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ORIGINAL ARTICLE

Living with high putrescine: expression of ornithine and arginine biosynthetic pathway genes in high and low putrescine producing poplar cells

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Abstract Arginine (Arg) and ornithine (Orn), both derived from glutamate (Glu), are the primary substrates for polyamine (PA) biosynthesis, and also play important roles as substrates and intermediates of overall N metabolism in plants. Their cellular homeostasis is subject to multiple levels of regulation. Using reverse transcription quantitative PCR (RT-qPCR), we studied changes in the expression of all genes of the Orn/Arg biosynthetic pathway in response to up-regulation [via transgenic expression of mouse *Orn decarboxylase* (*mODC*)] of PA biosynthesis in poplar (*Populus nigra* × *maximowiczii*) cells grown in culture. Cloning and sequencing of poplar genes involved in the Orn/Arg biosynthetic pathway showed that they have high homology with similar genes in other plants. The expression of the genes of Orn, Arg and PA biosynthetic pathway fell into two hierarchical clusters; expression of one did not change in response to high putrescine, while members of the other cluster showed a shift in expression pattern during the 7-day culture cycle. Gene expression of branch point enzymes (*N*-acetyl-Glu synthase, Orn aminotransferase, Arg decarboxylase, and spermidine synthase) in the sub-pathways, constituted a separate cluster from those involved in intermediary reactions of the pathway (*N*-acetyl-Glu kinase, *N*-acetyl-Glu-5-P reductase, *N*-acetyl-Orn aminotransferase, N_2-acetylOrn:*N*-acetyl-Glu acetyltransferase, N_2-acetyl-Orn deacetylase, Orn transcarbamylase, argininosuccinate synthase, carbamoylphosphate synthetase, argininosuccinate lyase, *S*-adenosylmethionine decarboxylase, spermine synthase). We postulate that expression of all genes of the Glu-Orn-Arg pathway is constitutively coordinated and is not influenced by the increase in flux rate through this pathway in response to increased utilization of Orn by mODC; thus the pathway involves mostly biochemical regulation rather than changes in gene expression. We further suggest that Orn itself plays a major role in the regulation of this pathway.

Keywords Arginine biosynthesis · Ornithine biosynthesis · Gene expression · Polyamines · Reverse transcriptase quantitative polymerase chain reaction · Hierarchical clustering

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Introduction

Arginine (Arg), proline (Pro) and glutamic acid (Glu), along with polyamines (PAs) and γ-aminobutyric acid (GABA) constitute a significant proportion of the non-protein soluble nitrogen (N) in plant cells. In addition to their roles as substrates and intermediates in N metabolism (Fig. 1), they all play important roles in abiotic stress in plants (Minocha et al. 1997, 2000; Kasukabe et al. 2004; Alcazar et al. 2006; Seki et al. 2007; Kusano et al. 2007). Thus their cellular homeostasis is subject to multiple levels

of regulation, including the induction and suppression of genes that code for key enzymes of their biosynthetic pathway. Genetic manipulation of a step that uses a shared substrate or yields a product used in multiple reactions can have wide-ranging effects on several pathways, thereby disturbing the entire homeostatic regulation of these metabolites (Mohapatra et al. 2010; Mattoo et al. 2010).

Polyamines (putrescine, Put; spermidine, Spd; spermine, Spm) are ubiquitous in living cells and play important roles in plant growth and development, responses to abiotic stress, and regulation of reduced N, particularly the cellular amino acids (Minocha et al. 1997, 2000; Kusano et al. 2007; Mattoo et al. 2010; Mohapatra et al. 2010). Although the pathways specifically related to PA biosyntheses from Arg and Orn have been well characterized with respect to enzymes and the expression of their genes in both prokaryotes and eukaryotes (Page et al. 2007; Jumtee et al. 2008 and references therein), analyses of the expression of genes encoding various enzymes regulating their substrates (Orn/Arg) have been lacking. Furthermore, while the Arg/Orn biosynthetic pathway in animals and microbes is well studied (Morris 2006, 2007), according to Slocum (2005) little is known about expression of the genes for various enzymes involved in Arg biosynthesis in plants; and not much has changed since then (Kalamaki et al. 2009a).

