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Ornithine: The Overlooked Molecule in the Regulation of Polyamine Metabolism[3]

Rajtilak Majumdar[1], Lin Shao[1], Rakesh Minocha[2], Stephanie Long[2] and Subhash C. Minocha[1,*]

[1]Department of Biological Sciences, University of New Hampshire, Durham, NH 03824, USA
[2]USDA Forest Service, Northern Research Station, 271 Mast Rd, Durham, NH 03824, USA
[3]This is Scientific Contribution Number 2449 from the New Hampshire Agricultural Experiment Station.
*Corresponding author: E-mail, sminocha@unh.edu; Fax: +1-603-862-3784.
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We overexpressed a mouse ornithine decarboxylase gene under the control of a constitutive and an estradiol-inducible promoter in *Arabidopsis thaliana* to increase our understanding of the regulation of polyamine metabolism. Of particular interest was the role of the substrate ornithine not only in the regulation of polyamine biosynthesis, but also in the accumulation of related amino acids in response to short-term induction of this enzyme. We hypothesized that the inducible expression of the transgene would mimic the natural responses of plants to changing conditions, e.g. under stress conditions and during rapid growth. Our results reveal that ornithine, even though present in relatively small quantities (compared with other amino acids of the glutamate–arginine–proline pathway), may not only be the key regulator of polyamine biosynthesis in Arabidopsis, but it may also regulate the entire subset of pathways for glutamate to arginine and to proline. Indirectly, it could also regulate putrescine catabolism, therefore contributing to the γ-aminobutyric acid content of the cells. Furthermore, the induction of mouse ornithine decarboxylase resulted in up- and down-regulation of several amino acids in the transgenic plants. It was learned that the turnover of putrescine in both the wild type and the transgenic plants occurs rapidly, with a half-life of 6–8 h.

Keywords: *Arabidopsis thaliana* • Polyamines • Metabolism • Ornithine • Putrescine.

Abbreviations: Ala, alanine; Arg, arginine; ADC, arginine decarboxylase; Cad, cadaverine; DAO, diamine oxidase; GABA, γ-aminobutyric acid; GAD, Glu decarboxylase; Gln, glutamine; Glu, glutamate; Gly, glycine; GM, germination medium; HP, high putrescine; Leu, leucine; Lys, lysine; Orn, ornithine; ODC, ornithine decarboxylase; PA, polyamine; PAO, polyamine oxidase; PCA, perchloric acid; Pro, proline; Put, putrescine; SAMDC, *S*-adenosylmethionine decarboxylase; Ser, serine; Spd, spermidine; Spm, spermine; SPDS, spermidine synthase; SPMS, spermine synthase; TCA, tricarboxylic acid; Thr, threonine; Trp, tryptophan; WT, wild type.

Introduction

Polyamines (PAs), which are found in all living organisms, are implicated in many physiological phenomena in plants relating to growth and development, and stress responses via a multitude of biochemical functions (Minocha and Minocha 1995, Bouchereau et al. 1999, Hyvonen et al. 2006, Peremarti et al. 2009, Landau et al. 2010, Mattoo et al. 2010, Mohapatra et al. 2010a, Quinet et al. 2010). The diamine putrescine (Put) is synthesized either by direct decarboxylation of ornithine (Orn) by Orn decarboxylase (ODC; EC 4.1.1.17) or indirectly from arginine (Arg) by Arg decarboxylase (ADC; EC 4.1.1.19) in most plants (**Fig. 1**) except Arabidopsis, where an *ODC* gene is apparently absent (Hanfrey et al. 2001). Put is sequentially converted into spermidine (Spd) and spermine (Spm) by combined actions of *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) and two different aminopropyltransferases [Spd synthase (SPDS) EC 2.5.1.16 and Spm synthase (SPMS) EC 2.5.1.22; reviewed in Shao et al. (2012)]. Another diamine, cadaverine (Cad), which is found frequently in animals and occasionally in plants, is produced from lysine (Lys) either by Lys decarboxylase (Tjandrawinata et al. 1994, Ohe et al. 2009, Kashiwagi and Igarashi 2011) or by ODC, which has a low affinity for Lys (Pegg and McGill 1979).

In animals, the production of Put through ODC is considered the key step for limiting PA biosynthesis, whereas in plants ADC performs this role. A hypothesis emphasizing a regulatory role for Orn rather than ODC has also been proposed for animals (Morris 2006, Morris 2007). In spite of the fact that (i) Orn is an intermediate in the glutamate (Glu) to Arg pathway in plants; (ii) both Glu and Arg are often present in large quantities; and (iii) in most plants the cellular concentration of Orn is much lower compared with Arg or Glu, its significance in the regulation of Put production has rarely been discussed (Mayer and Michael 2003). Using genetic manipulation of Put production directly from Orn, we demonstrate here that not only does the cellular Orn concentration play a key role in Put production but also its own biosynthesis responds

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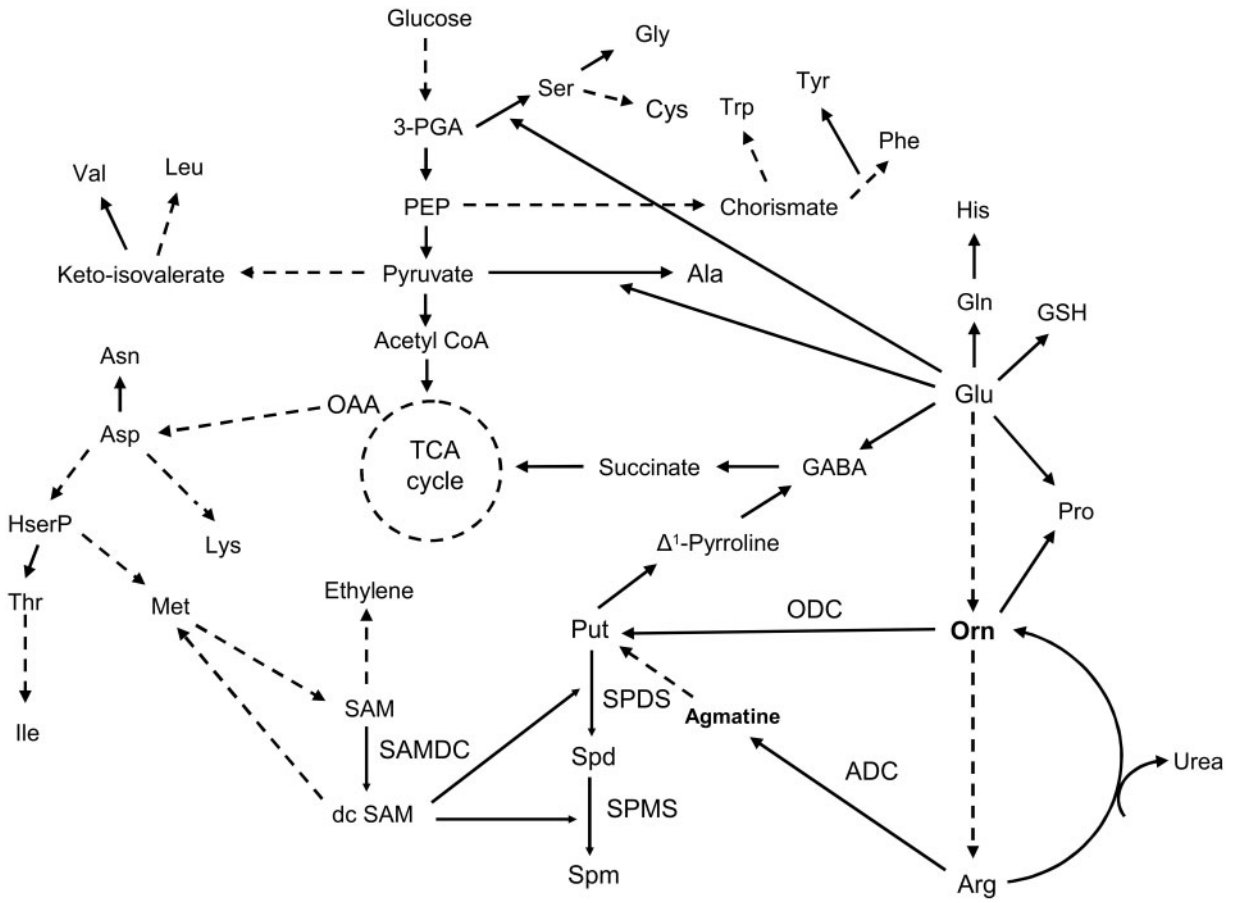

Fig. 1 Abbreviated pathway for the biosynthesis of polyamines and amino acids. Abbreviations (PA enzymes): ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SAMDC, *S*-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase (modified from Mohapatra et al. 2010b).

actively to its demand in the PA biosynthetic pathway. Based on the results presented here, we invoke the occurrence in plants of an Orn-sensing (monitoring) and signal transduction mechanism, which regulates its own biosynthesis and also plays a role in regulating Arg, Pro (proline) and GABA (γ-aminobutyric acid) production. Additionally, we demonstrate that the catabolic breakdown of Put is rather rapid [turnover rate, i.e. half-life ($T_{1/2}$) of about 6–8 h] and keeps pace with its production without a change in its $T_{1/2}$.

