Amino Acids (2012) 42:519–527
DOI 10.1007/s00726-011-1029-5

ORIGINAL ARTICLE

Plant ornithine decarboxylase is not post-transcriptionally feedback regulated by polyamines but can interact with a cytosolic ribosomal protein S15 polypeptide

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Received: 8 April 2011 / Accepted: 30 May 2011 / Published online: 4 August 2011

Abstract The formation of putrescine by ornithine decarboxylase (ODC) is a key regulatory step in polyamine biosynthesis in metazoa and fungi. Excess polyamines post-transcriptionally induce the synthesis of a unique non-competitive protein inhibitor of ODC, termed antizyme. Binding of antizyme to an ODC monomer subunit results in enzymatic inhibition, rapid ubiquitin-independent degradation of ODC by the 26S proteasome and recycling of antizyme. Plants possess an additional route for synthesizing putrescine via arginine decarboxylase (ADC). No homologue of ODC antizyme has been detected in plant genomes but several biochemical studies have reported plant ODC antizyme proteins of 9 and 16 kDa. Here we show that plant cells grown in liquid culture do not exhibit any substantial post-transcriptional, polyamine-responsive feedback regulation of ODC or ADC. However, using the yeast two hybrid system, a plant ODC-binding polypeptide was detected: the C-terminal 84-87 amino acids of cytosolic ribosomal protein (rp) S15. The *Arabidopsis* rpS15 polypeptide interacted specifically with plant ODC but not with human or *Saccharomyces cerevisiae* ODCs. Co-expression of either the full length or C-terminal rpS15 polypeptides with a plant ODC in yeast did not reduce ODC enzymatic activity. Only the full length mRNA encoding rpS15 was detected in *Arabidopsis* cells, suggesting that the C-terminal rpS15 polypeptide is encoded by a low abundance mRNA or the polypeptide is not physiologically relevant in plants. These results confirm the primacy of S-adenosylmethionine decarboxylase as the key regulatory enzyme in plant polyamine biosynthesis.

Keywords Polyamine · Antizyme · Ornithine decarboxylase · Post-transcriptional · *Arabidopsis* · Pea

Abbreviations

ODC	Ornithine decarboxylase
BY-2	Bright Yellow-2 tobacco cells
rpS15	Cytosolic ribosomal protein S15

Introduction

Unlike other eukaryotes, plants are able to synthesize polyamines from arginine as well as from ornithine. This ability is due to the acquisition of an arginine decarboxylase gene from the cyanobacterial progenitor of the chloroplast (Fuell et al. 2010). Ornithine decarboxylase (ODC) retains an important polyamine biosynthetic role in plants (Nolke et al. 2005; Deboer et al. 2011), however, some plant species, members of the Brassicaceae, and prominently Arabidopsis *thaliana* (*Arabidopsis*), have lost the ODC gene (Hanfrey et al. 2001). The ODC enzyme found in eukaryotes is a head to tail homodimer (Jackson et al. 2003) (Almrud et al. 2000) with two active sites formed across the dimer interface (Tobias and Kahana 1993). When polyamine levels become excessive, ODC activity is repressed by the binding of antizyme protein, made by polyamine-induced frameshifting of the antizyme

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mRNA, to the ODC monomer, which then targets the monomer for ubiquitin-independent degradation by the 26S proteasome (Heller et al. 1976; Matsufuji et al. 1990, 1995; Murakami et al. 1992; Rom and Kahana 1994; Li and Coffino 1992, 1993, 1994; Zhang et al. 2003).

The *Escherichia coli* ODC enzyme has evolved from an entirely different protein fold compared to the eukaryotic ODC. Whereas eukaryotic ODC is a head to tail homodimer from the alanine racemase fold, the *E. coli* ODC is related to the *Lactobacillus* sp. 30a ODC, which is a dodecamer of identical subunits, formed of two, doughnut-like hexamer rings, the monomers resembling the aspartate aminotransferase fold (Momany et al. 1995). Two basic proteins proposed to be putative antizyme proteins, i.e. non-competitive protein inhibitors regulated by polyamines, were detected that bound and inhibited the *E. coli* ODC (Heller et al. 1976). Surprisingly, the antizymes also inhibited the *E. coli* arginine decarboxylase (ADC), which is from a different protein fold (alanine racemase) and which is likely to be a tetramer of two head to tail dimers (Deng et al. 2010). These two *E. coli* antizyme proteins were subsequently shown to be ribosomal proteins S20/L26 and L34 (Panagiotidis and Kyriakidis 1985). The physiological role of the ribosomal proteins as ODC antizymes was called into question by the finding that a variety of *E. coli* ribosomal proteins can inhibit the *E. coli* ODC in vitro but that intact ribosomes did not inhibit ODC (Kashiwagi and Igarashi 1987; Ivanov et al. 1998). However, the in vivo role of ribosomal proteins that were not incorporated into ribosomes was not addressed in this study. The role of a non-ribosomal protein antizyme in *E. coli* has also been called into question (Ivanov et al. 1998). Recently, a more compelling example of a prokaryotic ODC antizyme has been shown in the soil bacterium *Selenomonas ruminantium*. A bifunctional ODC/lysine decarboxylase (alanine racemase fold) resembling the human ODC is present in *S. ruminantium* and was identified as an orthologue of ribosomal protein L10 (Yamaguchi et al. 2006). The *S. ruminantium* ODC antizyme is induced by putrescine but not cadaverine and binds to ODC, thereby accelerating its degradation. Two small peptide segments of the ribosomal protein L10 orthologue were found to be required for binding to the *S. ruminantium* ODC/LDC enzyme and the mouse ODC (Yamaguchi et al. 2008).

