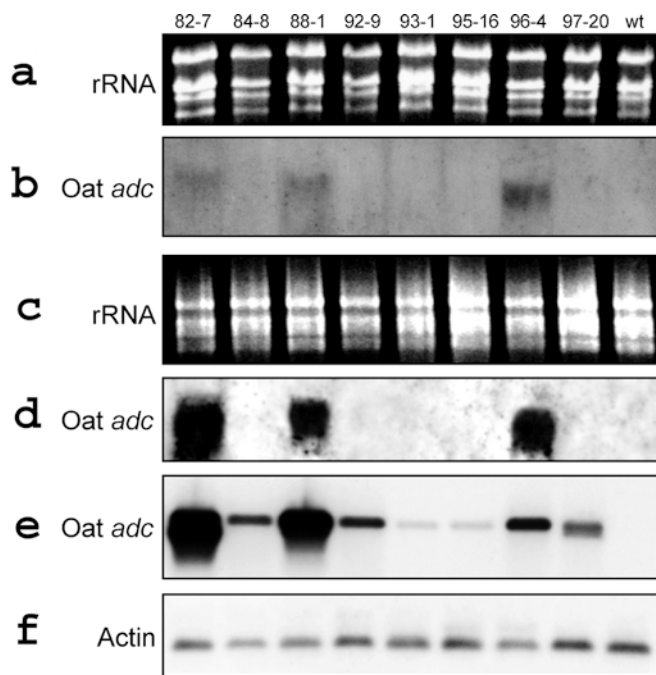
Fig. 4a–f RNA gel blots of total RNA extracted from wild-type (*wt*) rice and R1 progeny containing *35S:adca*. Numbers above gels represent one sibling from each line. b, c, d Blots were probed with the oat *adc* cDNA 1.5-kb DIG-labelled PCR product. a Ethidium bromide gel demonstrating equal loading of total RNA extracted from leaves. b Oat antisense *adc* expression in leaf tissue. Exposure time was 10 min. c Ethidium bromide gel demonstrating equal loading of total RNA extracted from roots. d Oat antisense *adc* expression in roots. Exposure time was 10 min. e RT–PCR analysis of 35S:*adc*a transcripts from leaf tissue. Set of primers, RT–PCR conditions, hybridisation and blotting, as described in Materials and methods. Exposure time was 15 min. f RT–PCR amplification of rice *actin-1* after 25 cycles (internal control). RT–PCR and re-probing of the blot were carried out as described in Materials and methods. Exposure time was 20 min

plant N97-20 a 50% reduction (89.98 ± 18 nmol g^{-1} fw, $P < 0.05$) when compared to control levels (162.15 ± 10 nmol g^{-1} fw, representative examples are shown in Fig. 6d).

Discussion

In plants, co-suppression and antisense inhibition have been widely used to elucidate gene function, to enhance the quality of essential oils through metabolic engineering, to alter flower color, etc. (Wang and Wagner 2003). One goal of expressing constitutively the oat *adc* cDNA in antisense orientation in rice was to determine whether this could influence the cellular free polyamine content through down-regulating the rice ADC enzyme. Prior to this investigation only one report on the modulation of enzymes involved in the polyamine pathway by down-regulating a potato *samdc* gene using a homologous transgene in antisense orientation was described. Engineered potato plants exhibited an abnormal phenotype that was correlated with altered levels of the
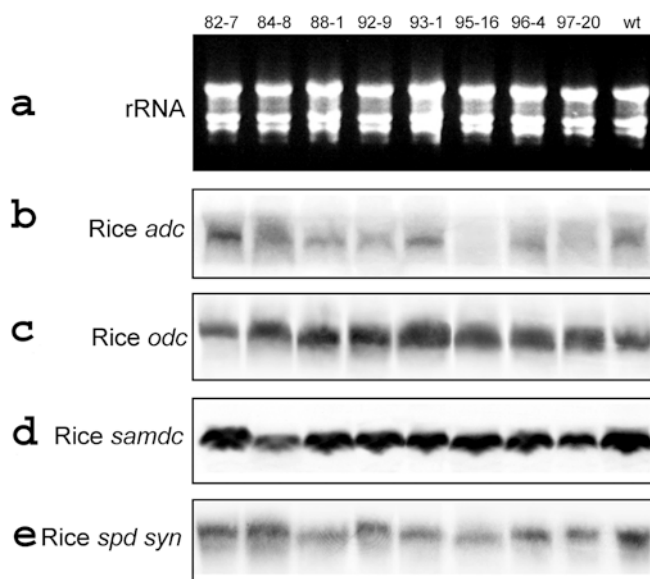