Manipulation of PA contents through transgenic expression of the key biosynthetic genes (*ODC*, *ADC*, *SAMDC* and *SPDS*) has been achieved in plants by several laboratories (Thu-Hang et al. 2002, Mayer and Michael 2003, Rea et al. 2004, Alcázar et al. 2005, Nölke et al. 2008, Wen et al. 2008). Most studies on transgene expression have used constitutive promoters, which is analogous to using mutants since in both cases the manipulated pathway is subject to homoeostatic adjustments over time. Such a system is not amenable to analysis of short-term perturbations in PA metabolism. Previous studies have shown that long-term adjustments to up-regulation of PA biosynthesis involve metabolic consequences that go far beyond the PA pathway, including effects on several amino acids, sugars, sugar alcohols, organic acids and inorganic ions (Minocha et al. 2004, Page et al. 2007, Mattoo et al. 2010, Mohapatra et al. 2010a, Mohapatra et al. 2010b, Nambeesan et al. 2010, Page et al. 2012). The physiological effects of Put overproduction also include changes in the oxidative state of transgenic cells and alterations in their carbon (C) and nitrogen (N) balance (Mohapatra et al. 2009, Mohapatra et al. 2010b). However, in most cases of natural responses of a plant to environmental or developmental stimuli, short-term changes in PA metabolism are perhaps more common, and conceivably the most critical events in its life. In this regard, inducible transgenic expression systems (such as the conditional mutants) permit us to mimic turning on a gene for a limited duration, as would occur in nature. Thus inducible expression systems should provide a better understanding of the regulation of a metabolic pathway under conditions of transient (short-term) expression of genes, and, consequently, quick changes in cellular metabolic flux associated with the induced step. Furthermore, overexpression of a transgene encoding a regulatory enzyme is often used to achieve genetic manipulation of metabolism with the assumption that its substrate(s) may not be limiting, which is not always the case (Galili and

Höfgen 2002, Goff and Klee 2006, Schnee et al. 2006, Bartels and Hussain 2008).

The primary goal of the present study was to address the potential role of substrates in regulation of the PA biosynthetic pathway under conditions of short-term (via induction) as well as long-term (constitutive) manipulations in Arabidopsis. We also studied the interaction of PA up-regulation with the accumulation of amino acids and the role of Put turnover in relation to its biosynthesis. Four specific questions were addressed here which pertain to the anticipation that induction of a heterologous *ODC* gene would quickly deplete cellular Orn (its substrate) with associated consequences of affecting PA levels, as well as the levels of Orn, Glu and Arg, and presumably the associated amino acids (**Fig. 1**). In order for cells to prevent excessive accumulation of a specific PA, it was hypothesized that its catabolism will also be affected. The four questions were as follows (i) Will siphoning away Orn from the Glu → Orn → Arg pathway affect the production of Arg and its utilization for Put production? (ii) Will overutilization of Orn (by the newly introduced ODC step) increase its production from Glu? (iii) If the answer to the latter question is affirmative, will the increased flux of the Glu → Orn → Arg pathway affect accumulation of other amino acids? (iv) Will the catabolism of Put be affected under conditions of its excessive production? The results revealed some remarkable and unique aspects of the regulation of PA metabolism, especially regarding the function of the substrate Orn and, also, the rate of Put turnover. We believe that an understanding of the effects of cellular metabolic adaptations in response to short-term (e.g. up to 48 h) changes in gene expression on the pathways related to the PA metabolic pathway would lead to improved design of approaches for metabolic engineering of plants (i) for food and feed improvement; (ii) for chemotherapies involving PA inhibitors (Tavladoraki et al. 2011); and (iii) for use in increased biomass or bioenergy production.

In *Arabidopsis thaliana*, Orn is synthesized from Glu through participation of at least five enzymes. Arg is then produced from Orn in two steps and serves as the main source of Put biosynthesis by ADC. Orn also serves as an intermediate for Pro metabolism, which itself has several functions in plants (Sharma and Verslues 2010). Using a combination of constitutive and inducible transgene expression systems, we demonstrate that under normal conditions, Orn plays a critical role in the regulation of Put production in Arabidopsis as opposed to Arg and/or Glu. We also present evidence that under conditions of increased Orn utilization to produce Put (i.e. overexpression of transgenic ODC), the biosynthesis of Orn (from Glu) is increased commensurate with its demand. We propose that the pathway of Glu → Orn is regulated via a mechanism that involves monitoring of cellular Orn concentration. On the other side of the equation, which determines cellular Put accumulation, there is an increase in Put catabolism, creating a high-flux pathway of Glu → Orn → Put → GABA → tricarboxylic acid (TCA), which compensates for increased Orn utilization. This flux neither changes the production of Arg from Orn nor affects the production of Put from Arg by ADC. The rate of Put turnover in the control [uninduced or wild type (WT)] as well as the high Put- (HP) producing transgenic plants (constitutive or induced) occurs with a $T_{1/2}$ of about 6–8 h despite the fact that the amount of Put catabolism on a fresh weight basis (g^{-1} FW) is 3- to 4-fold higher in the latter.

Results

Overexpression of mouse *ODC* cDNA and changes in polyamines

The present study involved transgenic expression of a mouse *ODC* cDNA in Arabidopsis to increase the biosynthesis of Put and investigate its effects on cellular PAs and soluble amino acids, and to examine the role of Orn in PA biosynthesis and accumulation. Since Arabidopsis lacks the ODC gene for Put biosynthesis (Hanfrey et al. 2001), we used the coding sequence of a mouse ODC (m*ODC*) to achieve two goals: (i) to introduce the Orn→Put step to manipulate PAs; and (ii) to study the biochemical effects of depleting Orn (which is the essential intermediate in Arg biosynthesis from Glu) on cellular Arg, on the native ADC pathway (for Put production) and on other related amino acids. The aim was to better understand the role of Orn in the regulation of cellular PAs and the subsequent effects on other amino acids in plants. This gene was selected based on previous studies which showed its effectiveness in increasing Put production in several plant species, including tobacco, carrot and poplar (DeScenzo and Minocha 1993, Bastola and Minocha 1995, Bhatnagar et al. 2001, Kumria and Rajam 2002).

Two types of m*ODC* transgenic plants were produced in this study: those expressing the m*ODC* coding sequence under the control of a constitutive ($2 \times 35S$) promoter and those in which the same open reading frame was induced by estradiol. The two plasmids used for transformation are shown in **Fig. 2**. From 5–8 putative transgenic lines (single copy of the transgene as determined by segregation analysis) selected on hygromycin for each construct (and confirmed for the presence of the m*ODC* sequence), 3–4 lines were used to produce homozygous plants (tested by segregation analysis), which were further tested for Put accumulation through each generation. Plants of the T_3 and T_4 generation from one or two selected lines were used for the experiments presented here. As a result of the effects of transgene expression, a 10- to 50-fold increase in Put accumulation was observed.

Cellular Put + Cad content was significantly higher in different transgenic lines constitutively expressing the m*ODC* sequence than in the WT, but to a variable extent (**Supplementary Table S1**). The transgenic plants could have as much as 20–30% of the total diamine fraction as Cad, whereas the WT plants did not have any Cad (see later). Taking that into account, the calculated increase in Put was estimated to be 40-fold (vs. the WT) in the m*ODC*-1-7 line and approximately 24-fold in the m*ODC*-18-2 line; Spd and Spm

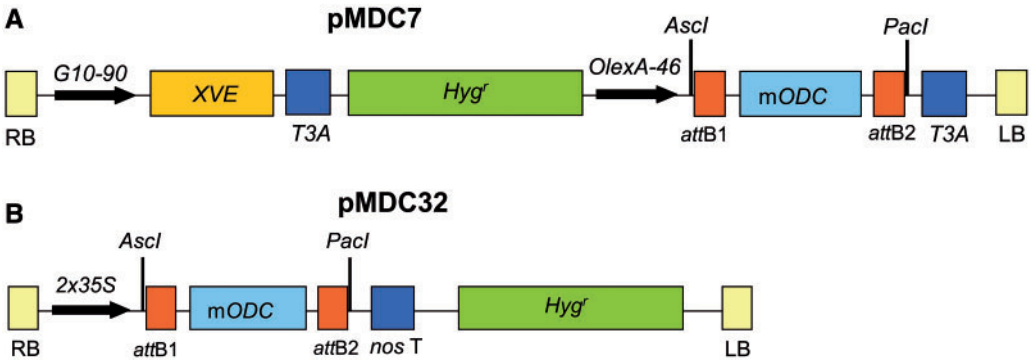

Fig. 2 Vectors used for (A) inducible expression (pMDC7) and (B) constitutive expression (pMDC32) of m*ODC*. $2 \times 35\,S$ and *G10-90* are constitutive promoters; RB, right border; LB, left border; *att*B1 and *att*B2, recombination sites after LR clonase; *Hyg*^{r}, hygromycin resistance gene; XVE, estradiol-responsive transcription factor; *OlexA-4*, XVE-responsive promoter; *T3A* and *nos T*, terminators. (Curtis and Grossniklaus 2003).

changed only slightly in the transgenic plants. Also in the inducible plants, a >20-fold increase in Put was seen upon induction with estradiol, again with little change in Spd and Spm and a significant production of Cad (**Fig. 3**; **Supplementary Fig. S3**).