There have been reports of ODC antizyme proteins in plants: a cytosolic antizyme of approximately 16 kDa and a chromatin-bound antizyme of 9 kDa found in barley (Panagiotidis and Kyriakidis 1985). These ODC-binding proteins did not behave like normal antizyme proteins: inhibition of ODC activity by the chromatin-bound antizyme was relieved by polyamines in vitro (Koromilas and Kyriakidis 1987), suggesting a different regulatory paradigm to the fungal and metazoan antizyme. In tobacco cell suspension cultures, spermidine and spermine did not repress ODC activity but it was not known whether there was any post-transcriptional buffering of activity (Hiatt et al. 1986).

The identity of the plant ODC binding proteins was of interest to us because of the different evolution of polyamine biosynthesis in plants compared to other eukaryotes (Fuell et al. 2010). In particular, the presence of the alternative ADC pathway for putrescine biosynthesis means that the plant ODC binding proteins are likely to be evolutionarily and functionally distinct from the antizyme found in fungi and metazoa. We had previously shown, using the yeast two hybrid system, that the interaction between the human ODC and human antizyme can be detected readily but no interaction between human antizyme and ODC from either yeast or the solanaceous plant *Datura stramonium* was detectable (Illingworth and Michael 1998). Furthermore, the interaction between the two ODC monomers forming the active ODC dimer was not detected in the yeast two hybrid system (Illingworth and Michael 1998), possibly due to the weak interaction and rapid equilibrium between active dimers and inactive monomers (Pegg 2006).

Materials and methods

Tobacco BY-2 cell cultures and treatment with polyamines and methyljasmonate

A suspension culture of tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) was grown with gentle shaking at 27°C in the dark and subcultured every 7 days. The cells were grown in BY-2 medium with 2,4-dichlorophenoxyacetic acid (0.2 mg l^{-1}) as previously described (Imanishi et al. 1998). Methyljasmonate was dissolved in 1% dimethylsulphoxide and added to cultures at a concentration of 100 μM. Control cells were treated with the same amount of 1% dimethylsulphoxide without dissolved methyljasmonate. Aqueous solutions of polyamine were added to the growth medium at a concentration of 500 μM. Seven day-old cell cultures were centrifuged, the cells were washed and resuspended in fresh growth medium. For experiments with polyamines, these were present from the start of the subculture. For experiments with methyljasmonate, subcultured cells were grown for 24 h before additional of methyljasmonate for 4 h.

Tobacco cell culture total RNA and polyamine biosynthetic enzyme analysis

RNA and enzymatic analyses were performed as previously described (Mayer and Michael 2003).

Yeast two hybrid libraries

An *Arabidopsis* cv. Columbia two hybrid library in the GAL4 activation domain prey plasmid pGAD10 was purchased from Clontech Laboratories Inc. (Palo Alto, CA, USA). The library was constructed from 3 week old green vegetative tissues and was reported to comprise 3.0×10^6 independent clones with inserts ranging from 0.6 to 4.0 kbp. A pea (*Pisum sativum* line JI813) cDNA library prepared from 4 week old 3 mm shoot apices was prepared by Trine Juul and Julie Hofer (John Innes Centre, Norwich, UK) using the HybriZAP 2.1 two hybrid system (Stratagene, UK). The library consisted of inserts of 400 bp or longer and was comprised of 1.5×10^5 independent clones. Inserts were transferred into the pAD-GAL-2.1 plasmid for yeast two hybrid analysis. Library DNA was transformed into yeast reporter strains using the lithium acetate method (Ito et al. 1983). Single purified plasmids were transformed into yeast strains using the 'PLATE' method (Elbe 1992).

Yeast strains

Strain Y190 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal80Δ, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*). Strain CG-1945 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3,112, gal4-542, gal80-538, cyh^{r}2, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA::GAL4$_{17\text{-}mers(x3)}$-CYC1$_{TATA}$-lacZ*). Strain J69-4A (*MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ.* YW5-1B (*MATa, trp1, ura3-52, leu2-3,112*).