Fig. 5a–e RNA gel blots of total RNA extracted from leaf tissue of wild-type (*wt*) rice and R1 progeny containing *35S:adca*. Numbers above gels represent one sibling from each line. a Ethidium bromide gel demonstrating equal loading of RNA extracted from leaves. b Membrane probed with the rice *adc* 200 bp DIG-labelled PCR product. Exposure time 30 min. c Membrane re-probed with the rice *odc* 289 bp DIG-labelled PCR product. Exposure time 50 min. d Membrane re-probed with the rice *samdc* 0.7-kb DIG-labelled PCR product. Exposure time 30 min. e Membrane re-probed with the rice *spd syn* 0.9-kb DIG-labelled PCR product. Exposure time 1 h. Re-probing of the membrane, cycling conditions for DIG-labelling of the probes, primers used and sequences are described in Materials and methods

samdc transcript, SAMDC activity and polyamine content (Kumar et al. 1996). In the current investigation, we down-regulated the rice *adc* gene by using its full-length oat ortholog in antisense orientation. The oat and the rice *adc* cDNAs share a high sequence homology (Fig. 1). The alignment of the oat *adc* cDNA sequence (X56820) to the rice putative *adc* cDNA (GI6006369 from GI6006355) indicates a 71% identity, reflecting a close evolutionary relationship between them (http://www2.ebi.ac.uk/clustalw). This provides adequate levels of homology between the two species for the antisense effect to occur and results from genomic DNA gel blot analysis between the two orthologous genes confirm this (Fig. 1).