Since Orn occupies a pivotal position in the biosynthesis of Put, Arg and Pro, and its concentration is already quite low (see later), we hypothesized that in the transgenic plants the siphoning away of Orn by mODC would quickly limit Put biosynthesis. Furthermore, since Put is solely produced via Arg in the WT Arabidopsis, the draining away of Orn by mODC would (potentially) deprive the cells of the substrate to make Arg. Therefore, we designed an experiment in which three amino acids of this pathway (Orn, Glu and Arg) were added to the medium during induction. Within 12 h of induction (+estradiol), Put content increased in the transgenic plants by >10-fold; the increase was sustained for at least 24 h (**Fig. 3A**) and even beyond (**Supplementary Fig. S3**). No change in PAs was seen in the WT plants treated with estradiol (data not shown) or in uninduced (−estradiol) plants. Addition of Orn concomitant with induction caused a further 4-fold increase (up to a total of 40-fold at 12 h and ∼50-fold at 24 h). Once again, without induction, Orn had little effect on Put accumulation. The addition of Arg or Glu without induction also had no effect on Put.

The content of Spd was slightly higher at 12 h (not statistically significant) upon induction, with no further enhancement in its production by exogenous Orn (**Fig. 3B**). No significant change in Spd was seen with other treatments, except for Orn or Arg, which caused an increase in Spd in the uninduced plants. Similar to Spd, changes in cellular Spm in response to induction or the addition of Orn, Arg or Glu were always small (< 2-fold; **Fig. 3C**). In induced plants, a significant amount of Cad was seen in all cases (**Fig. 3D**). Its content increased further at 24 h of induction (**Supplementary Fig. S3**); however, the increase in Cad was smaller in the presence of Orn as lysine is the alternative substrate for induced mODC to produce Cad only when the Orn concentration is lower than normal.

Overexpression of m*ODC* and changes in amino acids

Since Orn and Arg (the substrates of Put) are derived entirely from Glu, and Glu also serves as a substrate for the biosynthesis of most other amino acids (**Fig. 1**), it is logical to postulate that increased utilization of Orn by mODC would affect the connected pathways in which Orn, Arg or Glu are involved. Thus in addition to the PAs, we analyzed the contents of perchloric acid (PCA)-soluble amino acids in HP plants (constitutive and induced) in the presence as well as the absence of exogenous Orn, Glu and Arg.

Upon induction of m*ODC*, the content of Glu was not significantly affected (**Fig. 4A**) even though its consumption for Orn production must have increased several fold. This would indicate that Glu was being continuously replenished either by its increased biosynthesis or by a reduction in its use for other amino acids. Exogenous Orn surprisingly caused a doubling of the Glu content in both induced and uninduced seedlings. Such an increase was not seen by the addition of either Arg or Glu itself, except in uninduced seedlings at 24 h. Thus, it appears that exogenous Orn not only met the demand (as substrate) of mODC, but also stimulated Glu biosynthesis. The effect of exogenous Orn on Glu was similar (i.e. a significant increase) in the uninduced plants as well, again showing a stimulation of Glu production. Glutamine (Gln), which constituted >50% of the total soluble amino acid pool in the seedlings, increased with time between 12 h and 24 h, and its cellular content increased further at 24 h of induction (**Fig. 4B**). Exogenous Orn (with or without estradiol) effectively prevented the increase in Gln at 24 h, but Arg had no effect.

In view of the fact that Orn was being consumed heavily by mODC, other predicted effects would include a potential reduction in Arg biosynthesis (and its availability to ADC) and Pro biosynthesis, as both are the direct products of Orn (Sharma and Verslues 2010). However, this did not occur during the short-term induction of m*ODC*. On the contrary, a significant increase in Pro content was observed at 24 h upon

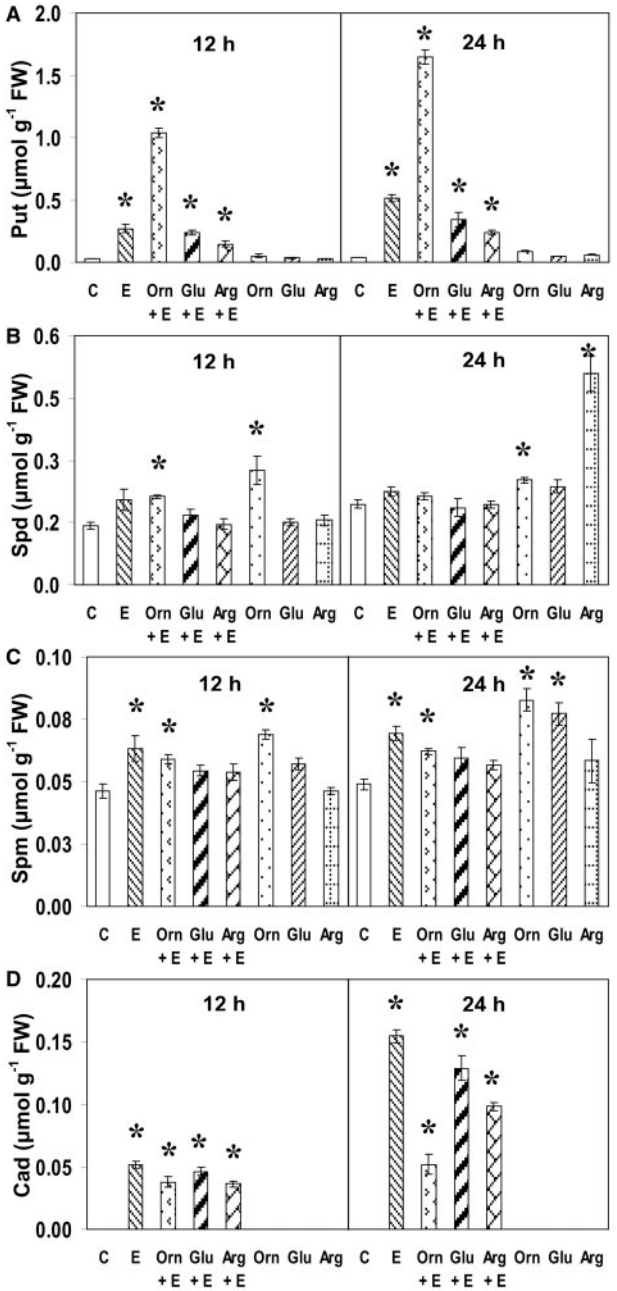

Fig. 3 Cellular contents of (A) putrescine, (B) spermidine, (C) spermine and (D) cadaverine after 12 and 24 h in induced (+E) and uninduced (C = control) 12-day-old m*ODC*-10-1 transgenic seedlings with or without 0.1 mM Orn, 1.0 mM Glu or 0.5 mM Arg. Data are the mean ± SE of four replicates; each replicate consists of 6–7 seedlings. *$P \leq 0.05$ for a significant difference between treated and untreated seedlings at a given time.

induction as well as in the uninduced seedlings treated with exogenous Glu or Arg (**Fig. 4C**). An increase in the combined peak of Arg + threonine (Thr) + glycine (Gly) (>70% of the peak was Arg; data not shown) at 24 h in both uninduced and induced plants was perhaps due to Arg absorbed from the medium (**Fig. 4D**).

In the seedlings, Orn was present in relatively small amounts ($\leq$ 0.5% of the total soluble amino acids) as compared with Arg, Glu or Gln (**Fig. 4E**). An exogenous supply of Orn in the medium caused its increased accumulation only in the absence of induction, showing its rapid utilization by mODC upon induction. Exogenous Arg or Glu did not affect Orn.