Arabidopsis cell suspension culture

An *Arabidopsis* Landsberg erecta cell suspension culture was kindly provided by J. Murray, Cambridge, UK. The cell cultures were maintained in 0.44% (w/v) Murashige and Skoog medium (Duchefa, Harlem, NL), 3% (w/v) sucrose, 0.5 mg/l 1-naphthaleneacetic acid and 50 ng/l kinetin, pH 5.8. Cultures of 50 ml were grown in 250 ml erlenmeyer flasks in the dark at 25°C with shaking at 120 rpm, and subcultured every 7 days.

Assay of ODC activity from yeast cells

Yeast cells (10 ml) were grown overnight in appropriate selective medium at 30°C. One hundred ml of the same medium was then inoculated to an $OD_{600\ nm}$ of 0.3 and grown for 3 h under the same conditions. Cells from 50 ml of the culture were then centrifuged, washed with sterile water and then resuspended in 1 ml of 25 mM Tris–HCl pH 8.0, 0.1 mM EDTA pH 8.0, 1.0 mM $MgCl_2$, 0.02% Brij 35 and 2.0 mM DTT. Approximately 0.5 g of acid washed glass beads (425–600 μm) were then used to disrupt the cells by vortexing four times for 1 min and placing on ice between each vortex cycle. Ornithine decarboxylation was measured using a standard radiolabel L-[1-^{14}C]ornithine hydrochloride CO_2 release assay with the ^{14}C CO_2 being trapped with 1.0 M KOH (Mayer and Michael 2003).

Quantification of β-galactosidase activity

Single colonies of the yeast two hybrid reporter strains were grown in 10 ml of suitable selective medium for approximately 16 h at 30°C. Pelleted cells were resuspended in 1.0 ml of cold Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2-PO_4.7H2O, 10 mM KCl and 1 mM $MgSO_4 \cdot 7H_2O$). After centrifugation, the cells were resuspended in 0.3 ml of Z buffer and disrupted by vortexing with 0.5 g of glass beads. The supernatant was removed to a fresh tube and protein content was measured using Bradford reagent (Biorad). Activity of β-galactosidase was determined using a Galactolite-plus kit (Tropix Inc., Bedford, MA) following manufacturer's instructions. Luminescence was detected in a Lumat LB 9501 luminometer (Berthold, Germany). The reading was adjusted for protein content and results presented as relative light units (RLU)/mg protein.

Results

Ornithine decarboxylase activity is not post-transcriptionally repressed by polyamines in tobacco BY-2 cells

The presence of 500 μM exogenous putrescine in liquid-grown tobacco cell suspension cultures caused a decrease in ODC steady-state mRNA level but ODC enzymatic activity increased approximately 30% after 28 h exposure (Fig. 1). Exogenous agmatine at the same concentration also had little effect on ODC mRNA level or activity, and a similar pattern was also observed with spermidine and spermine. Small decreases in ADC enzymatic activity were reflected by small decreases in ADC mRNA level upon exposure to exogenous putrescine, agmatine, or spermine, but there was a detectable post-transcriptional inhibition of ADC activity with spermidine.

In contrast, the highly specific enzyme-activated ODC suicide inhibitor α-difluromethylornithine (DFMO) reduced ODC activity by 45-fold (Fig. 1), with no effect on ADC activity. In agreement with other findings (Hanfrey et al. 2005), spermine reduced AdoMetDC activity to barely detectable levels. Whereas methyljasmonate increased ODC activity by 6.3- to 7-fold and increased ODC mRNA

