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ORIGINAL ARTICLE

Putrescine overproduction does not affect the catabolism of spermidine and spermine in poplar and Arabidopsis

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Abstract The effect of up-regulation of putrescine (Put) production by genetic manipulation on the turnover of spermidine (Spd) and spermine (Spm) was investigated in transgenic cells of poplar (*Populus nigra* × *maximowiczii*) and seedlings of *Arabidopsis thaliana*. Several-fold increase in Put production was achieved by expressing a mouse *ornithine decarboxylase* cDNA either under the control of a constitutive (in poplar) or an inducible (in Arabidopsis) promoter. The transgenic poplar cells produced and accumulated 8–10 times higher amounts of Put than the non-transgenic cells, whereas the Arabidopsis seedlings accumulated up to 40-fold higher amounts of Put; however, in neither case the cellular Spd or Spm increased consistently. The rate of Spd and Spm catabolism and the half-life of cellular Spd and Spm were measured by pulse-chase experiments using [^{14}C]Spd or [^{14}C]Spm. Spermidine half-life was calculated to be about 22–32 h in poplar and 52–56 h in Arabidopsis. The half-life of cellular Spm was calculated to be approximately 24 h in Arabidopsis and 36–48 h in poplar. Both species were able to convert Spd to Spm and Put, and Spm to Spd and Put. The rates of Spd and Spm catabolism in both species were several-fold slower than those of Put, and the overproduction of Put had only a small effect on the overall rates of turnover of Spd or Spm. There was little effect on the rates of Spd to Spm conversion as well as the conversion of Spm into lower polyamines. While Spm was mainly converted back to Spd and not terminally degraded, Spd was removed from the cells largely through terminal catabolism in both species.

Keywords Arabidopsis · Poplar · Polyamines · Polyamine oxidase · Catabolism · Putrescine · Spermidine · Spermine · Turnover · Uptake

Introduction

Cellular contents of soluble polyamines (PAs) at a given time reflect a balance of their biosynthesis, degradation, transport and conjugation. However, the regulation of these processes is poorly understood (Cohen 1998; Handa and Mattoo 2010; Igarashi and Kashiwagi 2010). An important piece of information often lacking in most studies is about the fraction of total cellular PAs that are physiologically active in a cell, i.e. available for degradation, transport and conjugation. In animals, spermine (Spm) is easily converted into spermidine (Spd), and Spd into putrescine (Put), via acetylation by Spd/Spm N^1-acetyl-transferases (SSATs; EC 2.3.1.57) followed by oxidation via PA oxidases

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(PAOs; e.g. spermine oxidase-EC 1.5.3.16).[1] It is known that the expression of SSAT genes is subject to regulation by PAs (Casero and Pegg 2009); and the acetyl-PA pathway in animals is believed to serve both as a means of regulating cellular Spd and Spm levels, and a source of Put under conditions of its depletion (Tavladoraki et al. 2011 and references therein). The occurrence of acetylated PAs is rare or non-existent in plants (Moschou et al. 2008a, b). The plants, on the other hand, catabolize Spd and Spm using two groups of PAOs (EC 1.5.3.3 and 1.5.3.16): one that has apoplastic localization and the other which is localized in the cytoplasm or the peroxisomes (Moschou et al. 2008a, b, 2012). The former brings about terminal catabolism by converting Spd and Spm into 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, which in turn cyclise into $^{\Delta}$1-pyrroline and 1,5-diazabicyclononane, respectively (Fig. 1). The additional byproducts of the reaction are H_2O_2 and 1,3-diaminopropane. The second group of plant PAOs, which are present either in the cytoplasm or in the peroxisome, catalyze the back-conversion of Spm into Spd and/or Spd into Put. These enzymes appear to catabolize Spd and Spm with equal efficiency and they utilize flavin adenine nucleotide as a co-factor. They resemble the mammalian PAOs in function except that their substrates are typically non-acetylated PAs.

Other than intercellular and inter-organ transport, a significant proportion of PAs in plant cells can be channeled away from physiological activity by conjugation with soluble phenolic acids or with macromolecules (Grienenberger et al. 2009; Fellenberg et al. 2012). However, not much is known about the actual transporters and their substrate specificities (Mulangi et al. 2012a, b). Likewise, the enzymes involved, their specificities, and the driving forces that determine PA conjugation have not been characterized.

It is commonly observed that the cellular levels of Spd and Spm in plants are maintained within a narrower range than those of Put. This conclusion comes from studies (1) where cellular Put was enhanced via genetic manipulation by transgenic expression of either ornithine (Orn) decarboxylase (ODC) or arginine (Arg) decarboxylase (ADC) coding sequences (DeScenzo and Minocha 1993; Bastola and Minocha 1995; Bhatnagar et al. 2001, 2002; Nölke et al. 2008; Alet et al. 2011; Majumdar et al. 2013); (2) where Put levels increased in response to abiotic stress (Wargo et al. 2002; Prabhavathi and Rajam 2007; Alet et al. 2011); and (3) where attempts were made to genetically manipulate Spd and Spm levels directly by transgenic expression of either *S*-adenosylmethionine decarboxylase (SAMDC) or a Spd synthase (SPDS) coding sequence (Noh and Minocha 1994; Thu-Hang et al. 2002; Franceschetti et al. 2004; Kasukabe et al. 2004; Wi et al. 2006; Wen et al. 2008; Nambeesan et al. 2010; Fu et al. 2011). In all cases, only small (<fivefold) increases in Spd and/or Spm were seen vs. as much as 10- to 50-fold increases in Put (e.g. Majumdar et al. 2013). It is also apparent from the literature that the cellular content of Spm is quite low in plants, leading to speculation that catabolism of Spd, rather than its conversion into Spm, may be a major means of regulating its homeostasis. Whereas the metabolic pathways of Spd and Spm catabolism are reasonably well characterized, their regulation is poorly understood.

In earlier studies on Put turnover in non-transgenic (NT or Wild type, WT) and transgenic cells of poplar (*Populus nigra* × *maximowiczii*-Bhatnagar et al. 2002) and seedlings of *Arabidopsis thaliana* (Majumdar et al. 2013) that overexpressed a mouse ODC (m*ODC*) coding sequence, we demonstrated that Put loss was proportionately higher in the high Put producing (HP) cells/plants than their NT/WT counterparts. In Arabidopsis, the results were similar whether the transgene was expressed constitutively or under the control of an inducible promoter (Majumdar et al. 2013). The half-life ($T_{1/2}$) of Put turnover in the NT as well as the HP cells/plants in both species was calculated to be 6–8 h, with >70 % of it being terminally degraded (vs. converted to Spd or transported out of the cells). It was further seen that increased catabolism of Put was neither accompanied by concomitant increase in the activity of diamine oxidase (DAO-the enzyme primarily responsible for Put catabolism) nor the expression of its gene (Bhatnagar et al. 2002; Page et al. 2007). The cellular Spd contents in both species showed little correlation with their Put contents, indicating that Spd production in the HP cells/plants in these species was neither limited by nor regulated by the substrate Put. This is in contrast to the role

[1] The enzymes commonly called polyamine oxidases, which oxidize Put, Spd, Spm, acetyl-Spd and acetyl-Spm, have been assigned different EC numbers depending upon the source or characterization of the substrate preference; the terminology sometimes appears confusing. A few examples of the confusion about nomenclature of different polyamine oxidases are cited here: Spermine oxidase (EC 1.5.3.16), http://www.brenda-enzymes.org/php/result_flat.php4?ecno=1.5.3.16; Polyamine oxidase (EC 1.5.3.3), http://www.ebi.ac.uk/intenz/query?cmd=SearchEC&ec=1.5.3.3; AtPAO1 (EC# 1.5.3.16), http://www.uniprot.org/uniprot/Q9FNA2; AtPAO2 (EC# 1.5.3.?), http://www.uniprot.org/uniprot/Q9SKX5, http://enzyme.expasy.org/EC/1.5.3; AtPAO3 (EC# 1.5.3.17), http://www.uniprot.org/uniprot/Q9LYT1; AtPAO4 (EC# 1.5.3.16), http://www.uniprot.org/uniprot/Q8H191; AtPAO5 ((EC# 1.5.3.?), http://www.uniprot.org/uniprot/Q9SU79, http://enzyme.expasy.org/EC/1.5.3; ZmPAO1 (EC# 1.5.3.14/1.5.3.15?), http://www.ncbi.nlm.nih.gov/nuccore/NM_001111636; Non-specific polyamine oxidase (EC 1.5.3.17), http://enzyme.expasy.org/EC/1.5.3.17; *N*8-acetylspermidine oxidase, http://www.brenda-enzymes.org/php/result_flat.php4?ecno=1.5.3.15; *N*(1)-acetylpolyamine oxidase (EC 1.5.3.13), http://enzyme.expasy.org/EC/1.5.3.13; *N*-acetylpolyamine oxidase EC 1.5.3.11 (deleted entry), http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/5/3/11.html, http://www.brenda-enzymes.org/php/result_flat.php4?ecno=1.5.3.11.