The non-protein amino acid GABA is a product of Put catabolism via diamine oxidase [DAO; also known as copper-containing amine oxidases (CuAO), Moschou et al. (2008)] and is also produced directly from Glu by Glu decarboxylase (GAD; Shelp et al. 2012; also **Fig. 1**). The carbon skeleton of Glu and Put is recycled through the reactions of GABA→succinate→TCA cycle. The cellular content of GABA increased significantly on induction of m*ODC* parallel to the changes observed in Put (**Fig. 4F**). Its content was also higher when uninduced plants were treated with exogenous Arg (at 24 h) or Glu.

Changes in the cellular contents of other amino acids, which are not direct products of the Glu → Orn → Arg → Pro → Put pathway, are discussed in **Supplementary data (Figs. S1, S2; Supplementary Tables S1–S3)**.

In the constitutive HP seedlings also, the abundant (>2% of the total) amino acids were Gln (~60% of total), Glu, Pro, Arg, Ser (serine), Gly and Ala (alanine); others, except histidine, were present at <1% each. The amino acids whose concentrations were lower in the HP vs. the WT seedlings were Orn, Lys and tryptophan (Trp), while GABA, Gly, methionine, Ala, valine, Pro and Thr were slightly higher in them (**Supplementary Table S1**).

Exogenous supply of polyamines and changes in amino acids

Since significant differences in amino acids were seen between the WT and the HP (inducible and constitutive) transgenic plants, it was hypothesized that if the changes in amino acids were due to the presence of high Put, it should be possible to mimic them by treatment with exogenous PAs in the WT plants. On the other hand, if changes in the HP plants were due to the depletion of Orn, the effects on amino acids would be different. For this experiment, WT seeds were germinated and grown in the presence of Put, Spd or Spm for 7 d before analysis of PAs and amino acids.

As shown in **Supplementary Table S4**, exogenous Put was taken up by the seedlings, leading to a 30-fold increase (vs. the untreated); however, there was a slight decrease in Spd and Spm. Treatments with exogenous Spd resulted in its own accumulation, and also that of Put (~5-fold) but not Spm. Exogenous Spm also increased cellular Put along with its own accumulation. Among changes in amino acids between the untreated and the Put-treated seedlings were: reductions in Pro (~68%) and phenylalanine (~47%) and increases in Orn (~33%), Ser (~28%) and Trp (~133%). Exogenous Spd had little effect on most amino acids, except for Orn (~100%), Arg + Thr + Gly (~30%) and isoleucine (~20%), which were

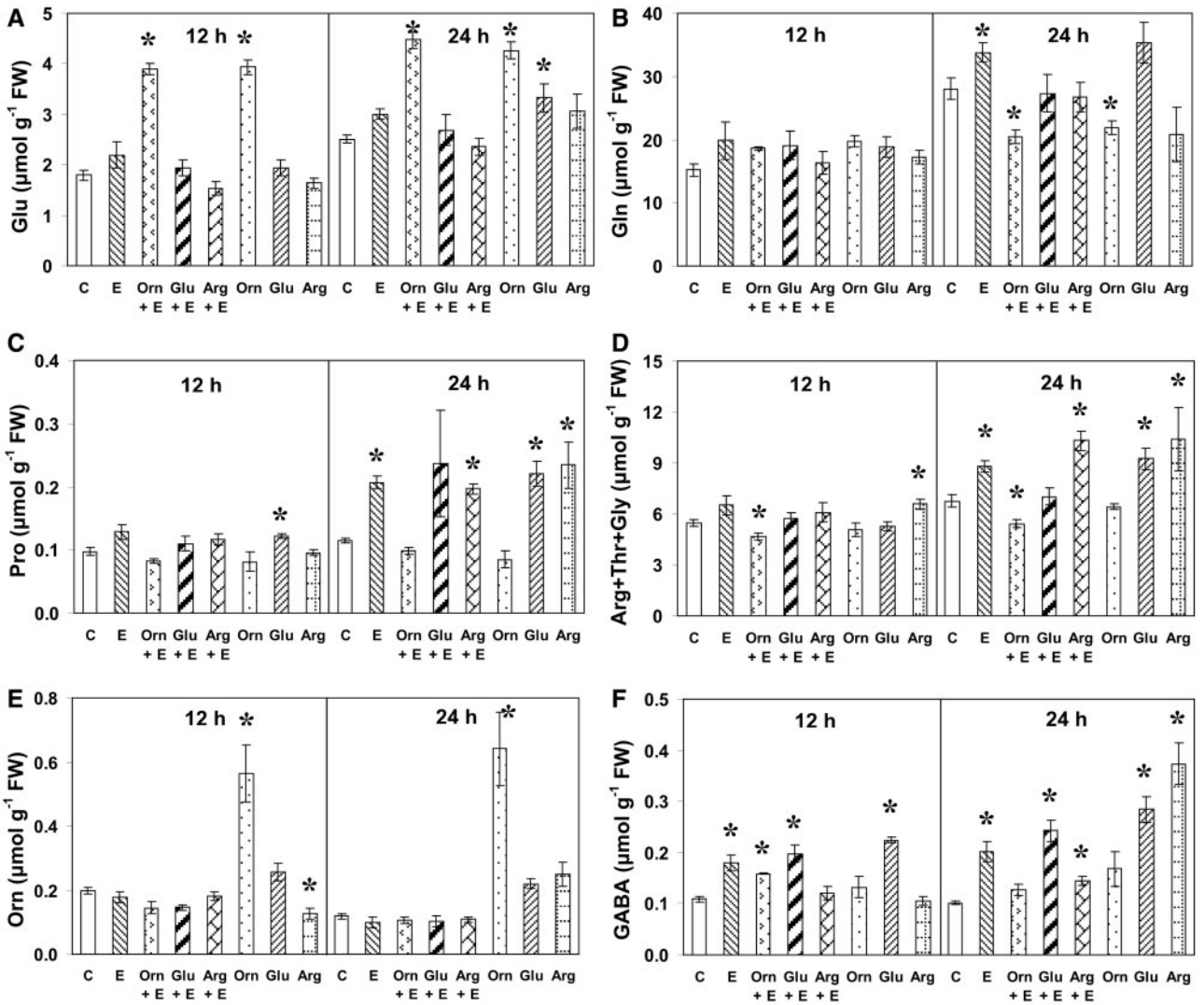

Fig. 4 Cellular contents of (A) glutamate, (B) glutamine, (C) proline, (D) arginine + threonine + glycine, (E) ornithine and (F) γ-aminobutyric acid after 12 and 24 h in induced (+E) and uninduced (C = no estradiol) 12-day-old m*ODC*-10-1 transgenic seedlings with or without 0.1 mM Orn, 1.0 mM Glu or 0.5 mM Arg. Data are the mean ± SE of four replicates; each replicate consists of 6–7 seedlings. *$P \leq 0.05$ for a significant difference between treated and untreated seedlings at a given time.

higher. The effects of Spm were similar to those of Spd. The results imply that the changes in amino acids were not affected by exogenous PAs in the same way as those produced in higher quantities within.

The effects of m*ODC* induction on ADC activity

In order to measure mODC activity in the transgenic plants directly and its possible compensatory effects on ADC activity, 14-day-old seedlings were induced with estradiol (for 2 h) and supplied with [U-^{14}C]Orn or [U-^{14}C]Arg to study their incorporation into PAs. The data presented in **Fig. 5A** show that the induction of m*ODC* almost tripled the incorporation of [U-^{14}C]Orn into PAs during 8 h of incubation, but had no effect on the incorporation of [U-^{14}C]Arg into PAs. More than 70% of radioactivity in the toluene fraction was found in Put as determined by thin-layer chromatography (TLC; data not shown). On the other hand, the aqueous fraction (**Fig. 5B**) had higher levels of radioactivity in the uninduced vs. the induced seedlings for [^{14}C]Orn and similar amounts for [^{14}C]Arg. The total amount of [^{14}C]Orn and [^{14}C]Arg taken up by the plants (the sum of the toluene and the aqueous fractions) was comparable in the induced and the uninduced plants, showing that their utilization rate did not affect their uptake. The [U-^{14}C]Orn incorporation into Put in the uninduced plants (**Fig. 5A**) is conceivably due to its conversion into [^{14}C]Arg and subsequently into Put by ADC.