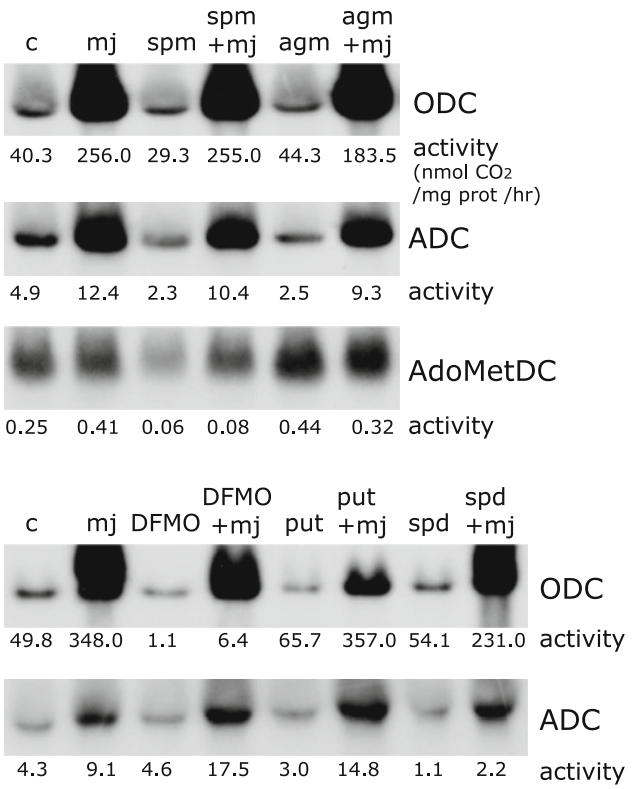

Fig. 1 Effects of polyamines, methyljasmonate and α-difluoromethylornithine on polyamine biosynthetic enzyme steady-state mRNA levels and enzymatic activities in tobacco BY-2 cell suspension cultures. Polyamines were applied at 500 μM, methyljasmonate at 100 μM and α-difluoromethylornithine at 5 mM. All enzyme activities represent nmol CO_2/mg protein/h. Polyamines were present from time 0 h. Methyljasmonate was added after 24 h of growth for 4 h. *ODC* ornithine decarboxylase, *ADC* arginine decarboxylase, *AdoMetDC* *S*-adenosylmethionine decarboxylase, *c* control cells, *mj* methyljasmonate, *spm* spermine, *agm* agmatine, *DFMO* α-difluoromethylornithine, *put* putrescine, *spd* spermidine

accumulation to an even greater extent, the presence of either putrescine, agmatine, spermidine or spermine simultaneously with methyljasmonate had little effect on the increased ODC mRNA level and ODC activities caused by the presence of methyljasmonate. In clear contrast, the presence of spermine, but not agmatine, prevented the methyljasmonate-induced increase of AdoMetDC activity. The methyljasmonate-induced increased activity of ODC was prevented by the simultaneous application of DFMO. Biosynthesis of putrescine in tobacco cells does not appear to be subject to a post-transcriptional negative feedback system typical of fungi and mammals.

Identification of *Arabidopsis* and pea S15 ribosomal proteins as ODC binding proteins

The solanaceous plant *Datura stramonium* is related to tomato, potato, chilli pepper, aubergine and tobacco and the *D. stramonium* ODC protein (acc. no. P50134) is composed of a sequence of 341 amino acids exhibiting 59%, 43% and 43% identities respectively with the ODC proteins of rice (NP_00106386), *Saccharomyces cerevisiae* (NP_012737) and *Homo sapiens* (NP_002530). A cDNA encoding the *D. stramonium* ODC was used as the bait for a yeast two hybrid screen. The *D. stramonium* cDNA was cloned as a fusion with the GAL4 DNA binding domain in the yeast two hybrid plasmid pGBT9 and then introduced into the *S. cerevisiae* two hybrid reporter strain J69-4A (James et al. 1996). In the J69-4A strain, two levels of selection, growth on medium without histidine or adenine, are available to detect interactions between the bait and prey proteins. We decided to search for ODC interacting clones in cDNA libraries from actively dividing tissue (pea shoot tips) and from *Arabidopsis* 3 week old green vegetative tissue (*Arabidopsis* does not possess an ODC gene). The *Arabidopsis* cDNA library was cloned into the prey plasmid pGAD10 (leucine selection), such that some of the insertions would be in-frame fusions with the GAL4 transcription activation domain of the pGAD10 plasmid. The *D. stramonium* ODC cDNA in pGBT9 was transformed into the yeast reporter strain J69-4A and grown on SCD minimal medium minus tryptophan. Transformants were regrown on fresh minimal medium lacking tryptophan to ensure all internal supplies of tryptophan were exhausted. The transformed strain was then retransformed with the *Arabidopsis* cDNA library in pGAD10. Transformed cells were plated onto SCD plates containing 100 μM spermidine and spermine but lacking tryptophan and leucine to select for the bait and prey plasmids, respectively and lacking histidine to select for interactions between the bait and prey plasmid-encoded proteins. A total of 425,000 colonies were screened and 230 colonies grew on SCD plates with spermidine and spermine and lacking tryptophan, leucine and histidine. A second selection was used (growth without adenine) to more stringently select for the bait-prey protein interactions. The 230 colonies were then replica-plated on plates lacking adenine and 18 colonies grew. Cell extracts from each of the 18 colonies were then assayed for β-galactosidase activity, which is the reporter activity for bait-prey interactions, and total soluble protein content was determined. Eight of the colonies exhibited higher β-galactosidase activity than the positive control, i.e., human ODC in pGBT9 and permanently frameshifted human antizyme in pGAD424. After sequencing the cDNA inserts of 14 of the 18 clones, all insertions were identified as identical 575 bp fragments encoding 84 amino acids of the C-terminal half of the *Arabidopsis* S15 cytosolic ribosomal protein in-frame with the GAL4 DNA binding domain of the pGAD10 plasmid.