The overall pathway for PA biosynthesis from Glu comprised more than 20 steps (Fig. 1), beginning not far downstream of N assimilation via Gln synthetase (GS), Glu synthase (GOGAT) and Glu dehydrogenase (GDH), and ending with branches producing several key metabolites including Pro and GABA, in addition to PAs. The PA biosynthetic pathway has been the target of numerous successful manipulation attempts through mutagenesis, transgene expression and the use of inhibitors (Kumar and Minocha 1998; Bhatnagar et al. 2001, 2002; Roy and Wu 2002; Capell et al. 2004; Franceschetti et al. 2004; Kasukabe et al. 2004). Using RT-qPCR, earlier we reported changes in the expression of genes of the core PA biosynthetic pathway in response to up-regulation of Put biosynthesis in poplar cells (Page et al. 2007). Here we describe the expression of genes coding for enzymes involved in the biosynthesis of the two substrates of PA biosynthetic pathway, namely, Orn and Arg. The present study builds upon our earlier work (Bhatnagar et al. 2001, 2002; Quan et al. 2002; Page et al. 2007; Mohapatra et al. 2009, 2010) on pleiotropic changes associated with enhanced accumulation of Put in the high Put (HP) cells versus the control cells, focusing on quantification of the transcripts of 17 genes that encode enzymes in the pathway from Glu to Orn and Arg (Fig. 1).

The two transgenic cell lines used here are isogenic except for a single gene that affects PA biosynthesis, thus they mimic the situation provided by an up-regulated mutant. The HP line, which expresses a mouse *Orn decarboxylase* (m*ODC*) gene, produces several-fold greater amounts of Put than the control line, which has the *GUS* (*β-glucuronidase*) gene; each gene is controlled by an identical 2x35S CaMV promoter (Bhatnagar et al. 2001). The two cell lines have had

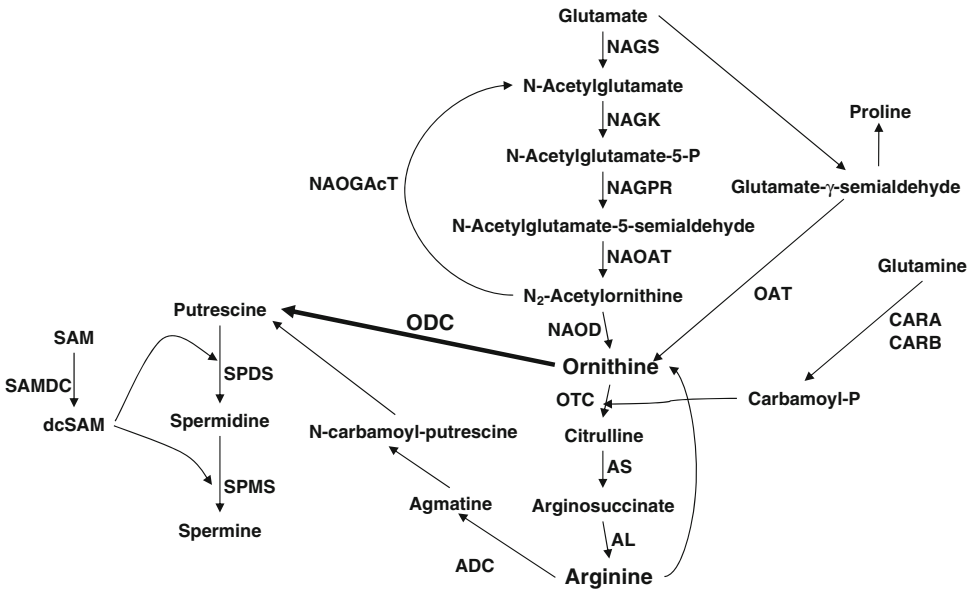

Fig. 1 Pathway for the biosynthesis of polyamines and related metabolites from glutamate. *ADC* Arg decarboxylase, *AL* argininosuccinate lyase, *AS* argininosuccinate synthase, *CARA* carbamoylphosphate synthase large subunit, *CARB* carbamoylphosphate synthase small subunit, *NAGK* *N*-acetyl-Glu kinase, *NAGPR* *N*-acetyl-Glu-5-P reductase, *NAGS* *N*-acetyl-Glu synthase, *NAOAT* N_2-acetyl-Orn aminotransferase, *NAOD* N_2-acetyl-Orn deacetylase, *OAT* Orn aminotransferase, *ODC* Orn decarboxylase (over-expressed in HP cells), *OTC* Orn transcarbamylase, *SAMDC* *S*-adenosylmethionine decarboxylase, *SPDS* Spd synthase, *SPMS* Spm synthase

an identical history of culture over several years and have been checked periodically with respect to similarity of growth pattern in culture, and cellular PA, amino acids, and soluble protein contents over the week-long culture period. These parameters act as experimental markers of stability of these cell lines over the years in culture.