Turnover of cellular putrescine

For determination of the turnover rates of Put ($T_{1/2}$ = loss of 50% of cellular Put), 12-day-old WT, the constitutive m*ODC* and

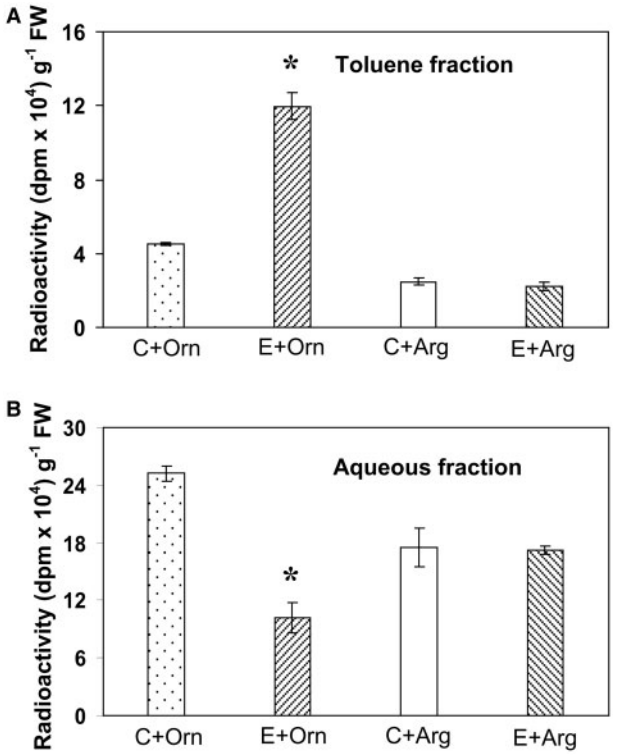

Fig. 5 Distribution of ^{14}C into (A) the toluene (total polyamines) and (B) the aqueous (non-polyamine) fractions from [U-^{14}C]Orn or [U-^{14}C]Arg incubation for 8 h (following 2 h of induction) in 14-day-old m*ODC*-10-1 transgenic seedlings. Data are the mean ± SE of three replicates; each replicate consists of 12–14 seedlings. $^{*}P \leq 0.05$ for a significant difference between uninduced (C) and induced (+E) seedlings.

the induced (for 8 h) seedlings were incubated with [U-^{14}C]Orn for 4 h and then transferred to label-free medium for 0, 2, 4, 8, 22 (or 24) and 46 (or 48) h before analysis of radioactive and total soluble PAs. For up to 60 h, the cellular Put content of induced plants increased steadily but remained unchanged in the uninduced plants; Spd and Spm content showed only minor changes (**Supplementary Fig. S3**). As expected, the diamine Cad was always seen in the induced plants (**Supplementary Fig. S4**), increasing up to 8-fold between 12 and 60 h of induction (**Supplementary Fig. S3**). The content of Cad in the induced plants at different times was as much as 20–30% of Put, showing that adequate amounts of Orn were not available for mODC, which then used Lys, the less favored substrate, to produce Cad (Pegg and McGill 1979).

The $T_{1/2}$ of Put was measured in the three genotypes for two sources of Put; one produced endogenously from [U-^{14}C]Orn and the other that was supplied exogenously as [1,4-^{14}C]Put. This experimental design allowed us to investigate further if the two pools of Put were catabolized differently. The dansyl-PAs were separated by TLC (**Supplementary Fig. S4**) and the spots of the three PAs counted for radioactivity. As shown in **Fig. 6A**, the uptake of [U-^{14}C]Orn in the uninduced control and the induced as well as the constitutive HP seedlings were quite similar. In the toluene fraction, which contains all three dansyl-PAs, radioactivity was several fold higher in the two HP lines at any time of analysis. The total radioactivity in this fraction declined with time (**Fig. 6B**).

Seedlings of both the HP lines showed a similar trend of decline in the radioactive Put fraction over time, with about 50% loss occurring by 6–8 h after being transferred to the label-free medium (**Fig. 6C**). The $T_{1/2}$ of Put produced from [^{14}C]Orn (**Fig. 6C** inset) in the constitutive HP line was calculated to be about 8 h (**Fig. 6D**), which includes Put loss by conversion into Spd, its catabolism and its secretion out of the cells. The control line had very low [^{14}C]Put (data not shown); thus the information was not used for calculation of $T_{1/2}$. The only radioactive Put in these seedlings would come via ADC after [^{14}C]Orn was converted into Arg. In the HP seedlings, up to 75% of ^{14}C in the toluene fraction (that contains all dansyl-PAs) was present in the Put fraction (**Fig. 6C**). The radioactivity in Spd and Spm derived from endogenously produced [^{14}C]Put was also higher in the two HP lines than in the control but did not show a clear trend of change with time (**Supplementary Fig. S5B, C**).

In the experiment involving [1,4-^{14}C]Put, its uptake was slightly (<15%) higher in the control than the induced HP seedlings (**Fig. 7A**). While the amount of radioactivity in the toluene fraction remained almost 2-fold higher in the control seedlings than in the induced seedlings, the trend of changes over time was similar between the two (**Fig. 7B**). Similar to the [U-^{14}C]Orn experiment, a fast decline in [^{14}C]Put was observed for both the control and the induced plants, with >50% loss occurring by about 6–8 h (**Fig. 7C** and inset). The calculated $T_{1/2}$ of Put was similar to that for endogenously produced Put (**Fig. 6D**).

From the data on the loss of [^{14}C]Put and measurement of the respective PAs in the same seedlings (**Supplementary Fig. S3**), we were able to estimate the total amount of Put loss in the seedlings at different times. While the control (uninduced) plants lost about 20 nmol Put g^{-1} FW within 2 h, the induced plants lost >50 nmol g^{-1} FW during the same period (**Table 1**); by 8 h, these numbers were >4-fold in the induced vs. the uninduced plants. This translates into an average rate of catabolism of 5–10 nmol g^{-1} FW h^{-1} in the control and 20–30 nmol g^{-1} FW h^{-1} in the HP plants. Of the total Put lost during the first 2 h, as a percentage of the cellular content, >75% was converted into Spd in the control and <40% in the HP plants; thus it appears that the majority of Put in HP plants was lost to its catabolism.

Discussion

Genetic manipulation of plant metabolism can be used to improve the nutritional value of crop plants and also to understand the regulation of a metabolic pathway. In contrast to the mutants and the constitutive transgenic expression systems for genetic manipulation, the inducible transgenic expression

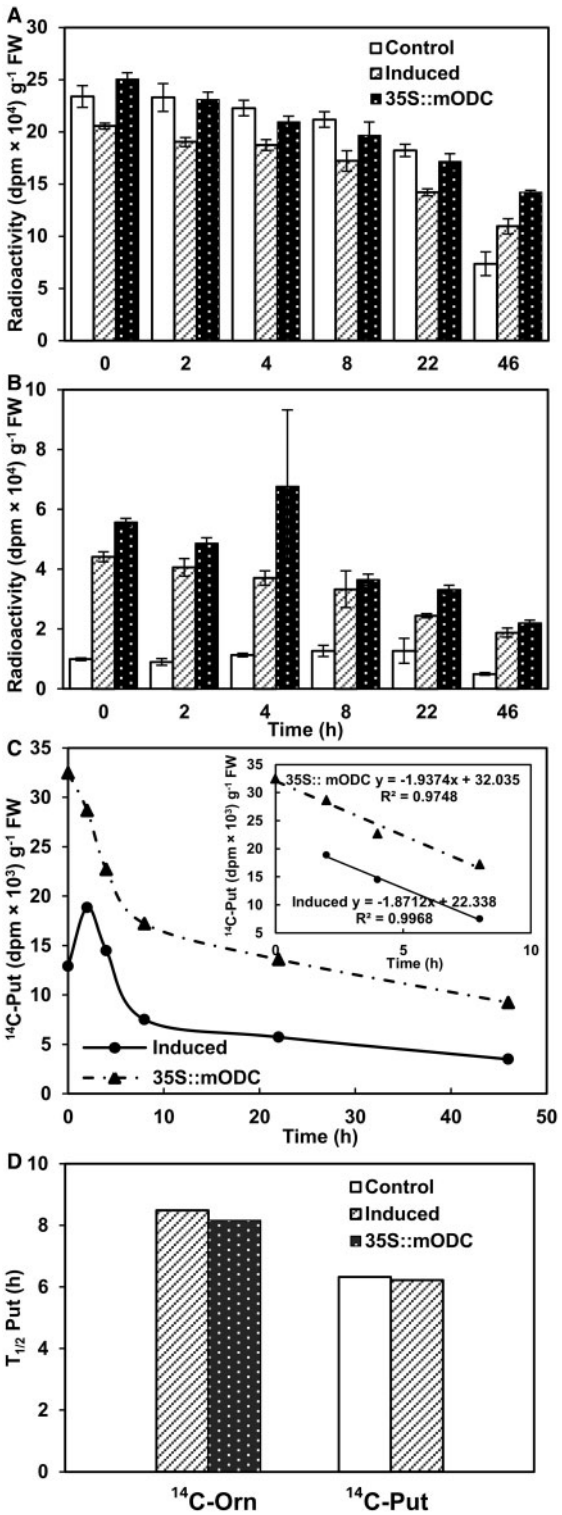

Fig. 6 Amount of radioactivity in the ^{14}C (A) PCA extract (showing uptake) and (B) toluene fraction (total polyamines) of uninduced (control), induced m*ODC*-10-1 (for 8 h) and constitutive 2 × 35 *S*::m*ODC*-1-7 seedlings (2 weeks old) at different incubation times in label-free medium following incubation with [U-^{14}C]Orn for 8 h (= 0 time). Data are the mean ± SE of three replicates. (C) Radioactivity in Put at different incubation times in the label-free medium; the inset shows the regression curve for the loss of