The screen of the *Arabidopsis* library was repeated and this time 3×10^6 colonies were screened. After the final selection on SCD medium lacking adenine, 15 colonies

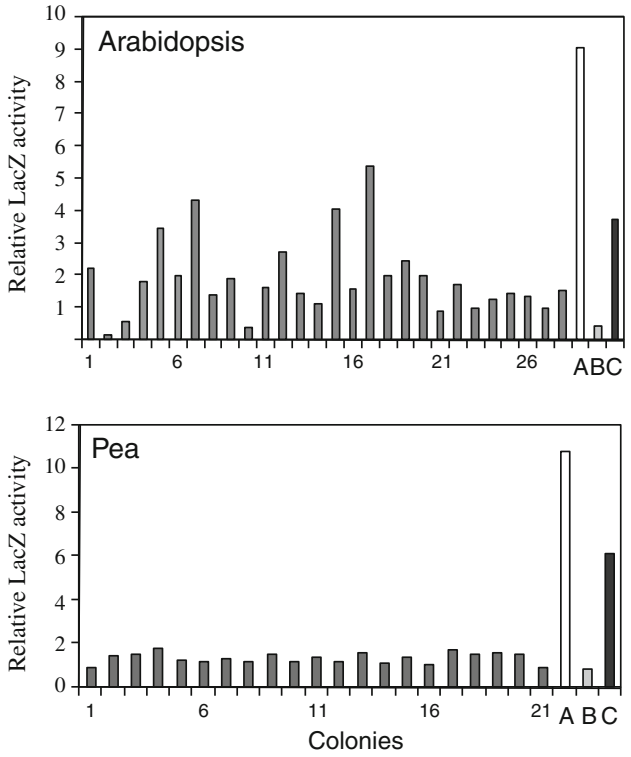

Fig. 2 Relative β-galactosidase activities in the two hybrid system, from independent J69-4A yeast colonies, conferred by *Arabidopsis* and pea cDNAs interacting with the *Datura stramonium* ODC bait plasmid. The *D. stramonium* ODC ORF in the bait plasmid pGBT9 interacting with *Arabidopsis* cDNA-encoded proteins expressed from the prey plasmid pGAD10 or with pea cDNA-encoded proteins expressed from the prey plasmid pAD-GAL4-2.1. Colonies were grown on SCD growth medium containing 100 μM spermidine and spermine but lacking histidine and adenine to select for interacting proteins. *A Arabidopsis* C-terminal ribosomal protein S15 cDNA (isolated from first screen) in pGBT9 and *D. stramonium* ODC in pGAD424; *B Arabidopsis* C-terminal ribosomal protein S15 antisense cDNA in pGBT9 and *D. stramonium* ODC in pGAD424; *C D. stramonium* ODC in pGBT9 and *Arabidopsis* C-terminal ribosomal protein S15 cDNA in pGAD10. Values represent the average of duplicate assays

grew. Each colony was assayed for β-galactosidase activity and the activity was similar to the activity of a positive control containing the *Arabidopsis* S15 ribosomal protein fragment in pGAD10 and the *D. stramonium* ODC in pGBT9 (Fig. 2). An antisense S15 ribosomal protein fragment in pGAD10 abolished β-galactosidase activity. Inserts from 5 of the 15 positive colonies were sequenced and all were identical to the 575 bp S15 ribosomal protein cDNA found in the previous screen. The relative β-galactosidase activities of all 19 colonies from both screens, identified by sequencing as containing the S15 ribosomal protein fragment, is compared with the positive and negative (antisense S15) controls in Fig. 2.

A *Pisum sativum* (pea) cDNA library of mRNA from highly meristematic 3 mm shoot tips was cloned into pAD-GAD4-2.1 and introduced into J69-4A containing the *D. stramonium* ODC in pGBT9. The original library contained 1.5×10^5 independent clones and 3.7×10^6 colonies were screened in the yeast two hybrid assay, i.e. a 25-fold coverage of the original library. A total of 197 colonies grew from the original library transformation plated onto plates lacking leucine, tryptophan and histidine. After replica plating the 197 colonies onto similar medium lacking adenine, 19 colonies grew. The β-galactosidase activities of these clones were not quite as high as those detected from the *Arabidopsis* library (Fig. 2), although it should be noted that a different expression plasmid (pAD-GAD4-2.1) was used for the library. Plasmids from 8 of the 19 colonies were sequenced and all contained an identical 485 bp fragment encoding 87 amino acids of the C-terminal half of cytosolic ribosomal protein S15 in-frame with the DNA binding domain. Comparison of the *Arabidopsis* and pea peptides encoded by the prey cDNAs indicates that they exhibit 98.8% similarity (Fig. 3). The *Arabidopsis* cDNA encodes amino acids 69–152 of the 152 amino acid S15 ribosomal protein. An almost identical polypeptide with three more amino acids at the N-terminal end of the ribosomal protein fragment is encoded by the pea cDNAs.