We hypothesized that increased production of Put via mODC in the HP cells will necessitate a concomitant increase in production of the substrate Orn, which is among the least abundant amino acids in these cells (Mohapatra et al. 2010). Here we address the question of whether redirection of high amounts of Orn towards Put production by transgenic ODC will cause a change in the expression of genes encoding enzymes involved in its (Orn) biosynthesis. The results reveal that even though flux of metabolites for most steps (from Glu to Arg/Orn) is expected to be increased several-fold to meet the demand for its utilization in Put biosynthesis, overall changes in expression of these genes are relatively small. Furthermore, there is a temporal coordination of the expression of most of the genes involved in the Orn/Arg and the PA pathways in both HP and the control cells. These data are consistent with the existence of a strong homeostatic regulation of transcription of these genes, and the regulation of Orn/Arg metabolism occurring via biochemical control at the enzyme level. We discuss the possibility of this regulation being responsive to cellular Orn.

Experimental procedures

Cell growth and cDNA preparation

The HP cell line (expressing m*ODC* cDNA) and the control cell line (expressing a *GUS* gene) of *Populus nigra* × *maximowiczii* used here have been described earlier (Bhatnagar et al. 2001, 2002; Page et al. 2007; Mohapatra et al. 2010). Suspension cultures of the two lines were maintained under identical growth conditions. Cells were harvested by vacuum filtration on different days of the 7-day culture period, flash-frozen in liquid nitrogen, and stored at −80°C.

Total RNA (3 µg), isolated using the RNeasy Kit (Qiagen, Valencia, CA), was treated with DNAse (Promega, Madison, WI) and subjected to phenol:chloroform:iso-amyl alcohol (25:24:1) treatment. Precipitated RNA was dissolved in RNase-free water and reverse-transcribed using SuperScript™ III Reverse Transcriptase using oligo(dT)$_{22}$ (Invitrogen, Carlsbad, CA).

Sequence acquisition

Fragments of each gene were amplified, cloned and sequenced (at the UNH Hubbard Center for Genome Studies) to confirm their identity, then used to make probes for screening a cDNA library made from our poplar suspension cultures (Creator™ SMART Kit; BD Biosciences, Palo Alto, CA). Sequences obtained from the *Populus trichocarpa* genome database (Tuskan et al. 2006; http://www.phytozome.net/poplar.php) were used to design additional primers for amplification, cloning and sequencing of the corresponding gene fragments (for accession numbers, see Table 1). The m*ODC* (Accession # M10624) used in transformation has been described previously (DeScenzo and Minocha 1993; Bhatnagar et al. 2001).

Quantitative RT-PCR

Taqman® probes were designed using Primer Express™ (Applied Biosystems) in such a way that each primer/probe combination would amplify all expressed paralogues of each gene. Because of the lack of introns in some sequences, it was not feasible to use exon–exon boundaries to prevent signal contamination from genomic DNA; hence we relied on DNase treatment of RNA. Effectiveness of DNAse treatment was confirmed by lack of amplification of glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) in RNA. Probes were 5′-labeled with 6′FAM and 3′-labeled with Iowa Black FQ (IDT, Coralville, IA). Each primer/probe combination was tested using templates for individual paralogues to ensure specificity and satisfactory amplification.

RT-qPCR reactions were set up as described earlier (Page et al. 2007) using two master mixes for each reaction in order to maintain inter-sample consistency; the first contained Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and template (0.5 µL) and the second contained forward and reverse primers (0.3 µM final concentration) and the probe (0.2 µM final concentration). RT-qPCR was performed in a GeneAmp 5700 (Applied Biosystems) using 40 cycles of 95°C for 15 s, 55°C for 30 s and 65°C for 45 s. Baseline cycles were set at 6–15 and threshold at 0.05; validation was performed by inspection of a plot of cycle number versus fluorescence using both log and linear axes for fluorescence. Relative starting template concentrations were calculated using efficiency and critical cycle number (C_t) values (Page et al. 2007). PCR efficiency for each gene was calculated from a series of ten dilutions of matching template over three orders of magnitude. Each experiment was repeated in its entirety three times with similar results.