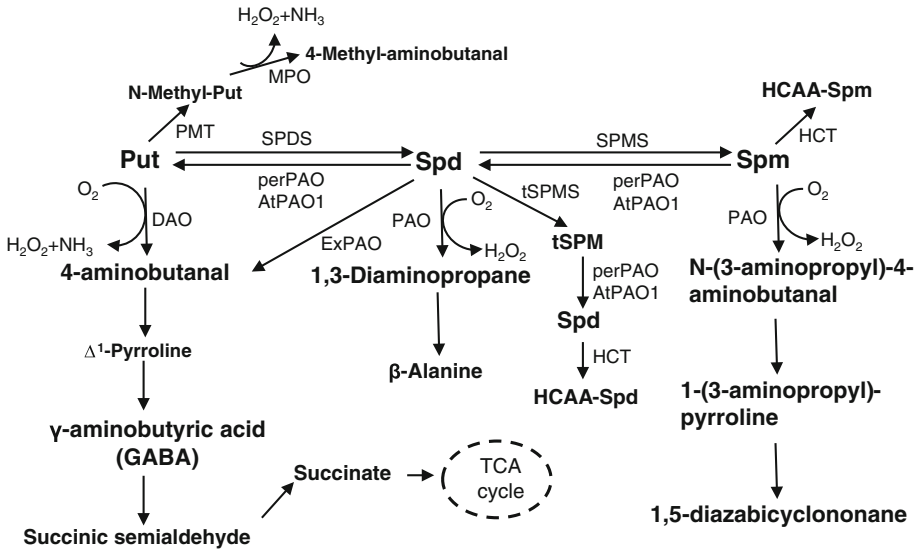

Fig. 1 The metabolic fate of putrescine, spermidine and spermine in plants. *DAO* diamine oxidase, *HCAA* hydroxycinnamic acid amide, *HCT* hydroxycinnamoyl transferase, *MPO* *N*-methyl-putrescine oxidase, *PAO* polyamine oxidase (*per* peroxisomal, *ex* extracellular); *Spd* spermidine, *Spm* spermine, *PMT* putrescine *N*-methyl transferase, *Put* putrescine, *SPDS* spermidine synthase, *SPMS* spermine synthase, *tSpm* thermospermine, *tSPMS* thermospermine synthase (modified from Moschou et al. 2012)

of Orn in Put production in the HP cells during early stages of up-regulation of Put biosynthesis (Majumdar et al. 2013). Although a competition for SAM between PA biosynthesis and ethylene production has been discussed (Minocha 1988; Quan et al. 2002), and more recently demonstrated in tomato (Gupta et al. 2013), studies with HP and control poplar cells had shown little or no evidence of competition between these two pathways. This is indicative of ample supply of dcSAM (Quan et al. 2002), which is the co-substrate (along with Put) for Spd and Spm biosynthesis. In the study of Gupta et al. (2013), inhibition of ACC synthase genes by RNAi-mediated silencing led to increased PA accumulation in the transgenic fruits presumably via altered SAM allocation for PA and ethylene biosynthesis. The explanation for the lack of increase in Spd in the HP poplar cells could then be either the enzyme SPDS and/or SAMDC being limiting or Spd catabolism being increased concomitant with its increased biosynthesis (Bhatnagar et al. 2001; Page et al. 2007). A similar argument can be made for the lack of increase in Spm in the HP poplar and Arabidopsis. In these studies, the transport to other organs would not be important because of the experimental design, which involved the submergence of all cells/plants in the growth medium.

The present study was aimed at analyzing the effects of increased Put production, in the ODC-transgenic poplar cells and Arabidopsis seedlings, on the catabolism of Spd and Spm. The specific questions being addressed here were: (a) Does increased accumulation of Put affect Spd and Spm uptake and their catabolism? (b) Are the poplar cells and Arabidopsis plants capable of back converting Spd into Put and/or Spm into Spd? (c) Are Spd and Spm catabolized differently in whole plants of Arabidopsis vs. cell cultures of poplar? In addition to answering the above questions, the results presented here provide further insight into the turnover rates and the flux of Put, Spd and Spm into various sub-pathways in plant cells.

Materials and methods

Poplar cell cultures

The two poplar cell lines used in the present study, non-transgenic (NT) and (transgenic, a.k.a. high putrescine-HP) have been described previously; the latter expresses a m*ODC* coding sequence under the control of a 2× 35S CaMV promoter (Bhatnagar et al. 2001, 2002). The original sequence of Kahana and Nathans (1985) was used as the source of amplification. The cloned sequence lacks both the 5′ and 3′ UTRs, and also the C-terminus PEST region, which is involved in a rapid turnover of this enzyme. More details about the processing of this construct and the production of poplar cell lines used here are given in Bhatnagar et al. (2001).

The cultures were maintained as suspension in MS (Murashige and Skoog 1962) medium at a weekly subculture regimen involving the transfer of 7 mL of 7-day-old cells into 50 mL fresh medium. Longer-term callus cultures were maintained on solid medium at 4–5 week intervals. For experimental setup, at 3 days of incubation in the fresh medium, about half of the medium was decanted and to the remaining suspension (about 25 mL volume), 1.0 μCi of [1–4, ^{14}C]Spd.triHCl (specific activity 112 mCi mmol^{-1}, Amersham Life Science, Elk Grove, IL, USA) or [1–4, ^{14}C]Spm.tetraHCl (specific activity 110 mCi mmol^{-1}, Amersham) was added. Following 2 h incubation on a shaker, the cells were washed with label-free MS medium by centrifugation (1,000 rpm for 2 min) and re-suspended in 50 mL of fresh MS medium in

125 mL Erlenmeyer flasks. The flasks were placed back on the shaker and samples were collected at different times, e.g. 0, 2, 4, 8, 24 and 72 h. For sample collection, the suspension was vacuum-filtered through Miracloth and the cells washed 3× with label-free and sucrose-free medium. Total cell mass was determined, and known amounts of cells were mixed with 7.5 % perchloric acid (0.5 g FW cells in 1.0 mL of PCA). The samples were frozen at −20 °C for up to 4 weeks before PA analysis.

Arabidopsis thaliana plants

The same m*ODC* cDNA that was used for poplar transformation (Bhatnagar et al. 2001) was PCR amplified and initially cloned into pENTR™/D-TOPO® vector (Invitrogen, Carlsbad, CA, USA). It was subsequently transferred into estradiol-inducible Gateway-compatible pMDC7 destination vector (see Majumdar et al. 2013) using LR clonase reaction (Invitrogen, Carlsbad, CA, USA). The resultant pMDC7 vector with the m*ODC* coding sequence was used to transform wild-type Arabidopsis plants to obtain inducible m*ODC* transgenic lines (Majumdar et al. 2013). Third or fourth generation (homozygous for the m*ODC* gene) seedlings (∼2.0 g FW) of estradiol-inducible m*ODC*-10-1 line, grown on germination medium (GM) for 2 weeks at 21 °C under 18 h photoperiod (Majumdar et al. 2013), were transferred into 15 mL of liquid GM in 250-mL beakers. For induction, 5.0 µM (final concentration) of 17β-estradiol (made in DMSO) was added to each beaker; the same line without estradiol served as the control. The beakers were covered with aluminum foil and kept on a shaker at 90 rpm. After 8 h of induction, 1.0 µCi of either [1–4, ^{14}C]Spd.triHCl or [1–4, ^{14}C]Spm.tetraHCl and additional 5.0 mL of GM (with/without 5.0 µM estradiol) were added into each beaker which was placed back on the shaker. Following 4 h of additional incubation (induction), seedlings were washed with 200 mL of non-radioactive GM with/without estradiol three times, transferred into 9-well cell culture plates with 2.0 mL GM (with/without estradiol), and placed back under normal growth conditions without shaking. About 300 mg of tissue samples were collected in 500 µL of 7.5 % PCA at different times and frozen at −20 °C for PA analysis. A parallel set of treatments was run without radioisotope for analysis of total PAs.