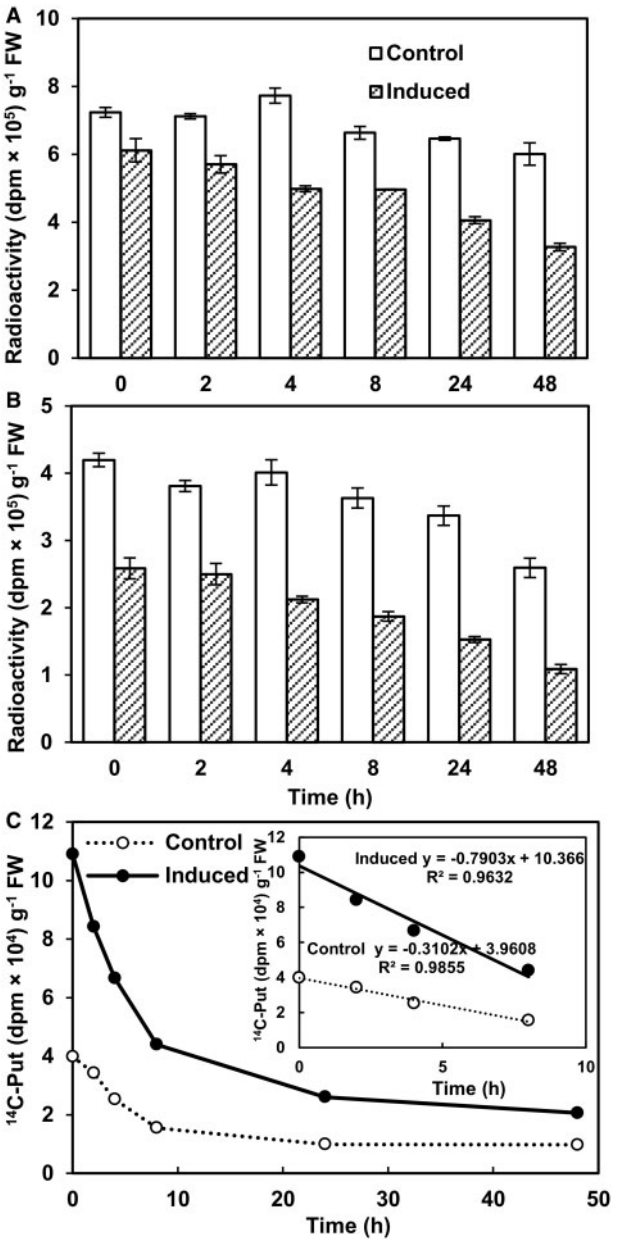

Fig. 7 Amount of radioactivity in (A) the PCA extract, (B) toluene fraction and (C) in putrescine of uninduced (control) and induced m*ODC*-10-1 (12-day-old) seedlings. The seedlings were incubated with [^{14}C]Put for 4 h (following induction for 8 h) and collected at different times after transfer to label-free medium. Data are the mean ± SE of three replicates. Inset: regression curve for loss of [^{14}C]Put over the 8 h period.

Fig. 6 Continued
[^{14}C]Put over the 8 h period. (D) The calculated half-life ($T_{1/2}$) of Put in the two high putrescine (HP) lines and the control (inducible line –E); Orn represents data from [^{14}C]Orn incorporation and Put represents data generated from [^{14}C]Put incorporaion. The $T_{1/2}$ was calculated by using data on the loss of [^{14}C]Put at various time during the first 8 h period.

Table 1 The amount of total Put lost (nmol g^{-1} FW) after 2 and 8 h and the amount of Put converted into Spd (nmol g^{-1} FW) at 2 h after the transfer of control (−E) and induced (+E) seedlings of m*ODC*-10-1 line to label-free medium following the incorporation of [1,4-^{14}C]Put

Line (treatment)	Put loss (nmol g^{-1} FW)		Put→Spd conversion (nmol g^{-1} FW) (% of total loss at 2 h)
	2 h	8 h	2 h
m*ODC*-10-1 (−E)	19.53	44.72	14.83 (76%)
m*ODC*-10-1 (+E)	50.84	173.28	19.48 (38%)

The seedlings were induced for 4 h prior to treatment with labeled Put.

systems (such as the conditional mutants) permit us to mimic gene expression for shorter periods, as it might occur in nature. While the primary goal of the present study was to better understand the regulation of PA metabolism, we discovered remarkable and unique aspects of the regulation of PA metabolism, especially in relation to the role of the substrate Orn and the rate of turnover of Put. Four important themes that we studied are discussed here briefly.

Diversion of Orn from the Glu → Orn → Arg pathway does not affect Arg production or its utilization in polyamine production

Since *A. thaliana* does not have its own *ODC* gene (Hanfrey et al. 2001), one can hypothesize that diversion of Orn from the Glu→Orn→Arg pathway would affect production of Put via ADC either by limiting its substrate (Arg) or via feedback inhibition of ADC by excess Put. However, our results clearly demonstrate that in the transgenic plants, up to a 12-fold increase in Put production from Orn by the transgenic mODC occurs without impacting its production from Arg by ADC. In fact, the production of Arg itself is not negatively impacted in spite of the removal of Orn in large amounts. This was demonstrated directly by the incorporation of [^{14}C]Arg and [^{14}C]Orn into Put following induction as well as from the direct measurement of Arg content. Moreover, it is evident that ADC activity is not feedback inhibited by even a >40-fold increase in cellular Put, under both constitutive and short-term (inducible) conditions. This is consistent with similar analysis of the HP poplar cells reported earlier (Bhatnagar et al. 2001, Bhatnagar et al. 2002).

The fact that the transgenic plants always had substantially greater amounts of Cad under inducible conditions than in the constitutive presence of high Put suggests that long-term homeostatic adjustments lead to equilibrium between the biosynthesis and the utilization of Orn in these plants. Furthermore, under conditions of short-term induction, Orn appears to become limiting for transgenic mODC, which then utilizes Lys as a substrate to produce Cad (despite it's high K_m for Lys); this is a well-known property of the mammalian ODC (Pegg and McGill 1979). It can also be argued that the pathway of Arg → Orn production (via arginase–urease activity; Slocum 2005) does not exist in Arabidopsis seedlings because the addition of exogenous Arg did not mimic the effects of exogenous Orn to affect either Orn incorporation into PAs or its cellular contents.

Diversion of Orn from the Glu → Orn → Arg pathway causes a compensatory increase in Orn production

At present we have little understanding of the regulation of Orn metabolism or its role in controlling the biosynthesis of PAs and other amino acids in plants, despite the fact that this question has attracted considerable attention in animals (Morris 2006, Morris 2007, Urschel et al. 2007). A part of the rationale may be that Orn is presumed simply to be an intermediate in the production of Arg and Put (and perhaps Pro) from Glu, with little regulatory function of its own. Ornithine is present in rather small amounts in Arabidopsis seedlings and in most plants (**Fig. 4E**; also see, Slocum 2005). In the present case, it quickly becomes limiting for meeting the demand of mODC in the transgenic cells, as is apparent from the observation that exogenous Orn stimulates Put accumulation far beyond that produced by induction alone. These observations imply that (i) Orn production (from Glu) is stimulated in response to its increased utilization by mODC; (ii) Glu is not limiting for Orn production; and (iii) its conversion into Arg (through Orn) continues to occur unabated. Glutamate is a well-known key intermediate in cellular N metabolism, serving as a substrate for many other amino acids and numerous other metabolites, including PAs (Forde and Lea 2007).

Based on the data presented here, we postulate a direct role for cellular Orn in regulating not only its own biosynthesis but also the entire pathway from Glu to Arg, including a vital role for regulating PA biosynthesis via both ODC and ADC routes. We contend that under certain conditions, cellular Orn levels could perhaps over-ride the control exerted by the PA biosynthetic enzymes. Furthermore, since the PA biosynthetic pathway is directly coupled to Glu utilization, it is not surprising that overconsumption of Orn in transgenic plants has major effects on the entire suite of soluble amino acids (see also Mohapatra et al. 2010b, Page et al. 2012). This would require stringent monitoring of Orn levels in the cells, and also a regulatory role for it in controlling the Glu → Orn → Arg and Glu → Orn/Arg → Put pathways in plants. The results indicate that similar mechanisms exist both under short-term induction and under the constitutive production of high Put.