Data analysis

Data were analyzed by a General Linear Model Analysis of Variance using Systat 8.0 with Tukey's post hoc tests for multiple comparisons. All differences noted as

Table 1 Summary of different gene paralogues, their location on the *Populus trichocarpa* (*Pt*) genome, the cloned fragment length of *Populus nigra* × *maximowiczii* (*Pm*), base pair differences between *Pt* and *Pm* sequences, and accession numbers of *Pm* clones

Gene name	Location in *Pt* genome	*Pm* clone length	Base pair difference	Accession #
NAGS1	C-VII	603	4	FJ899845
NAGS2	C-XIV	936	6	FJ899846
NAGK1	S-147	896	2	FJ899842
NAGK2	S-193	917	3	FJ899843
NAGPR	S-130	836	2	FJ899844
NAOAT	C-XVII	680	9	FJ899847
NAOGAcT	S-232	809	4	FJ899848
NAOD1	S-118	992	5	FJ899849
NAOD2	S-29	992	1	FJ899850
CARA1	C-III	843	3	FJ899839
CARA2	S29	843	6	FJ899840
CARB1	S-132	ND	ND	ND
CARB2	C-X	635	5	FJ899841
AS1	C-VIII	600	1	FJ899837
AS2	C-X	807	3	FJ899838
AL	C-II	683	8	FJ899836
OAT	C-XI	1,261	15	FJ899851

ND not detected

"significant" have $p < 0.05$. Data were also analyzed using Genesis (Sturn et al. 2002) by normalizing and then performing hierarchical clustering using a complete linkage algorithm. All data were analyzed using four time points during the culture period (days 1, 3, 5 and 7); however, because day 7 is the same as day 0 for the repeat experiment, data are graphed showing day 0 in order to reflect a complete subculturing cycle. The clustering analysis presented here also includes previously published data (Page et al. 2007) on *ADC,* m*ODC*, *SAMDC,* and *SPDS* genes. The inclusion of these data enabled us to analyze and discuss almost the entire pathway.

Results

The cells of the two lines used for RT-qPCR analyses were characterized with respect to PA and soluble protein content during the entire period of study and found to show consistency of results as reported previously (Page et al. 2007; Mohapatra et al. 2009). The cellular PAs in HP cells were about 4- to 5-fold higher and the growth rates of the two cell lines were comparable during the 7-day culture period.

Whereas the major regulatory step for PA biosynthesis in plants is the production of Put from Orn (by ODC) and/or Arg (by ADC); the primary source of all Put is Glu, which is the precursor of both Orn and Arg. The premise of the study is that: since Orn is present in rather small amounts as compared to either Arg or Glu in the cells (1–5 nmol g^{-1} FW in both cell lines; Arg:Orn ratios = 80:1 to 200:1 in control and about 100:1 in HP cells; Glu:Orn ratios = 400:1 in control and about 100:1 in HP cells; Mohapatra et al. 2010); its increased utilization for Put production in the HP cells (by mODC) must therefore be accompanied by its increased biosynthesis from Glu via steps outlined in Fig. 1. The question is: "Does this increase in Orn production involve increase in transcription of the enzymes of this pathway?" We compared changes in transcripts of each enzyme in the Orn/Arg biosynthetic pathway in the HP and the control cells to directly answer this question. In order to account for slightly different growth rates on different days within each line, we quantified transcripts on 4 days of the 7-day culture cycle. The results are presented for each enzyme in the order that it is involved in the pathway (Fig. 1). The annotated homologous genes of *P. trichocarpa* (*Pt*) that were used for comparison are shown in Table 1.