Analysis of free polyamines

The protocol used for determination of Spd and Spm turnover was similar to that described for determination of Put turnover earlier (Bhatnagar et al. 2002; Majumdar et al. 2013). Samples in PCA were frozen and thawed three times, vortexed for 1 min and centrifuged at 14,000×*g* for 10 min. For poplar cells, 1.0 mL of the PCA extract was dansylated according to Bhatnagar et al. (2002) in glass Reactivials (Kontes, Vineland, NJ, USA). Following dansylation, the dansyl-PAs were partitioned into 1.0 mL of toluene, from which an 800 µL aliquot was transferred to a new microfuge tube and dried in a Speed-Vac. The residue containing dansyl-PAs was dissolved in 50 µL of 100 % methanol. For Arabidopsis, the procedure described by Minocha and Long (2004) as modified by Majumdar et al. (2013) was used. In microfuge tubes, 200 µL of PCA extract was mixed with 200 µL of saturated Na_2CO_3 and 200 µL of dansyl-chloride (20 mg/mL in acetone) and incubated at 60 °C. After 80 min, 100 µL of 20 mg/mL asparagine was added and the tubes were incubated again at 60 °C for 30 min. Acetone in the samples was evaporated in a Speed-Vac, and the dansyl-PAs were extracted in 400 µL of toluene by centrifugation. Toluene fractions of triplicates from the same time and treatment were combined, dried under vacuum and then re-dissolved in 90 µL of methanol. An aliquot of 20 µL from PCA extract, toluene fraction and aqueous fraction of each replicate was counted for radioactivity separately. A 40 µL methanol sample for poplar cells or 60 µL for Arabidopsis was spotted on TLC plates (Whatman LK6D silica gel 60; Whatman Inc., Clifton, NJ, USA). The plates were developed in a solvent mix of chloroform:triethylamine (5:1, v/v). The respective PA bands were marked under UV light, scraped, and counted for radioactivity. The information obtained, along with the actual amounts of PAs determined in the cells by HPLC (Minocha et al. 1994; Minocha and Long 2004) was used to calculate the amount (nmol g^{-1}FW) of each PA lost from the cells within a certain time period as described in Bhatnagar et al. (2002) and Majumdar et al. (2013).

Calculations and statistical analysis

The formulae used for calculations were:

Loss of Spd/Spm over X h = [(dpm in Spd/Spm at 0 h−dpm in Spd/Spm at X h)/dpm in Spd/Spm at 0 h] × Mean amounts of Spd/Spm during 0–X h.

Amount of Spd converted into Put in X h = [(dpm in Put at X h−dpm in Put at 0 h)/Mean dpm in Spd at 0–X h] × Mean amounts of Spd during 0–X h.

Amount of Spd converted into Spm in X h = [(dpm in Spm at X h−dpm in Spm at 0 h)/Mean dpm in Spd at 0–X h] × Mean amounts of Spd during 0–X h.

Amount of Spm converted into Spd in X h = [(dpm in Spd at X h−dpm in Spd at 0 h)/Mean dpm in Spm at 0–X h] × Mean amounts of Spm during 0–X h.

Each treatment included at least three replicates and each experiment was done at least three times for poplar cells. Combined data from three experiments (total nine

replicates) are presented here except where specified. Experiments with Arabidopsis were repeated twice, each with three replicates (until the last step for radioactive Spd and Spm, where due to low ^{14}C counts, the three toluene fractions were combined into one sample), and data from one representative set of experiments are presented. The data were subjected to one-way analysis of variance using Systat version 9. Student's t test was used to determine significance at $p \leq 0.05$. The statistical comparisons were usually made between control (NT for poplar and uninduced for Arabidopsis) and HP cells of poplar and induced seedlings of Arabidopsis only at a given time, except when stated otherwise. Half-lives ($T_{1/2}$) of Spd and Spm were calculated by plotting the radioactivity data at different times of collection and performing regression of the slope.

Results

The metabolic status of poplar cells and Arabidopsis seedlings

Important biochemical and molecular properties of the transgenic poplar cells used here have been reported earlier (Bhatnagar et al. 2001; Mohapatra et al. 2009, 2010a, b; Page et al. 2007, 2012); the most relevant ones to this study are that the HP cells produced several-fold (as much as tenfold) higher amounts of Put, had 20–50 % increase in Spd and no significant difference in their Spm contents. Their overall growth rates during the one-week culture period were quite similar. On induction, the transgenic Arabidopsis seedlings produced as much as a 40-fold increase in Put, again with only small changes in Spd and Spm. The growth rates of the induced and the uninduced seedlings were also comparable during the experimental period (Majumdar et al. 2013).

Spermidine turnover in poplar and Arabidopsis

Following the incubation of samples with [^{14}C]Spd for 2 h (poplar cells) or 4 h (Arabidopsis seedlings), washing with label-free medium, and transferring them to label-free fresh medium, samples were collected at different time points for analysis. The tissues were analyzed for the contents of individual [^{14}C]PAs by TLC, and in parallel experiments (in the absence of radioisotopes) for total PAs by HPLC. These two measurements were used to calculate the specific activities (dpm ^{14}C-PA nmol total PA) of the three PAs in the cells; from this information we estimated the actual amounts of Spd conversion into other PAs. The differences in cellular contents of each of the three common PAs between the NT and the HP cells of poplar were consistent with the earlier reports on these cell lines. The HP cells at all times of analysis had >eightfold more Put and about up to 50 % more Spd; their Spm contents were similar or slightly lower than the NT cell (data not presented).

Total radioactivity in the PCA extracts (0 time following incubation in ^{14}C-Spd—this value represents the uptake of ^{14}C-Spd), the toluene fractions (representing all PAs) and the aqueous fractions (representing amino acids and charged catabolic products of ^{14}C-Spd) were similar at most times in the respective control and the induced Arabidopsis plants (Fig. 2). A clear trend of decline in radioactivity with time was observed in both the PCA (Fig. 2c) and the toluene (Fig. 2a) fractions while an increase was seen in the aqueous fraction (Fig. 2b) after 24 h; the latter indicates a steady accumulation of non-PA catabolic products of ^{14}C-Spd in the cells. The radioactivity lost from the PCA extract with time was the sum of radioactivity that leaked out and/or was incorporated into insoluble pellet (perhaps conjugated and bound). In poplar cells, the radioactivity accumulated from [^{14}C]Spd in the toluene fraction was always higher (sometimes up to twofold) in the NT cells than the HP cells; however, differences were not always significant ($p \leq 0.05$) (Fig. 2d, e). Higher amounts of radioactivity at time zero in the toluene fraction of NT cells as compared to HP cells represented higher [^{14}C]Spd uptake in the former. There was a decline with time in radioactivity associated with the toluene fraction in both cell lines; the decline was not statistically significant up to 4 h for either. During the 72-h period of study, the pattern of loss of radioactivity in this fraction was comparable in the two cell lines. The counts in the aqueous fraction showed no significant trend with time.