The *Arabidopsis* S15 ribosomal protein polypeptide does not interact with the human and *S. cerevisiae* ODC proteins

The *Arabidopsis* S15 ribosomal protein cDNA isolated from the yeast two hybrid screen was recloned from the prey vector into the bait plasmid pGBT9. Similarly, the *D. stramonium* ODC ORF was recloned into the prey vector pGBT9. An antisense version of the S15 DNA was also cloned into pGBT9. Different yeast two hybrid reporter strains exhibit different β-galactosidase activities when the same interacting clones are present. Therefore, we introduced the new plasmids into the yeast reporter strains J69-4A, CG-1945 and Y190. The β-galactosidase activities observed with the bait and prey plasmids are shown in Fig. 4. No β-galactosidase activity was detected when only the antisense ribosomal protein S15 fragment was expressed in any of the reporter strains. The CG1945 and Y190 strains co-expressing the sense S15 cDNA and the *D. stramonium* ODC manifested much higher relative β-galactosidase activities than the J69-4A strain. Interaction between the S15 ribosomal protein C-terminal polypeptide and the *D. stramonium* ODC was not dependent on the bait or prey plasmid. We then tested the interaction of the *Arabidopsis* ribosomal protein S15 C-terminal peptide expressed in pGAD10 with the ODCs of *D. stramonium*, human and *S. cerevisiae* expressed in pGBT9 in each of the J69-4A, CG1945 and Y190 yeast two hybrid reporter strains. The ribosomal S15 C-terminal peptide interacted only with the plant ODC (Fig. 5).

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Arab : MADVEPEVAAAGVPKKRTFKKFAFKGVDLDALLDMSTDDLVKLFSSRIRRR :  51
Pea  : -------------------------------------------------- :   -

Arab : FSRGLTRKPMALIKKLRKAKREAPQGEKPEPVRTHLRNMIIVPEMIGSIIG : 102
Pea  : --------------KLRKAKREAPPGEKPEPVRTHLRNMIIVPEMIGSIIG :  37

Arab : VYNGKTFNQVEIKPEMIGHYLAEFSISYKPVKHGRPGVGATHSSRFIPLK : 152
Pea  : VYNGKTFNQVEIKPEMIGHYLAEFSISYKPVKHGRPGIGATHSSRFIPLK :  87
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Fig. 3 Comparison of the C-terminal regions of the *Arabidopsis* and pea S15 ribosomal proteins. The polypeptides encoded by the two hybrid library cDNAs are highlighted in black. The absent N-terminal half of the S15 ribosomal protein is shown for the *Arabidopsis* sequence. *Arab Arabidopsis* S15 ribosomal protein C-terminal fragment obtained from the two hybrid screen using the *D. stramonium* ODC as bait

Arabidopsis S15 ribosomal protein does not inhibit the enzymatic activity of the plant ODC when co-expressed in yeast

The cDNA encoding the C-terminal polypeptide of *Arabidopsis* ribosomal protein S15 isolated from the yeast two hybrid screen was cloned into the galactose-inducible yeast expression plasmid pYX243. A cDNA encoding the entire S15 ribosomal protein was also cloned into pYX243. The *D. stramonium* ODC cDNA was cloned into the galactose-inducible yeast expression plasmid pYES2. Co-transformation of the yeast wildtype strain YM51-B with various combinations of plasmids yielded strains containing both pYES2 and pYX243 plasmids, as empty vectors, or with inserts of ODC, and the partial or full length S15 ribosomal protein. Expression of the *D. stramonium* ODC increased ODC activity 50-fold above the endogenous ODC activity of the yeast strain. However, co-expression of either the *Arabidopsis* S15 ribosomal protein C-terminal half or the full length protein did not inhibit the increased ODC activity conferred by expression of the *D. stramonium* ODC (Fig. 6).

Only full length ribosomal protein S15 mRNA is detectable in *Arabidopsis* cells

Arabidopsis Landsberg erecta suspension cultures were treated with the cytokinin 6-benzylaminopurine to induce cell division (Fig. 7). Total RNA was extracted from samples taken at different time points after the addition of cytokinin. An RNA gel blot of the total RNA from different time points indicated that only one mRNA species hybridised to the S15 ribosomal protein probe, with a size calculated to be approximately 700 bases. This is equivalent to the 668 bp full length S15 ribosomal protein cDNA initially isolated by Gantt and colleagues (Sangwan et al. 1993). No shorter transcript potentially encoding the S15 C-terminal polypeptide was detected at any time point.