N-Acetylglutamate synthase (NAGS)

Acetylation of Glu by NAGS (EC 2.3.1.1) is the first step committing Glu to Orn/Arg biosynthesis. Two sequences were identified in *Pt* (*Populus trichocarpa*) genome database for NAGS (Table 1); both were expressed in cell cultures of *Pm* (*Populus nigra* × *maximowiczii*). Cloning and partial sequencing of the two cDNAs showed that Pm*NAGS*1 differed from its homologue Pt*NAGS*1 by only 4 bp over the 603 bp region that was amplified; the second (Pm*NAGS*2) differed from Pt*NAGS*2 by 6 bp (out of 936 bp region cloned). The expression of Pm*NAGS* (both genes together; Fig. 2a) increased significantly within 24 h of transferring control cells to fresh medium. After 3 days, the Pm*NAGS* transcripts declined to their lowest level seen

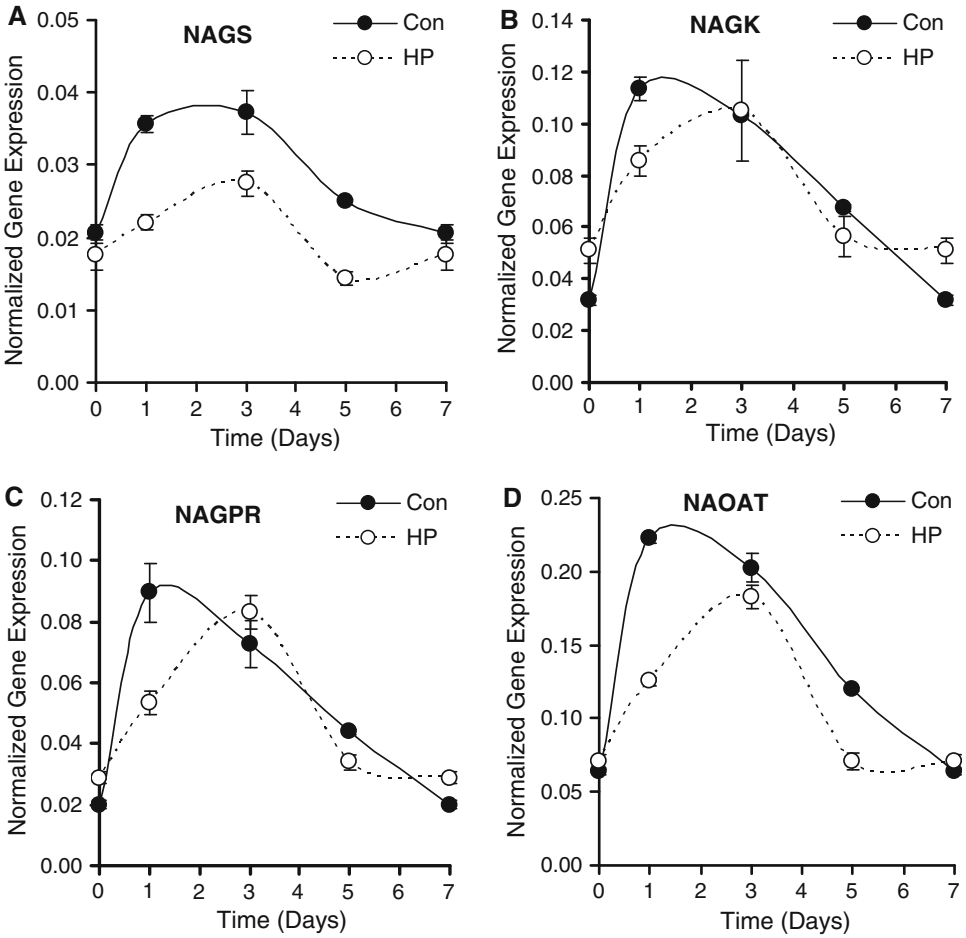

Fig. 2 Changes in relative expression of *NAGS*, *NAGK*, *NAGPR* and *NAOAT* in control and HP cells on different days of the 7-day culture cycle. $n = 6$ from two different experiments

on day 7. In HP cells, the increase in transcripts between days 1 and 3 following transfer to fresh medium was rather small but showed a decline between days 3 and 5 parallel to the control cells. As with control cells, there was little change in relative expression of *NAGS* genes between days 5 and 7 in HP cells. Although there was no significant difference in *NAGS* expression between the two cell lines on day 7 (the same as day 0 in Fig. 2a), on days 1, 3 and 5, HP cells showed lower expression of *NAGS* genes relative to control cells.