Radioactivity present specifically in Spd in the control and the induced Arabidopsis plants was similar at all times (Fig. 3a, b), and both showed a decline after transfer of plants to label-free medium with about 50 % loss ($T_{1/2}$) occurring around 50 h (Fig. 4). Likewise, no difference in [^{14}C]Spm made from ^{14}C-Spd was observed between the two genotypes; however, the absolute dpm present in Spm were several-fold lower than those in Spd (Fig. 3b, d). While the radioactivity in Put did not differ much between the induced and the uninduced plants or with time during the first 24 h, by 48 and 72 h the induced plants had several-fold higher [^{14}C]Put in them (Fig. 3c). These plants also had >40-fold higher amounts of total Put being produced by m*ODC*, which would cause a dilution of [^{14}C]-Put catabolism. In contrast to Arabidopsis, the counts in each of the three PAs were higher in the poplar NT cells than the HP cells at all times of analysis (Fig. 3e–h). The [^{14}C]Spd fraction declined in both cell lines with time (Fig. 3e, f); the kinetics of losses being different in the two. The initial losses in the NT cells (up to 24 h) were faster than in the HP cells. While [^{14}C]Put increased slightly

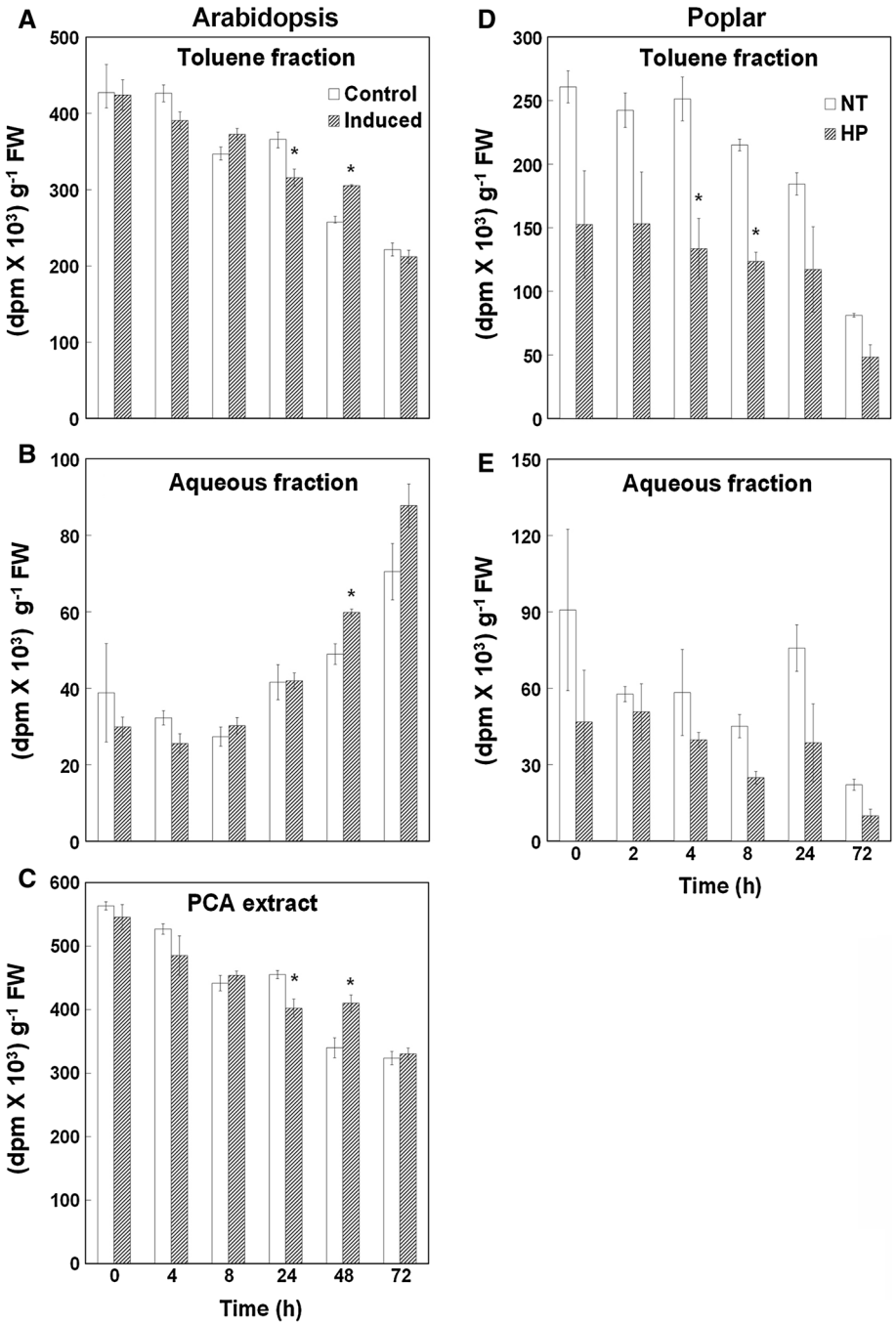

Fig. 2 Amount of radioactivity from [^{14}C]Spd present in the toluene fraction (**a**, **d**), the aqueous fraction (**b**, **e**) and PCA extract (**c**) in 2-week-old Arabidopsis seedlings and 3-day-old poplar cells at different times following transfer from [^{14}C]Spd to label-free medium. The Arabidopsis seedlings were incubated with [^{14}C]Spd for 4 h and poplar cells for 2 h and transferred to label-free medium after washing, and collected for analysis at different time periods. Data are mean ± SE of $N = 3$ (for Arabidopsis) and $N = 9$ for polar. An *asterisk* indicates that the values in the two cell lines/seedlings within the same species are significantly different ($p \leq 0.05$) at a given time

within 2 h of transfer to label-free medium, it remained largely unchanged thereafter in both cell lines (Fig. 3g). On the other hand, [^{14}C]Spm showed a substantial increase until 8 h, its levels also declined significantly between 24 and 72 h (Fig. 3h). Although the trends for the HP and the NT cells were similar, the amounts of [^{14}C]Spm made from ^{14}C-Spd in the HP cells was always less than half of that in the NT cells.

Linear regression was done on first 48 h data for Arabidopsis (Fig. 3b) and 24 h data for poplar cells (Fig. 3f) in order to determine the $T_{1/2}$ (i.e. loss of 50 % radioactivity) of [^{14}C]Spd, as described in Bhatnagar et al. (2002). The calculated $T_{1/2}$ of Spd was 52 h for the control and about 56 h for the induced Arabidopsis plants (Fig. 4). On the other hand, the calculated $T_{1/2}$ of [^{14}C]Spd in the poplar NT and HP cells was about 22 and 32 h, respectively (Fig. 4). It should be pointed out here that the calculated values for $T_{1/2}$ of Spd are based primarily upon the loss of radioactivity and include a sum total of losses due to secretion into the medium, conversion into Spm and Put, as well as its terminal catabolism. For poplar, the amount of [^{14}C]Spd incorporated into the insoluble (conjugated) fraction was

generally <1 %; for Arabidopsis this fraction was not determined.

Spermine turnover in Arabidopsis seedlings and poplar cells

The turnover of [^{14}C]Spm in Arabidopsis seedlings, followed a trend very similar to that for Spd. The uptake of [^{14}C]Spm (Fig. 5a) and the radioactivity in the toluene and the aqueous fractions were comparable in the induced and the control Arabidopsis plants at all times of analysis. While radioactivity in the toluene fraction decreased with time (Fig. 5b), the aqueous fraction showed an increase with time (Fig. 5c), showing the accumulation of charged metabolic products. At time zero, the aqueous fraction only contained a small proportion (~5 %) of the total radioactivity; this fraction increased to a maximum of 20 % by 72 h (Fig. 5c). In contrast to the uptake of [^{14}C]Spd, the uptake of [^{14}C]Spm (total counts in the PCA extract and the toluene fraction) in poplar cells were significantly higher in the HP as compared to the NT poplar cells (Fig. 4d, e). There was little loss of radioactivity with time from the cells as revealed by the total extractable counts at various times over the 72-h period of incubation in the label-free medium. The digested pellet (representing PCA-insoluble a.k.a. 'bound' products of labeled Spm) contained ≤2 % of the total radioactivity at any time of analysis in both poplar cell lines (data not shown); similar analyses were not done in Arabidopsis. Following dansylation and partitioning into toluene, the counts in the aqueous fraction as well as the toluene fraction increased with time reaching a peak around 24 h; the aqueous fraction contained about 20 % of the total radioactivity in the cells by 24 h. The amount of radioactivity in the aqueous fraction was generally higher in the HP than the NT cells, but the trend of changes with time in the two cell lines were similar.