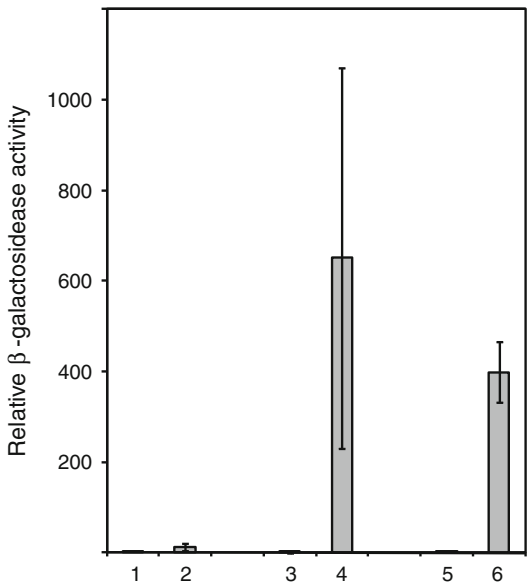

Fig. 4 Interaction of sense and antisense S15 ribosomal protein C-terminal fragment with *D. stramonium* ODC in different yeast two hybrid system host strains. The ODC and S15 ribosomal protein cDNAs were swapped from bait (pGBT9) to prey (pGAD424) plasmids and prey (pGAD424) to bait (pGBT9) plasmids, respectively. An antisense version of the S15 fragment cDNA was cloned into the pGAD424 plasmid. For the Y190 and CG-1945 strains, 3-aminotriazole was added to the growth medium at 35 and 5 mM, respectively, to suppress the leaky histidine selection. Interaction between the bait and prey proteins was selected for by growth in the absence of histidine, except for J69-4A where adenine selection was used. The interaction is reported as relative β-galactosidase activity. *1* antisense ribosomal protein S15 in pGBT9 and *D. stramonium* ODC in pGAD424 in yeast two hybrid strain J69-4A; *2* sense ribosomal protein S15 in pGBT9 and *D. stramonium* ODC in pGAD424 in yeast two hybrid strain J69-4A; *3* antisense ribosomal protein S15 in pGBT9 and *D. stramonium* ODC in pGAD424 in yeast two hybrid strain CG-1945; *4* sense ribosomal protein S15 in pGBT9 and *D. stramonium* ODC in pGAD424 in yeast two hybrid strain CG-1945; *5* antisense ribosomal protein S15 in pGBT9 and *D. stramonium* ODC in pGAD424 in yeast two hybrid strain Y190; *6* sense ribosomal protein S15 in pGBT9 and *D. stramonium* ODC in pGAD424 in yeast two hybrid strain Y190. The standard error of quadruplicate assays is shown

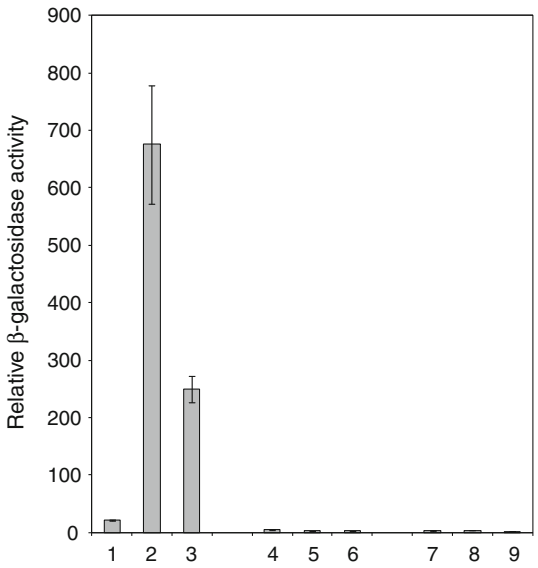

Fig. 5 Interaction between the *Arabidopsis* ribosomal protein S15 C-terminal polypeptide and the plant, human and yeast ODC in different yeast two hybrid strains. The *D. stramonium*, *H. sapiens* and *S. cerevisiae* ODCs were expressed from pGBT9, the *Arabidopsis* S15 C-terminal ribosomal protein fragment was expressed in pGAD10. Ribosomal protein S15 and *D. stramonium* ODC in J69-4A (*1*); CG-1945 (*2*); Y190 (*3*); ribosomal protein S15 and *H. sapiens* ODC in J69-4A (*4*); CG-1945 (*5*); Y190 (*6*); ribosomal protein S15 and *S. cerevisiae* ODC in J69-4A (*7*); CG-1945 (*8*); Y190 (*9*). The relative *β*-galactosidase activities are shown with standard errors of quadruplicate assays

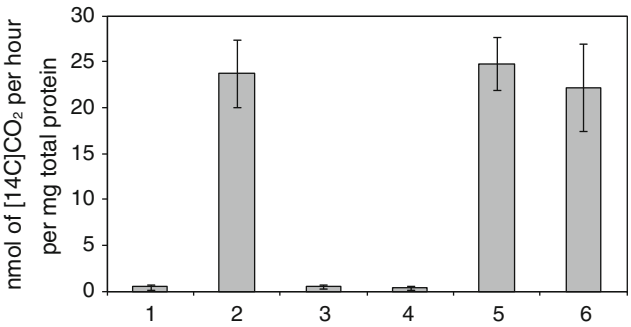

Fig. 6 ODC activity in yeast after co-expression of the *D. stramonium* ODC and full length and C-terminal region *Arabidopsis* S15 ribosomal proteins. The *D. stramonium* ODC was expressed from a galactose-inducible plasmid in the yeast strain YW5-1B and the effect of galactose-induced co-expression of the full length or C-terminal fragment of the *Arabidopsis* S15 ribosomal protein cDNA on ODC activity was assessed. *1* pYX243 (empty, leucine selection) and pYES2 (empty, uracil selection); *2* pYX243 (empty) and *D. stramonium* ODC in pYES2; *3 Arabidopsis* full length S15 ORF in pYX243 and pYES2 (empty); *4* C-terminal partial S15 ORF in pYX243 and pYES2 (empty); *5 Arabidopsis* full length S15 ORF in pYX243 and *D. stramonium* ODC in pYES2; *6* C-terminal partial S15 ORF in pYX243 and *D. stramonium* ODC in pYES2. Results shown are the mean of four independent sets of duplicate assays