Molecular analysis of the transformed plants (Fig. 2) showed that the oat transgene was stably integrated into the rice genome and was transmitted to progeny as a Mendelian trait, consistent with its integration in a single genetic locus (Kohli et al. 1998; Fu et al. 2000). RT–PCR analysis showed mRNA expression in six of the eight lines we analyzed (Fig. 3). Five of these contained an intact copy of the transgene, whereas the remaining expressing line, N88 contained a rearranged copy (Fig. 2b, c). Interestingly, lines N82 and N97 that contained the intact coding region of the transgene expressed two mRNA species, one of which was shorter than the expected size. The aberrant RNAs in these lines
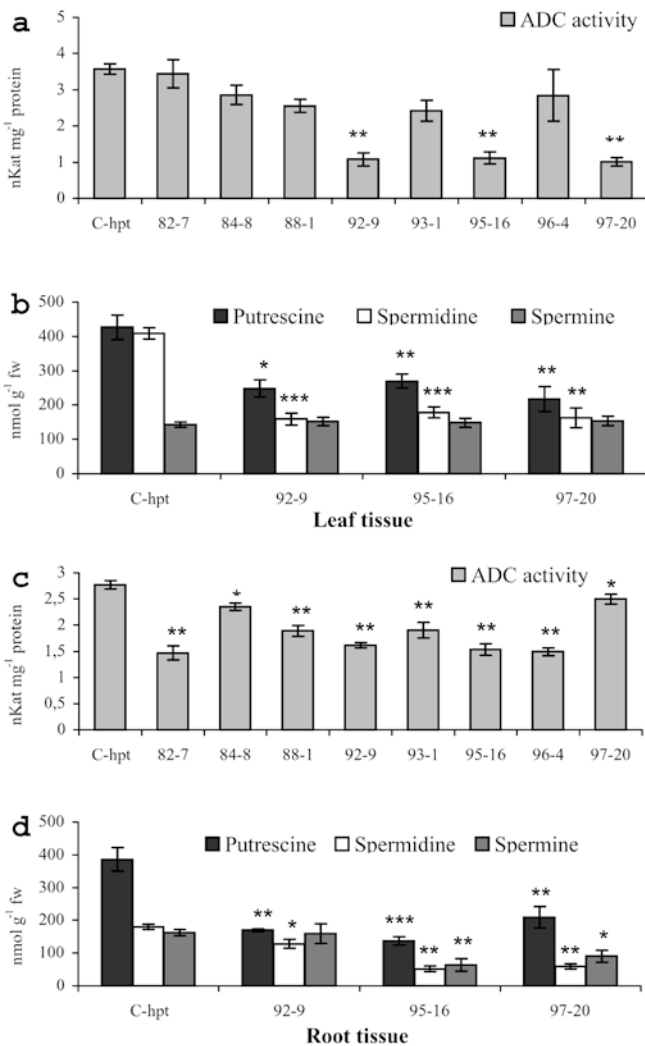
Fig. 6a–d Biochemical characterisation of R1 progeny of rice expressing *35S:adca*. Values are mean ± SE for control lines (*n* = 36) and mean ± SE in transgenic lines (*n* = 3). Significance of data is as follows: ***: $P < 0.001$; **: $0.01 > P > 0.001$; *: $0.05 > P > 0.01$. *C-htp* Control harboring the *htp* selectable marker gene. **a** ADC enzyme activity in leaves from different transgenic lines and wild type. **b** Cellular polyamine content in leaf tissue. **c** ADC enzyme activity in roots. **d** Cellular polyamine content in roots.

presumably resulted from a truncated copy (inside the open reading frame) of the transgene. This RNA profile was detected in leaves, roots and tillers in both lines (Fig. 3).

After comparing the normalized hybridization signals from the RNA gel blots for leaves and roots we observed that the intensity of the transcript signal from the transgene in root tissue was higher compared to leaves (Fig. 4b, d). Transgene expression resulted in a reduction of the steady-state rice *adc* mRNA in leaves (Fig. 5b). In one of these lines (N95), steady-state rice *adc* mRNA was not detectable. Some of the down-regulated antisense plants had a significant reduction in ADC activity in leaves. A maximum of 60% reduction in

activity was detected in plant N95-16 (Fig. 6a). Interestingly a significant reduction in ADC activity in roots was detected in all eight lines (Fig. 6c). Plants with reduced levels of ADC activity were indistinguishable from wild type at all stages of development. Even plant N95-16, which had a 60% reduction in ADC activity in leaves and a 50% reduction in roots, was indistinguishable from the rest of the plants, exhibiting normal phenotype and fertility (Fig. 6a, c). Watson et al. (1998) isolated mutants of *Arabidopsis thaliana* that were deficient in ADC activity. From a population of 15,000 EMS M2 plants they identified 9 independently isolated alleles with low ADC activity. The most striking phenotypes of the individual mutants were in root development, where decreased ADC activity was correlated with increased lateral root branching and growth. None of the individual mutant alleles (*spe1-1* and *spe2-1*) abolished ADC activity completely, and even the strongest double mutant (*spe1-1 spe2-1*) decreased polyamine levels by only 10–20%. Chattopadhyay et al. (1997) investigated the role of polyamines in abiotic-stress tolerance in plants. Firstly, they measured ADC activity in shoots and roots from salt-sensitive and salt-tolerant rice. When salinity stress was applied, a significant increase in ADC activity was observed in shoots and roots, but roots showed a much higher induction, with most dramatic increases observed in the salt-sensitive cultivar. Our data and also the studies of Watson et al. (1998) and Chattopadhyay et al. (1997), indicate that the regulation of ADC in root tissue is not as tight as it is in leaves. Feirer et al. (1984), by using α-difluoromethylarginine (DFMA, a specific inhibitor of ADC activity), reported an 88% reduction in ADC activity in wild-type carrot cells. This resulted in a significant reduction in putrescine and spermidine content. Embryogenic capacity of these cells was compromised by 50%. Thus, it is apparent that none of the three strategies, mutants, inhibitors or molecular approaches involving transgenic plants, designed to shut down ADC enzyme activity completely resulted in null plants for the ADC phenotype. It is likely that a null mutation in ADC activity may be lethal. This suggests that ADC may play a role in plant development.