Control as well as the induced Arabidopsis plants showed similar trend of changes with time in the [^{14}C]Spm fraction (Fig. 6a, b) with a calculated initial $T_{1/2}$ of about 24 h in both (Figs. 4, 6b). The [^{14}C]Spd content was slightly higher in the control than the induced plants, and in both cases it increased up to 24 h and then declined (Fig. 6c). Radioactive Put (Fig. 6d), which was <5 % of [^{14}C]Spm at time zero, showed a major increase at 48 and 72 h in the induced plants but not in the controls. In poplar cells (Fig. 6e–h), the amount of [^{14}C]Spm during the first 24 h was significantly higher in the HP cells than in the NT cells; however, at 48 and 72 h, the situation was reversed. The [^{14}C]Spm fraction decreased slowly with time in both cell lines (Fig. 6e, f), whereas [^{14}C]Spd fraction increased steadily in the NT cells over the entire period of 72 h, the total increase was >fourfold during this period (Fig. 6g). The content of [^{14}C]Spd in the HP cells increased almost 8- to10-fold by 24 h, declining to about half the peak amount during the next 48 h. There was a statistically significant difference between the two cell lines at all times except time zero; the NT cells accumulating less [^{14}C]Spd than the HP cells. The content of [^{14}C]Put produced from [^{14}C]Spm also exhibited a gradual increase with time in both cell lines (Fig. 6h). While the increase in [^{14}C]Put in the NT cells was about 2- to 3-fold, in the HP cells >fivefold increase was seen over the 72-h period.

Based on the decrease in [^{14}C]Spm over time in the two species, the calculated $T_{1/2}$ of Spm in the two genotypes of Arabidopsis was about 24 h, and for poplar cells it was around 48 and 36 h in the NT and the HP cells, respectively (Fig. 4).

Rates of back-conversion of Spd and Spm into smaller polyamines

Tables 1 and 2 show the percentage of back-conversion of [^{14}C]Spd into Put and that of [^{14}C]Spm into Put and Spd at different time periods in the two poplar cell lines and Arabidopsis plants, respectively; the forward conversion of [^{14}C]Spd into Spm is also shown. It is apparent that the percentage of [^{14}C]Spd that was converted into Put in the two poplar cell lines (Table 1) was quite comparable and increased from about 3.3 % at time zero (2 h of incubation in [^{14}C]Spd prior to transfer to label-free medium) to >20 % at 72 h. However, in Arabidopsis (Table 2), the percentage of [^{14}C]Spd converted into Put was different in the two genotypes; it remained at <4 % in uninduced seedlings and increased from about 3 % to >16 % at 72 h in the induced seedlings. While the percentage of [^{14}C]Spd appearing as [^{14}C]Spm was higher in the poplar NT than the HP cells, the relative amounts converted into Spm by 72 h were lower than those converted into Put at that time. The maximum amount of [^{14}C]Spd converted into ^{14}C-Spm was always about 10–13 % in both poplar and Arabidopsis; the latter exhibited a comparable amount of [^{14}C]Spd conversion into [^{14}C]Spm in the induced and the uninduced plants.

For [^{14}C]Spm conversion into Put, the results were quite different in the two poplar cell lines in that the HP cells converted a higher percentage of Spm into Put than the NT cells; the maximum amount being about 11 % at 72 h (Table 1). In contrast, the conversion of [^{14}C]Spm into [^{14}C]Spd was similar in the two poplar cell lines at any given time, the total percentage being much greater than that for conversion into Put. In Arabidopsis (Table 2) a similar trend of changes with time was observed for conversion into Put (i.e. <4 % at all times except 72 h) except that the induced plants had somewhat lower % of [^{14}C]Spm appearing as Spd than control plants.

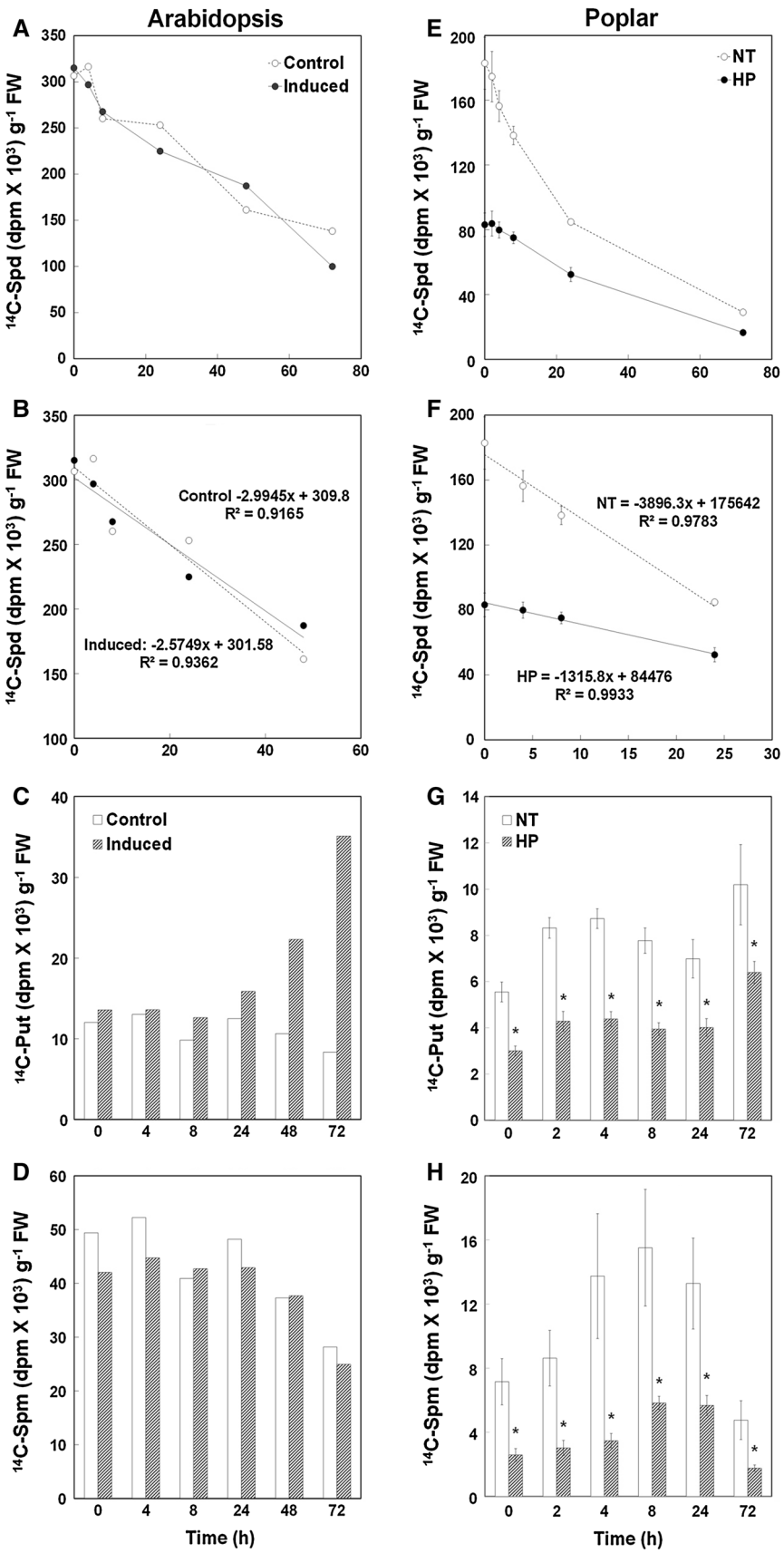

Fig. 3 Changes in the amount of [^{14}C]Spd (**a**, **e**), [^{14}C]Put (**c**, **g**) and [^{14}C]Spm (**d**, **h**) with time and the regression curves for the loss of [^{14}C]Spd over time (**b**, **f**) when 2-week-old induced and uninduced (control) Arabidopsis seedlings and NT and HP poplar cells were incubated with [^{14}C]Spd and grown in label-free medium for various lengths of time. The Arabidopsis and poplar cells were incubated in [^{14}C]Spd for 4 and 2 h, respectively, before washing and transfer to label-free medium. Data are mean ± SE of 9 replicates for poplar; for Arabidopsis a single combined sample of three replicates dansylated separately was run for TLC separation. An *asterisk* indicates that the values in the two cell lines of poplar are significantly different ($p \leq 0.05$) at a given time