Fig. 7 Steady-state mRNA levels of the *Arabidopsis* S15 ribosomal protein gene in suspension culture cells after treatment with cytokinin for different times. The C-terminal S15 cDNA obtained from the yeast two hybrid screen of *Arabidopsis* cDNAs was used to hybridise to the size-fractionated RNA. An *Arabidopsis* suspension culture was treated with 1 μM 6-benzylaminopurine at $t = -24$ h (*1*), 0 h (*2*), 1 h (*3*), 2 h (*4*), 4.5 h (*5*), 7 h (*6*), 10 h (*7*) and 24 h (*8*). *A* Ethidium bromide-stained total RNA; *B* RNA gel bot hybridised to the C-terminal S15 cDNA

Discussion

Unlike metazoa and fungi, plants usually have two different pathways to synthesize putrescine. The ODC pathway typical of other eukaryotes is present in most plants but an additional ADC pathway is present, derived from the original cyanobacterial endosymbiont that formed the chloroplast. There is convincing evidence ADC has been retargeted to the chloroplast (Bortolotti et al. 2004) although there may be also nuclear localisation. The presence of two parallel pathways for putrescine biosynthesis may explain why there is no clear orthologue of the ODC antizyme in plants: polyamine-responsive negative feedback regulation of polyamine biosynthesis in plants would require inhibition of both ODC and ADC if putrescine biosynthesis was subject to feedback control. We have shown here that there is little if any polyamine-induced post-transcriptional repression of ODC or ADC activity in tobacco BY-2 cells, ruling out any non-homologous, independently evolved antizyme-like feedback system. It is now clear that the main mechanism of homeostatic, autoregulatory polyamine-responsive repression of polyamine biosynthesis in plants is at the level of translational regulation of *S*-adenosylmethionine decarboxylase via a sequence-dependent upstream open reading

frame (Ivanov et al. 2010; (Hanfrey et al. 2002, 2005). Thus, although spermidine and spermine/thermospermine biosynthesis is likely to be repressed by excess polyamines, putrescine levels are not affected. Indeed, plants contain a much higher putrescine to spermidine ratio than mammalian cells, and the plant *S*-adenosylmethionine decarboxylase activity does not require putrescine for autocatalytic self-processing of the proenzyme or for full enzymatic activity (Xiong et al. 1997). Thus high putrescine levels have little effect on *S*-adenosylmethionine decarboxylase processing and activity in plants.

In the light of these findings, the reports of plant ornithine decarboxylase antizymes was intriguing. It might be that plant ODC binding proteins serve a novel function that regulates polyamine metabolism in response to unknown signals. We set out to identify plant ODC binding proteins using the yeast two hybrid system. *Arabidopsis* serves as an interesting comparator for the study of ODC binding proteins because it has lost its ODC gene.

The same ODC binding protein was the only protein identified from yeast two hybrid screens of pea and *Arabidopsis* tissues representing very different plant tissues. Remarkably, the same C-terminal fragment of the S15 cytosolic ribosomal protein was isolated in each case. Binding to the *D. stramonium* ODC was independent of whether the S15 polypeptide was fused to the GAL4 DNA binding domain or activation domain. However, the binding was specific for the plant ODC and no binding was detected with the yeast or human ODC. This is in contrast to the bacterial *S. ruminantium* ODC antizyme (ribosomal protein L10), which was able to bind the mouse ODC (Yamaguchi et al. 2008).

Neither the unfused full length *Arabidopsis* S15 ribosomal protein nor the C-terminal polypeptide isolated in the two hybrid screens was able to inhibit the *D. stramonium* ODC activity when they were co-expressed in yeast. If binding between the unfused S15 and *D. stramonium* ODC proteins occurs during co-expression in yeast, then clearly binding is not inhibitory for ODC enzymatic activity. The fact that no smaller-sized S15 ribosomal protein mRNAs could be detected in *Arabidopsis* cells raises the question of how the C-terminal S15 fragment is generated. It is formally possible that a very low abundance mRNA is present that could encode the partial S15 protein. Alternatively, the isolation of the partial cDNA may be an artifact of the yeast two hybrid system. Neither explanation supports a physiological role for the S15 ribosomal protein as an ODC-regulating protein. We also demonstrated that there is no detectable post-transcriptional negative feedback regulation of plant ODC in response to excess polyamines. Thus the original 16 kDa and 9 kDa ODC antizymes identified in barley (Panagiotidis and Kyriakidis 1985; Koromilas and Kyriakidis 1987) remain enigmatic.

Acknowledgments A. J. Michael is supported by UT Southwestern Medical Center. We would like to express our thanks to Susanne Sommer for analysis of the tobacco BY-2 cells.

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