Reduction in ADC activity (Fig. 6a) resulted in lower levels of putrescine and spermidine in leaves (Fig. 6b). Levels of putrescine and spermidine in roots were reduced in all eight lines (Fig. 6d). Two of these lines had also a significant reduction in spermine levels in roots (Fig. 6d). When polyamine levels were measured in mutants that had the lowest enzyme activity (*spe1-1* and *spe2-1*) or in double mutants (*spe1-1 spe2-1*) no significant variation was found in the aerial parts of the plant (Watson et al. 1998). Putrescine levels were only reduced in roots of the double mutants (*spe1-1 spe2-1*). Roots in these mutants had an altered morphology. Thus results from *Arabidopsis* and rice indicate that polyamine pools are altered more dramatically in roots than in leaves. It is interesting, however, that *Arabidopsis* and rice behave very differently in terms of phenotype when polyamine

levels are decreased. This may reflect evolutionary differences between the two species; however, it is not clear why *Arabidopsis* with two different *adc* genes (Watson et al. 1997) will behave in this manner compared to rice which only has one copy of the gene (Chattopadhyay et al. 1997).

A second goal of this investigation was to evaluate whether the rice *odc*, *samdc* or *spd syn* steady-state transcripts were influenced by the size of the free polyamine pool in the plants we generated. Our results indicate that steady-state mRNAs for the endogenous genes remain unaffected in the *adc* antisense transformants. This suggests that feedback regulation of the expression of the genes by polyamines is not a major regulatory mechanism. Thu-Hang et al. (2002) studied expression of the rice *samdc* and *spd syn* genes in rice plants transformed with a *Datura samdc* cDNA. Plants with a 2.5-fold increase in foliar spermidine as a result of expression of the introduced *samdc* transgene showed no variation in the rice *samdc* and the *spd syn* (Thu-Hang et al. 2002). White et al. (1990) reported an 18.8-fold increase in SAMDC activity in Swiss 3T3 cells that had depleted cellular polyamines by using the ODC inhibitor difluoromethylornithine (DFMO). The magnitude of the increase in SAMDC activity in these cells could not be accounted for by either the elevation of mRNA level or an increase in enzyme stability, suggesting increased efficiency of translation of the *samdc* message. These results indicate that the tight regulation at the level of mRNA expression of the genes involved in the polyamine pathway is not overcome by alteration of the size of the free polyamine pool.

Conclusions

By studying transgenic rice plants expressing the *35S:adca*, we have demonstrated that significant reduction in enzyme activity results in reduction in putrescine and spermidine content in leaf tissue, and putrescine, spermidine and spermine in roots. Expression of the transgene affected expression of its rice ortholog; however, expression of other endogenous genes involved in the pathway was not affected even though the size of the pool of the free polyamines was significantly reduced. By investigating all components in the polyamine pathway biosynthetic machinery, i.e. transgene integration, transcription and translation, and also how these affect end-product profiles in a range of lineages, we show that the pathway is tightly regulated. It appears that alteration of the size of the endogenous free polyamine pool does not act as a signal to induce changes in the transcription of other genes in the pathway. Such studies can now be extended to more complex pathways to unravel additional elements that control accumulation of end products in plants.

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