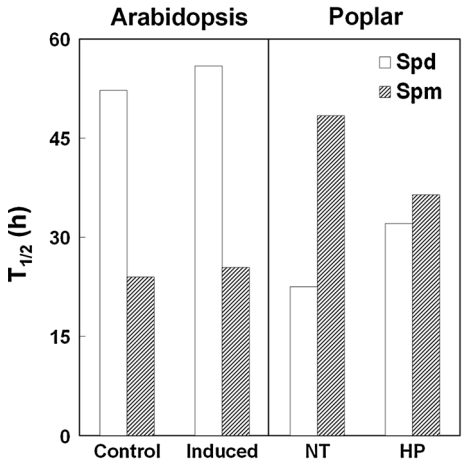

Fig. 4 Calculated half-life ($T_{1/2}$) of [^{14}C]Spd and [^{14}C]Spm in 2-week-old uninduced (control) and induced Arabidopsis seedlings and in NT and HP poplar cells. The $T_{1/2}$ was calculated by using data on the loss of [^{14}C]Spd and [^{14}C]Spm at various times after transfer to label-free medium—data are derived from Fig. 3b, f for Spd and Fig. 6b, f for Spm

From the combined data of the [^{14}C]-PA analysis and the total soluble PA analysis at different times, we estimated the actual amounts of Spd and Spm turned over (total loss) or converted into the other PAs on FW basis; the calculated estimates are shown in Table 3. Such numbers have rarely been published for various metabolites in plants and represent the actual flux rates within a cell in response to the availability of the substrates and the enzymes. Whereas the total amount of [^{14}C]Spd lost during the first 8 h of transfer to fresh medium was comparable in two treatments (induced and uninduced) for Arabidopsis, it was significantly different in the two poplar cell lines with different cellular contents of Put. On FW basis, the NT poplar cells lost twice as much Spd g^{-1} FW as the HP cells. Yet, the conversion of [^{14}C]Spd into [^{14}C]Spm and [^{14}C]Put was comparable in the two poplar cell lines; this was also the case in the uninduced and the induced Arabidopsis plants. The amount converted into Spm was >threefold higher than that converted into Put. Furthermore, either of the two pathways constituted only a small proportion of the total Spd loss. Also, it is apparent that the poplar cell cultures have a higher capacity for converting Spd to Spm and to Put than the Arabidopsis seedlings. It should be pointed out that the amount of Spd back-converted into Put is most likely underestimated for the HP poplar cells and the induced Arabidopsis plants due to the rapid turnover of Put in both cases (Bhatnagar et al. 2002; Majumdar et al. 2013). The total loss of Spm during the first 4 h in the control and the induced Arabidopsis plants was quite comparable, and almost all of it appeared to go towards Spd.

Discussion

The importance of Spd and Spm catabolism in plants

Although PA biosynthetic pathways play critical roles in regulating cellular PA titers, the contribution of PA catabolism to their homeostasis must not be underestimated (Moschou et al. 2012; Tavladoraki et al. 2012). The tight homeostatic control of PAs has significant roles in cellular metabolism since the PA catabolic pathways are on one side connected to the metabolism of several amino acids, and, on the other side, they produce important signal molecules like H_2O_2 and GABA (Moschou et al. 2012). In plants, catabolism of PAs is physiologically important in numerous developmental phases and differentiation processes (germination, cell wall strengthening/rigidity, root development, fruit ripening and senescence, etc.—Mattoo et al. 2010) as well as defense mechanisms against abiotic and pathogen stresses (Cona et al. 2006; Angelini et al. 2010; Tavladoraki et al. 2012). For example, GABA, a direct product of Put catabolism is an important metabolite rapidly synthesized in response to various abiotic stresses (Angelini et al. 2010; Shelp et al. 2012). It is also involved in amino acid C flux into the TCA cycle and cell signaling, and apparently plays a direct protective role against oxidative stress. The other by-product H_2O_2 produced from PA oxidation functions as (1) a signal molecule triggering a multiplicity of plant physiological responses; e.g. root xylem differentiation (Tisi et al. 2011); (2) potential determinant of the fate of cells in response to salinity leading to either programmed cell death (PCD) or tolerance to stress (Angelini et al. 2010; Tisi et al. 2011); and (3) a mediator in ABA signal transduction network in stomatal closure and the stress-responsive process (Angelini et al. 2010; Wimalasekera et al. 2011). Furthermore, the catabolic product of Spd/Spm oxidation 1,3-diaminopropane serves as the precursor for uncommon PAs (norspermidine and norspermine), once again associated with stress response (Vishnu Prasanth et al. 2012). Hence, the accumulation of PAs during stress response should not simply be attributed to their increased biosynthesis, but could also be the result of decreased catabolism. As a follow-up to our previous work on the catabolism of Put in control and high Put producing transgenic cells/plants of poplar and Arabidopsis (Bhatnagar et al. 2002; Majumdar et al. 2013), the present research was aimed at increasing our understanding of the catabolism of Spd and Spm under similar physiological conditions (i.e. high and low Put production). The specific questions being addressed here relate to the effects of increased Put production and accumulation on the uptake and catabolism of the two higher PAs. The increase in cellular Put was achieved through inducible and