N-Acetylglutamate kinase (NAGK)

Two NAGK (EC 2.7.2.8) sequences were identified in the *Pt* genome; again transcripts of both were detected in our *Pm* cells. Sequence comparison with the *Pt* database showed that Pm*NAGK*1 differed from Pt*NAGK*1 by 2 bp over the 896 bp cloned sequence, and Pm*NAGK*2 differed from Pt*NAGK*2 by 3 bp over the 917 bp cloned. The relative expression of Pm*NAGKs* (Fig. 2b) increased significantly in both cell lines to an apparent peak on day 1 in control and on day 3 in HP cells; thereafter, its expression declined by day 7 to about half of its peak expression. Although the two cell lines exhibited different timings of peak expression, there was no significant difference in *NAGK* transcripts between them at any time point tested (Fig. 2b).

N-Acetylglutamate-5-P reductase (NAGPR)

There is only one sequence in the *Pt* genome for NAGPR (EC 1.2.1.38). Comparison of Pm*NAGPR* and Pt*NAGPR* sequences revealed 2 bp difference over the 836 bp cloned. The expression of Pm*NAGPR* (Fig. 2c) followed a pattern similar to that of *NAGK*; a significant increase in expression between days 0 and 1 followed by a decline in expression to the lowest level on day 7. Again, the highest expression was seen on day 1 in control cells and day 3 in HP cells; this lead to a significant difference in transcript levels between the two cell lines on day 1, but not on any other day of the analysis.

N_2-Acetylornithine aminotransferase (NAOAT)

A single 680 bp fragment of *NAOAT* (EC 2.6.1.11) was amplified from our cells; this fragment differed from

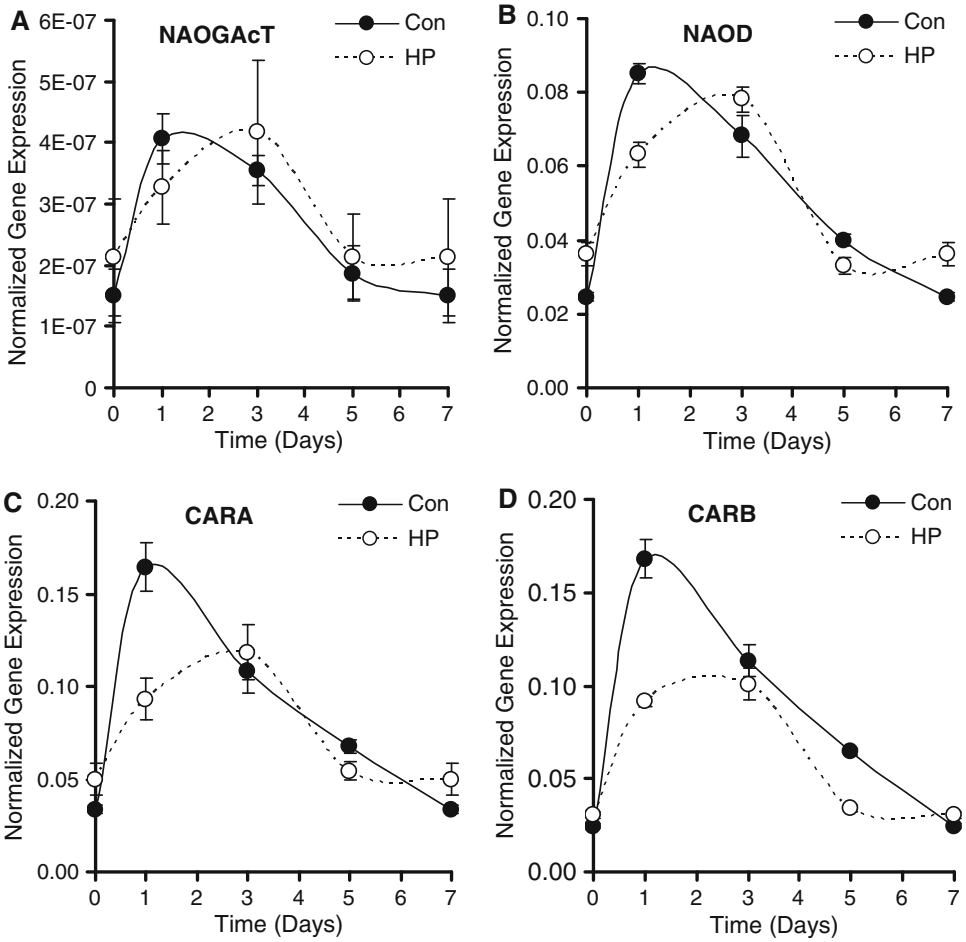

Fig. 3 Changes in relative expression of *NAOGAcT*, *NAOD*, *CARA* and *CARB* in control and HP cells on different days of the 7-day culture cycle. $n = 6$ from two different experiments

Pt*NAOAT* sequence by 9 bp. The expression of Pm*NAOAT* (Fig. 2d) was also similar to that of *NAGK* for both cell lines. The control cells showed a significant increase in expression within 24 h following subculture, thereafter declining to reach the lowest expression on day 7. In HP cells, a significant increase in *NAOAT* expression was observed up to day 3, followed by a substantial decline; these cells showed similar or somewhat lower expression of this gene compared to control cells on most days; the difference was significant only on days 1 and 5, but the pattern of change was quite similar.