Under conditions of its overconsumption in transgenic plants, what sensing mechanism is there to increase the biosynthesis of Orn (from Glu) is not yet understood. We believe that this non-protein amino acid itself is a part of the sensing as

well as the signal transduction mechanism to regulate its own cellular content (a situation analogous to that postulated for Arg in animals). Ornithine is synthesized from Glu by the action of several enzymes (Slocum 2005, Forde and Lea 2007, Kalamaki et al. 2009). The first, possibly a regulatory step in plants, seems to be *N*-acetyl-L-Glu synthase (NAGS). Constitutive overexpression of a tomato *NAGS1* in Arabidopsis led to higher accumulation of Orn and citrulline without a significant increase in Arg (Kalamaki et al. 2009). Unfortunately, no data on PAs were reported in this study. This is in contrast to the situation in animals where nutritional Arg is the primary source of Orn, and the sequence of reactions from Arg to Glu is reversed, the controlling enzyme for the pathway being arginase (Morris 2006, 2007). It can be envisaged that Orn could regulate the NAGS step either through interaction with the enzyme or through regulation of its transcription; however, the experimental evidence for either of these is not currently available.

The Orn/Arg → Put pathway affects the accumulation of many other amino acids

The observation that the induction of m*ODC* had no negative effect on cellular Arg, despite the fact that up to 20-fold more Orn was being siphoned into Put production, may indicate that Arg accumulation is driven more by its own content (as in animals) than by the availability of its substrate. It is, however, also consistent with the possibility that this step is regulated by Orn. This would happen only if a reduced Orn level increased its own production, which ensures its availability for Arg production.

Proline biosynthesis is also closely linked to the Glu–Orn–Arg pathway in more than one way. For example, its content typically changes parallel to changes in cellular PAs under conditions of abiotic stress (Jouve et al. 2004, Cvikrová et al. 2012). In the present study, Pro significantly increased in response to induction of m*ODC*, even though an exogenous stress was not applied. This suggests that Orn production keeps pace with its consumption not only for Put and Arg production but also for potential use in Pro biosynthesis. Our hypothesis would suggest that lowering of the cellular Orn level up-regulates the entire Glu → Orn pathway, thus causing all its products to be increased until a homeostasis in its cellular level is re-established. The fact that Glu content was not reduced under conditions of its being highly diverted to Orn (and Put) production is consistent with a net increase in its biosynthesis via additional N assimilation by the seedlings (Majumdar 2011).

Further experimental evidence for a critical function of cellular Orn in regulating the Glu → Orn/Arg → Put/Pro flux comes from the observation that in the presence of exogenous Orn, induced as well as uninduced plants had a higher content of Glu. We argue that this increase in Glu could not have come from back-conversion of Orn into Glu because: (i) the amount of Orn supplied in the medium was too small; and (ii) most of the Orn in transgenic seedlings was being channeled towards the production of Put (corroborated by the data from incorporation of [^{14}C]Orn into PAs). Supplementation with exogenous Arg did not cause a similar effect, showing that this amino acid could not substitute for Orn, neither was it converted into Orn. This is consistent with our assertion, and other reports (McKay and Shargool 1977, Slocum 2005, Kalamaki et al. 2009), that the major source of Orn in rapidly growing plant cells (e.g. seedlings) is Glu and not Arg. The exact mechanism by which the cellular Orn concentration is monitored and the signal is transduced from that system to increased assimilation of N is presently a matter of conjecture. In order for Orn to regulate this entire pathway, there must be an Orn monitoring system in the cells. This argument supports our earlier assertion (Mohapatra et al. 2010b) that in contrast to animals, where the primary source of Orn is dietary Arg, in plants the major source for Orn production is Glu and not Arg. The argument for up-regulation of N assimilation by Orn is further supported by the increased production of Gln upon m*ODC* induction, and its reversal by exogenous Orn.

It is also known that GABA and Pro typically co-accumulate in response to cold, drought, salinity, hypoxia, hormonal changes and pH change, all of which also promote PA (particularly Put) accumulation (Sharma and Verslues 2010, and references therein). In the present study, an increase in GABA in the induced plants (plus or minus Orn) is explicable since it is a direct product of Put degradation, which is several fold higher in HP seedlings. Since GABA is also produced from Glu by GAD (Shelp et al. 2012), it is however not clear if the proportion of GABA being produced by GAD vs. by Put degradation (by DAO) is changing in the HP cells. This question can be addressed by using specific precursors of Glu labeled differentially with ^{15}N and ^{13}C (Ricoult et al. 2006, Fait et al. 2011). It appears that Orn may play a critical role in regulating not only the metabolism of PAs but also that of Glu, Pro and GABA.

Putrescine catabolism increases concomitant with its increased biosynthesis

Although the general pathways for PA catabolism are well described, their regulation is mostly speculative. Moreover, while Put breakdown occurs by similar enzymes in plants and animals (DAO via the GABA shunt), the enzymes involved in Spd and Spm catabolism in the two kingdoms are different (Moschou et al. 2008, Casero and Pegg 2009). Often in attempts to up-regulate PA production through genetic engineering, little attention is given to their catabolic rates, which perhaps play a significant role in determining cellular accumulation of a particular PA. We experimentally addressed the question of "What effects does up-regulation of Put have on its conversion into higher PAs and its breakdown?" Using two different sources of Put ([^{14}C]Orn and [^{14}C]Put) further enabled us to compare the catabolism of Put that was produced within the cell vs. that supplied in the medium from the outside.

The results presented here lead us to conclude that the conversion rate of Put into Spd and Spm is not proportionate to the cellular Put content; rather it is limited by other factors,

e.g. the availability of decarboxylated SAM (dcSAM, from SAMDC activity), the activities of aminotransferases (SPDS and SPMS), and their feedback regulation by end-products. This conclusion is consistent with the frequent observations that in response to growth, stress or genetic manipulation, where Put contents can vary widely (up to 50-fold as in the present case), Spd and Spm contents do not show proportionate increases (Bastola and Minocha 1995, Masgrau et al. 1997, Minocha et al. 1997, Minocha et al. 2000, Capell et al. 2004, Nölke et al. 2008, Minocha et al. 2010). The results are also similar to earlier studies with poplar cell cultures (Bhatnagar et al. 2002). Based on the results of the two studies in our lab with very dissimilar experimental systems (cell cultures of poplar, a woody plant, and whole seedlings of Arabidopsis; the former under conditions of nutrient sufficiency and the latter somewhat limited for C), it can be argued that Put catabolism may also be regulated by cellular Orn (see further discussion below).

With the objective of determining if exogenous Put was being catabolized differently compared with the pool of endogenously produced Put in the cells, we used two distinctive sources of Put to compare their turnover; both showed identical results of a rapid turnover rate ($T_{1/2}$) of about 6–8 h. While the calculated initial $T_{1/2}$ of Put was rather similar in the HP and the control plants, the total amount of Put being removed from the cells per hour in the former was 3–4 times greater than that in the latter. Despite the fact that the total loss of Put via catabolism was proportionate to its rate of production, such was not the case for its conversion into Spd, again supporting the argument that Spd production was independently regulated regardless of the abundance of Put. As regards to different pathways for Put loss, while the control plants predominantly lost it to Spd, the HP plants lost most of it through degradation.

Assuming that the rate of Put catabolism is dependent on cellular Put production and/or its concentration, a key question that remains unanswered is "what controls this higher Put turnover?" In poplar cell suspensions, we had found that increased Put catabolism was not accompanied by induction of DAO activity or its gene expression, indicating that this enzyme was not a rate-limiting factor (Bhatnagar et al. 2002, Page et al. 2012); the answer in Arabidopsis could be complex. Arabidopsis has eight putative DAO genes, all of which show weak expression in the seedlings, and are apparently triggered by abiotic stress (Shelp et al. 2012), which suggests that Put catabolism in nature may also respond to higher endogenous Put (as seen under abiotic stress conditions). Similar observations have been reported in olive, barley and soybean where increased DAO activity corresponded to higher Put or Cad content (Asthir et al. 2002, Gomez-Jimenez et al. 2010, Quinet et al. 2010, Campestre et al. 2011). Neither the activity of DAO nor the expression of various *DAO* genes was analyzed in our study. The most common ingredient in all these studies would be Orn, which, as discussed above, may also be involved in regulating the catabolism of Put.