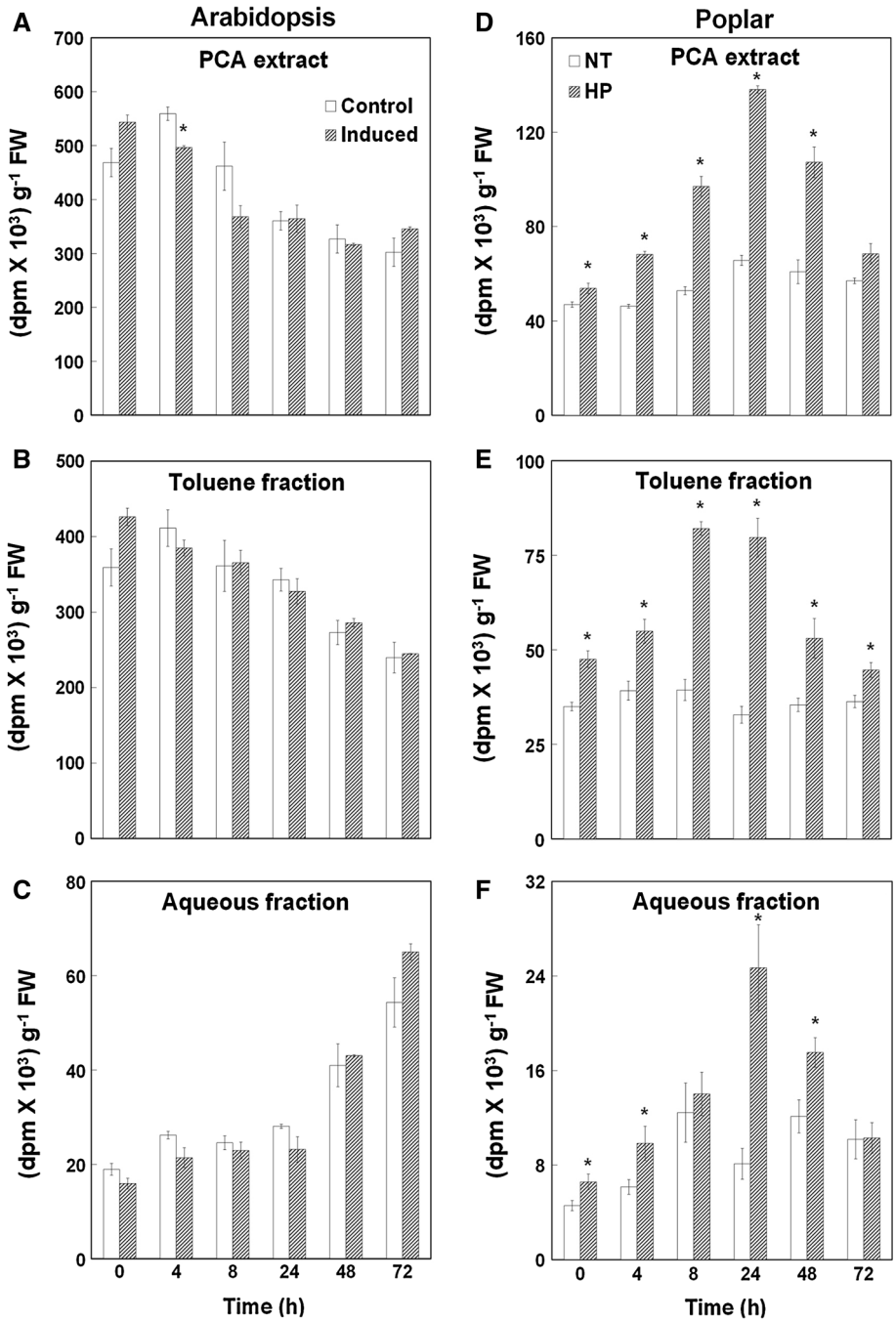

Fig. 5 Amount of radioactivity from [^{14}C]Spm present in the PCA extract (**a**, **d**), the toluene fraction (**b**, **e**) and the aqueous fraction (**c**, **f**) in 2-week-old Arabidopsis seedlings and 3-day-old poplar cells at different times following transfer from [^{14}C]Spm to label-free medium. The Arabidopsis seedlings were incubated with [^{14}C]Spm for 4 h and poplar cells for 2 h, transferred to label-free medium after washing, and collected for analysis at different time periods. Data are mean ± SE of $N = 3$ (for Arabidopsis) and $N = 9$ for poplar. An *asterisk* indicates that the values in the two cell lines/seedlings within the same species are significantly different ($p \leq 0.05$) at a given time

constitutive genetic manipulation of the ODC step in the PA biosynthetic pathway.

Distinct mechanisms regulate the uptake of polyamines

It is believed that the uptake (transport) of PAs is mediated through PA transporters even though information on specific transporters is rather scant in plants (Igarashi and Kashiwagi 2010, 2011; Mulangi et al. 2012a, b). An important question in this regard is the modulation of PA transporters or their kinetics by the cellular content of PAs (the one that is to be transported as well as the others). The results presented here suggest that the uptake of neither Spd nor-Spm is affected significantly by the endogenous Put content in Arabidopsis seedlings. This is in agreement with the results on Put uptake by the same plants in relation to their endogenous Put content (Majumdar et al. 2013). Bearing in mind that the cellular titers of Spd and Spm as well as the conversion of Arg into Put under conditions of high Put in Arabidopsis were not affected by excess Put production from Orn (Majumdar et al. 2013), one could argue that both the biosynthesis and the transport of

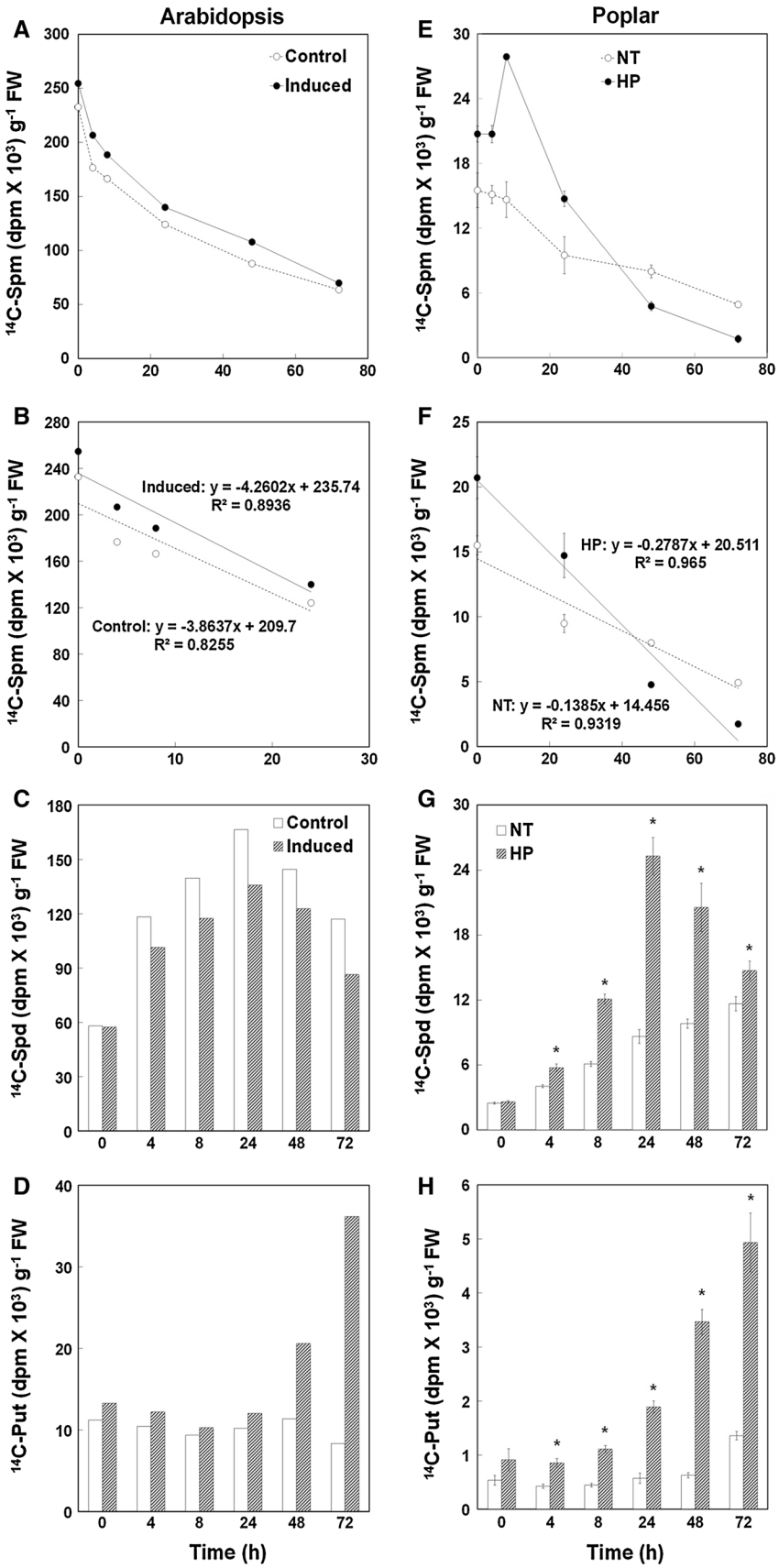

Fig. 6 Changes in the amount of [^{14}C]Spm (**a**, **e**), [^{14}C]Spd (**c**, **g**) and [^{14}C]Put (**d**, **h**) with time and the regression curves for the loss of [^{14}C]Spm over time (**b**, **f**) when 2-week-old induced and uninduced (control) Arabidopsis seedlings and NT and HP poplar cells were incubated with [^{14}C]Spm and grown in label-free medium for various lengths of time. The Arabidopsis and poplar cells were incubated in [^{14}C]Spm for 4 and 2 h, respectively, before washing and transfer to label-free medium. Data are mean ± SE of 9 replicates for poplar; for Arabidopsis a single combined sample of three replicates dansylated separately was run for TLC separation. An *asterisk* indicates that the values in the two cell lines of poplar are significantly different ($p \leq 0.05$) at a given time

Table 1 The calculated percentage of radioactivity accumulated in different polyamines within the toluene fraction at various times after NT and HP poplar cells were transferred to the label-free medium