N_2-Acetylornithine:*N*-acetyl-Glu acetyltransferase (NAOGAcT)

The enzyme NAOGAcT (EC 2.3.1.35) is involved in recycling of acetyl moiety from acetyl-Orn to Glu, generating Orn and acetyl-Glu. A partial sequence of 809 bp for Pm*NAOGAcT* was cloned that matched a single sequence for this gene in the *Pt* genome. The two sequences differed by 4 bp over the length of the clone. The relative abundance of Pm*NAOGAcT* transcript was the lowest observed for any of the genes measured. The two cell lines had almost identical amounts of *NAOGAcT* transcripts on any day of analysis (Fig. 3a). The control cells showed a small but significant increase during the first 24 h after transfer to fresh medium, while in HP cells the increase continued up to day 3. By day 5, both cell lines had a reduction in NAOGAcT transcripts with no change thereafter.

N_2-Acetylornithine deacetylase (NAOD)

Although according to Shargool et al. (1988), the enzyme NAOD (EC 3.5.1.16) is not found in plants; several ESTs have been reported in Arabidopsis (Accession Nos. AAK28643, E71448, CAB78785, CAA17126, CAB10562). Two *NAOD* sequences were identified in the *Pt* genome, and both were expressed in *Pm* cells; the cloned sequence for each being about 1 kb. Pm*NAOD*1 differed from its homologue Pt*NAOD*1 by 5 bp, while Pm*NAOD*2 differed by only 1 bp from Pt*NAOD*2 (Table 1). The combined expression pattern of the two *NAOD*s (Fig. 3b) over the 7-day culture period was similar to that observed for *NAGK* and *NAGPR* in that there was a sharp increase in transcripts on day 1 (in control cells) and up to day 3 (in HP cells), followed by a decline by day 5 and no change after that. The control cells

showed somewhat higher expression than HP cells only on day 1.

Carbamoyl phosphate synthetase (CARA and CARB)

The biosynthesis of Arg from Orn involves three enzymes: Orn transcarbamylase (OTC, EC 2.1.3.3), argininosuccinate synthase (AS, EC 6.3.4.5) and argininosuccinate lyase (AL, EC 4.3.2.1). Carbamoyl phosphate synthetase (CPS, EC 6.3.5.5) produces carbamoyl-P from Gln, which is used as a carbamoyl donor for the OTC reaction. Two sequences for CPS small subunit (a.k.a. CARA) as well as the large subunit (a.k.a. CARB) were identified in the *Pt* genome. Of the two 843 bp clones of Pm*CARA*, one differed from *Pt*CARA by 3 bp and the other by 6 bp. Of the two Pt*CARB* genes, only one was expressed in our cells. A 635 bp clone of this gene differed from its counterpart in *Pt* by 5 bp. The expression of *CARA* and *CARB* followed almost an identical pattern of changes in relative transcript abundance within each cell line; with a peak on day 1 in control cells and on day 3 in the HP cells (Fig. 3c, d). The relative transcript abundance of both genes on day 1 was different between the two cell lines; the control cells had more than twice the relative abundance of each as compared to the HP cells. Significant decreases in expression occurred between days 3 and 5.

Argininosuccinate synthase (AS)

Argininosuccinate synthase (AS, EC 6.3.4.5) is coded by two sequences in *Pt*; our *Pm* cells expressed both sequences. The Pt*AS*1 sequence differed from *Pm* cloned 600 bp fragment by 1 bp, and the Pt*AS*2 differed by 3 bp from the 807 bp fragment that was cloned from *Pm*. The relative transcript abundance of Pm*AS* in control cells was significantly higher than that in HP cells on days 1 and 5 but was similar on other days (Fig. 4a). The Pm*AS* transcripts increased significantly on transfer of cells to fresh medium and peaked at 24 h for control cells and at 3 days for HP cells. The lowest expression of this gene was on day 7.