The present study reveals additional information on the uptake of PAs in Arabidopsis, which is by and large poorly understood in plants. In *Escherichia coli* and yeast, apparently specific transporters mediate P uptake (Igarashi and Kashiwagi 2010). In mammalian cells, where the existence of a PA transporter is still debated, the uptake of PAs is stimulated by their low cellular content (Igarashi and Kashiwagi 2010). Studies on the uptake of PAs in plants have reported diverse PA transport mechanisms in different species (Kakkar et al. 1997, Theiss et al. 2004, Ohe et al. 2005, Mulangi et al. 2012). Recently, Mulangi et al. (2012) have identified a Spd-preferential transporter in rice whose heterologous expression in yeast showed that higher cytoplasmic Spd down-regulated its activity. The authors speculated that the feedback regulation was due to a conformational change in the transporter upon binding to Spd. Our results suggest that high Put in the induced as well as the constitutive m*ODC* plants had little effect on the uptake of [^{14}C]Put, [^{14}C]Arg or [^{14}C]Orn (and also [^{14}C]Spd and [^{14}C]Spm; L. Shao, unpublished data). This is similar to the results with poplar suspension cultures where Put uptake in HP and control cell lines was quite similar (Bhatnagar et al. 2002).

Conclusions

The present study demonstrates a potential key role for cellular Orn in regulating Put biosynthesis (and possibly also its catabolism), and reveals a complex but coordinated mechanism of regulation of the Glu–Orn–Arg–Pro–GABA subset of pathways. The coordinated regulation of these pathways is quite consistent with changes in these metabolites during development, stress responses, as well as genetic manipulations of their pathways in plants. Two additional revelations of this study are that: (i) Orn not only regulates its own biosynthesis but it also regulates the biosynthesis and accumulation of Glu in the cells, and (ii) the production of Put via ADC is not subject to any feedback inhibition. These conclusions together should help us design metabolic engineering strategies via transgenic expression of the PA biosynthetic genes to achieve optimal carbon and nitrogen assimilation in plants for use in increased biomass production and to achieve abiotic stress tolerance in plants.

Materials and Methods

Generation of inducible and constitutive m*ODC* plants

A 1,281 bp sequence including the open reading frame (423 amino acids, excluding the C-terminal PEST region) of the m*ODC* cDNA (NM_013614) was PCR amplified from the plasmid pCW122-*ODC* (Bhatnagar et al. 2001) using primers: F5′-CACCATGAGCAGCTTTACTAAGGA-3′ and R5′-CTACTACATGGCTCTGGA-3′. The amplicon, initially cloned into pENTR™/D-TOPO® vector (Invitrogen), was subsequently transferred into the estradiol-inducible Gateway-compatible pMDC7 destination vector (Curtis and Grossniklaus 2003) using the LR clonase reaction (Invitrogen). For constitutive expression, the

m*ODC* gene was amplified using primers F5′-GAACCATGGGC AGCTTTAC-3′ and R5′-CTACTACATGGCTCTGGA-3′ and cloned into pMDC32 containing the $2 \times 35S$ *Cauliflower mosaic virus* (CaMV) promoter via the pCR8.0/GW/TOPO vector. The final recombinant vectors are shown in **Fig. 2**. *Arabidopsis thaliana* (Columbia-0) plants were transformed with *Agrobacterium tumefaciens* (strain GV3101) containing the recombinant plasmids using the floral dip method (Clough and Bent 1998). Five to eight independent T_2 transgenic lines were selected that had a single insertion of the m*ODC* gene (tested by segregation analysis on hygromycin) and showed a significantly higher amount of Put under inducible or constitutive conditions as compared with the WT plants. These lines were grown to obtain T_3 generation seeds. Selected independent homozygous T_3 or greater lines were used for all experiments.

Induction of m*ODC*, PA treatments and sample collections

Arabidopsis seedlings were grown at 25 ± 1°C under a 12 h photoperiod (80 ± 10 μE m^{-2} s^{-1}) on solid germination medium (GM) containing Murashige and Skoog (1962) salts, 0.5 g l^{-1} MES, 1 g l^{-1} sucrose and 0.8% type A agar. For transgene induction, batches of 12- or 14-day-old seedlings were transferred into liquid GM in 12-well plates (8–12 seedlings in 1 ml of medium). A final concentration of 5.0 μM 17β-estradiol (Sigma/Aldrich) in dimethylsulfoxide (DMSO) was used for induction in the liquid medium. Other additives were added and samples collected as per the details provided in the Results or in the figure legends. To study the effects of exogenous PAs, WT seeds were germinated for 7 d in solid GM containing 1.0 mM Put or Spd or 0.5 mM Spm. For PA and amino acid analyses, seedlings were collected in 5% PCA (100 mg FW tissue in 900 μl of PCA) and processed according to Minocha et al. (1994) and Minocha and Long (2004).

Incorporation of labeled precursors and putrescine catabolism

For L-[U-^{14}C]Orn and L-[U-^{14}C]Arg incorporation experiments (**Fig. 5**), 14-day-old inducible m*ODC* transgenic seedlings grown on solid GM were transferred into 10 ml of liquid GM in 50 ml conical flasks, and 5 μM estradiol (final concentration) was added for induction. The flasks were incubated at 25 ± 1°C on a gyratory shaker at 150 r.p.m. Two hours later, 0.5 μCi of either L-[U-^{14}C]Orn (specific activity 257 mCi $mmol^{-1}$, Amersham Pharmacia Biotech) or L-[U-^{14}C]Arg (specific activity 272 mCi $mmol^{-1}$, Moravek Biochemicals) along with 0.1 mM (final concentration) of cold Orn or Arg were added to each flask and incubated for 8 h. Seedlings were washed three times with either 2.0 mM cold Orn or Arg, followed by three consecutive washes with de-ionized water. About 200 mg FW of tissue samples (12–14 seedlings) were collected in 500 μl of 5% PCA at various times (as indicated in the figure legends) in triplicate and stored at −20°C. Following three cycles of freezing and thawing, the samples were dansylated (Bhatnagar et al. 2001) and partitioned into toluene. An aliquot of the PCA extract, the toluene fraction (containing all dansylated PAs) and the aqueous phase (containing amino acids and other charged by-products) were counted for radioactivity in 10 ml of Scintilene (Thermo-Fisher) in a LSC-6000 liquid scintillation counter (Beckman). The amount of radioactivity in each fraction was expressed as d.p.m. g^{-1} FW. Different numbers of seedlings were used in different experiments in order to achieve the desired FW per sample.

For Put turnover experiments, seeds (T_3 or T_4) of $2 \times 35S$::m*ODC*-1-7 (constitutive expression) and estradiol-inducible m*ODC*-10-1 lines were germinated on solid GM for 2 weeks. Seedlings (~2 g FW) from three Petri dishes for each line were transferred into 15 ml of liquid GM in 250 ml beakers separately. Induction was performed as described above for the inducible line; the uninduced seedlings served as control. The beakers were covered with aluminum foil and kept on a shaker at 90 r.p.m. After 2–8 h of induction (see the figure legends for specifics), 1.0 μCi of L-[U-^{14}C]Orn-HCl or 1.0 μCi of [1, 4-^{14}C]Put-diHCl (specific activity 107 mCi $mmol^{-1}$; Amersham) and an additional 5 ml of GM (with/without estradiol) were added to each beaker. Following 4 h incubation, seedlings were washed with 200 ml of GM with or without estradiol three times, transferred into 9-well culture plates and incubated under normal growth conditions. About 300 mg of tissue samples were collected in 500 μl of 7.5% PCA at different times (0, 2, 4, 8, 22/24 and 46/48 h), and frozen at −20°C for PA analysis. The experiment was repeated twice, each time with three replicates. While the two experiments gave somewhat different absolute numbers, the trends of changes were quite similar and the results from only one experiment are presented here.

After three cycles of freezing and thawing, the samples were dansylated as per Bhatnagar et al. (2001, 2002). The toluene fraction (contains all dansyl-PAs) and the aqueous fraction (contains amino acids and other charged radioactive products) were counted separately for radioactivity. The three independently dansylated (biological replicates) samples of the toluene fraction from the same time and treatment were combined, dried in the Speed-Vac and dissolved in 90 μl of methanol. Following a recount of an aliquot of the methanol fraction, 60 μl of methanol extract was spotted on TLC plates (LK6D silica gel 60; Whatman Inc.). The plates were developed in a solvent mix of chloroform : triethylamine (5 : 1, v/v) for 45 min in a chromatography chamber (Bhatnagar et al. 2002). The respective PA bands were marked under UV light, scraped, and counted for radioactivity in 10 ml of Scintiverse. The information obtained, along with the actual amounts of PAs determined in the same samples by HPLC, was used to calculate the amount of catabolized Put.

Statistical analyses

The data were typically analyzed by Student's *t*-test using SigmaStat software, version 3.1. Significance was assigned at

$P \leq 0.05$. Each experiment was repeated at least twice (with three or four replicate samples; for each replicate, several seedlings were pooled to achieve the desired FW) and data from a single representative experiment are presented here in each case.

Supplementary data

Supplementary data are available at PCP online.

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