Substrate	Time (h)	% of Toluene fraction incorporated					
		Put		Spd		Spm	
		NT	HP	NT	HP	NT	HP
[^{14}C]Spd	0	3.35	3.28	–	–	4.32	2.82
	8	4.63	3.52	–	–	9.24	5.20
	72	18.54	20.90	–	–	8.64	5.77
[^{14}C]Spm	0	1.53	1.92	7.07	5.47	–	–
	24	1.74	2.37	26.30	31.75	–	–
	72	3.75	11.05	32.09	32.97	–	–

Three-day-old cells were incubated in [^{14}C]Spd or [^{14}C]Spm for 2 h before washing and transfer to label-free medium

Table 2 The calculated percentage of radioactivity accumulated in different polyamines within the toluene fraction at various times after uninduced (−E) and induced (+E) Arabidopsis seedlings were transferred to the label-free medium

Substrate	Time (h)	% of toluene fraction incorporated					
		Put		Spd		Spm	
		−E	+E	−E	+E	−E	+E
[^{14}C]Spd	0	2.81	3.21			11.56	9.91
	24	3.41	5.03			13.17	13.60
	72	3.76	16.55			12.73	11.76
[^{14}C]Spm	0	3.12	3.11	16.15	13.46		
	24	2.91	3.67	48.57	41.48		
	72	3.48	14.76	48.82	35.31		

Two-week-old seedlings were incubated in [^{14}C]Spd or [^{14}C]Spm for 4 h before washing and transfer to label free medium

different PAs in this species are independent of the cellular concentration of each other. Given that, one can then argue about other mechanisms, including catabolism to play a major role in PA homeostasis. On the other hand, the higher total PA content in HP poplar cells negatively impacted their ability to take up Spd from the medium. This, however, was not the case for the uptake of Put (Bhatnagar et al. 2002) or Spm in these cells, implying that there are distinct mechanisms for regulating the uptake of different PAs even in the same cells. The apparent difference in PA uptake between the two species may be either due to different sensitivities of their PA transporters to endogenous PAs or due to difference in the behavior of the intact seedlings vs. the cell culture system being used. Studies on PA uptake in other plants have also shown different kinetics of uptake for different PAs, confirming the occurrence of diverse PA transport mechanisms in different species (Kakkar et al. 1997; Theiss et al. 2004; Ohe et al. 2005; Mulangi et al. 2012a, b).

Different mechanisms regulate back-conversion of higher to lower polyamines

While the enzymes involved in actual catabolic conversions of different PAs and their breakdown are well known, their intracellular as well as apoplastic localization in plants points to their physiological importance more than a simple role in catabolizing the excess PAs. The question of inducibility of the catabolic enzymes in response to external factors and intracellular fluctuations in PA content have not been addressed; even less is known about the responses of plant PAOs to genetic manipulation of the PA pathway. Recent characterization of plant PAOs, which catalyze the back-conversion of PAs in a way parallel to that in animals, have provided some insight into the biological mechanisms by which Spd and Spm contents may be regulated in this kingdom (Moschou et al. 2008a; Tavladoraki et al. 2012). The lack of a clear understanding of the roles for various PAOs in plants and animals is further obvious from the confusion about their precise nomenclature, including information about their substrate specificity/preferences as cited in footnote 1.

The cellular Spd content is an outcome of its biosynthesis from Put, back-conversion from Spm, as well as its terminal catabolism. One must of course also consider its

Table 3 The total amount (nmol g^{-1} FW) of loss of Spd or Spm, and conversion of Spd → Put and Spm → Spd and Put, at different times after transfer of poplar cells and Arabidopsis seedlings to label-free medium

Plant/cell lines	Time (h)	Spd loss	Spd → Spm	Spd → Put	Spm loss	Spm → Spd
Poplar NT	8	441.64	92.81	24.68	NC	NC
Poplar HP	8	223.44	93.37	27.09	NC	NC
Arabidopsis (−E)	4	NC	4.62	1.62	15.31	18.68
	8	76.24	NC	NC	NC	NC
Arabidopsis (+E)	4	26.95	4.08	<1.00	12.49	12.67
	8	71.01	NC	NC	NC	NC

The poplar cells were incubated in [^{14}C]Spd/[^{14}C]Spm for 2 h and Arabidopsis seedlings for 4 h prior to washing and transfer to the label-free medium

NC not calculated

transport to other tissues and organs within the plant, and its conjugation and binding to macromolecules as contributing factors to cellular content at a given time. However, large-scale transport between different tissues and organs in plants is not presently indicated in the literature. Thus merely analyzing the total cellular PA content at a given time cannot reveal the dynamic contribution of individual sub-pathways in the overall cellular Spd titer in plants. The results reported here clearly show that taking into consideration all aspects of turnover referred to above, the calculated half-life ($T_{1/2}$) of cellular Spd is >50 h in both control (low Put) and the induced (high Put) Arabidopsis seedlings, which, is several-fold greater than the $T_{1/2}$ of Put in the same cells (estimated to be <8 h—Majumdar et al. 2013). Moreover, the total amount of Spd lost did not differ between the uninduced and the induced plants during the first 8 h of analysis. While the conversion of Spd to Put was much less (~5 % of total) than its conversion into Spm (~13 %), neither constituted a major pathway of Spd catabolism. These observations are consistent with the finding that the production of Spd from Put was not affected much by the endogenous content of Put (Majumdar et al. 2013). The results with poplar cells are also similar except that the $T_{1/2}$ of Spd was about 22 and 33 h in NT and HP cells, respectively; this also is at least 2- to 3-fold greater than the $T_{1/2}$ of Put. Taken together, it can be argued that the biggest contributor to Spd loss in plants is its terminal catabolism and not its conversion into Spm or Put. Also, the conversion of Put into Spd is often not limited by the substrate but via a complex interaction of the product and the enzymes.

Following a similar logic for Spm loss through various sub-pathways in Arabidopsis, our results show its $T_{1/2}$ in Arabidopsis is about half of that in poplar, and about 50 % shorter than that of Spd; still it is 3–4 times longer than Put. The total Spm loss (nmol g^{-1} FW) in the first 4 h was comparable in the control (uninduced) and the induced plants and the amount was close to that of Spm back-converted into Spd, again showing that terminal catabolism of Spm is not significant. On the other hand, the calculated $T_{1/2}$ of Spm in NT and HP poplar cells was 48 and 36 h, respectively; which is much longer than that of Spd. Thus it can be argued that for Spm turnover, back-conversion is the major route, which is consistent with the finding that Spm-to-Put back-conversion involves an effective recycling loop under drought stress in Arabidopsis (Alcázar et al. 2011). This is also in agreement with the reports that in Arabidopsis, four of the five *PAO* genes (*AtPAO1–AtPAO4*) have been suggested to favor the back-conversion reaction (Takahashi et al. 2010; Fincato et al. 2011). The only evidence favoring the terminal catabolic activity in this species was the production of small amount of 1,3-diaminopropane parallel to the major product of nor-Spd from the oxidation of nor-Spm by AtPAO1 (Tavladoraki et al. 2006). Furthermore, several authors have argued that in Arabidopsis Spm may not even be essential as long as tSpm was being produced (Imai et al. 2004; Rambla et al. 2010). These arguments then leave AtPAO5, whose properties have not been elucidated, as the only potential candidate for terminal catabolism of higher PAs.

In summary, the present study, while corroborating earlier reports on pathways for Spd and Spm catabolism, provides new and unique insights into the metabolism of higher PAs in Arabidopsis seedlings and poplar cells. The most important conclusions from this study are: (a) the uptake of Spd and Spm is affected differently by their total cellular PA contents, e.g. more in poplar cells than in Arabidopsis plants; (b) both species are capable of converting Spd and Spm back into lower PAs; (c) the rates of Spd and Spm catabolism in the cells are several-fold slower than that of Put; (d) overproduction of Put does not affect the overall rates of turnover of Spd or Spm, nor does it affect the rates of conversion of Spd and Spm into lower PAs; and (e) while Spm is mainly converted back to Spd and not terminally degraded, Spd is removed from the cells largely through terminal catabolism in both Arabidopsis and poplar cells. Finally, this study provides a direct measurement of the actual amounts of Spd and Spm catabolized g^{-1} FW, information that is not currently available in plant cells.

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Conflict of interest The authors declare that they have no conflict of interest.

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