Argininosuccinate lyase (AL)

One sequence corresponding to AL (EC 4.3.2.1) was identified in the *Pt* genome. Our cloned fragment (683 bp) of *AL* differed by 8 bp from the published Pt*AL* sequence. The expression of Pm*AL* (Fig. 4b) increased 4- to 6-fold in both cell lines by day 1 following transfer to fresh medium; thereafter, a rapid decrease occurred between days 1 and 3 in control cells, and between days 3 and 5 in HP cells. By day 5, both cell lines had comparable and low levels of

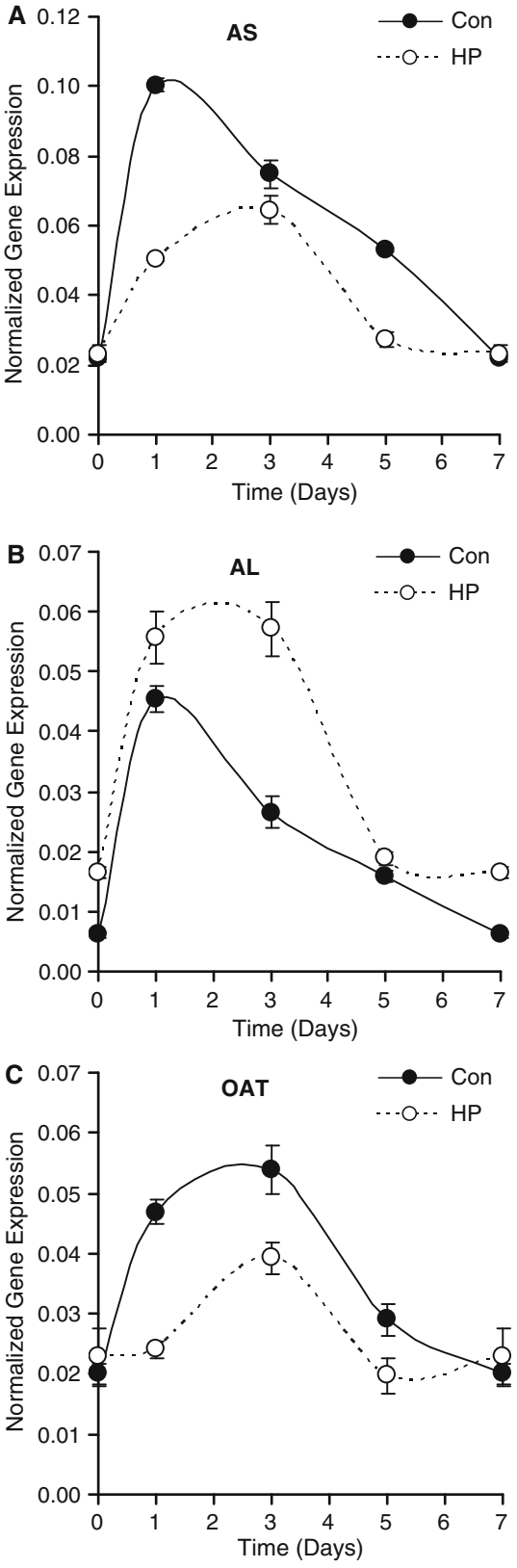

Fig. 4 Changes in relative expression of *AS*, *AL* and *OAT* in control and HP cells on different days of the 7-day culture cycle. $n = 6$ from two different experiments

transcripts of this gene; there was a further decline in *AL* transcripts in the control cells. In contrast to most other genes, the expression of AL was higher in the HP versus the control cells.

Ornithine aminotransferase (OAT)

Ornithine aminotransferase (δ-OAT, EC 2.6.1.13) is an enzyme whose function depends heavily on the cellular nitrogen (N) richness. Under high N (e.g., in germinating seeds), it presumably converts Orn (produced from Arg catabolism) into Glu-γ-semialdehyde (GSA), which spontaneously cyclizes into P5C and is used in Pro synthesis. On the other hand, under Arg-limiting conditions (e.g., fast growing cells, analogous to the situation here), OAT provides an alternate pathway for producing Orn from GSA (Ventura et al. 2009). Only one *OAT* sequence was identified in the *Pt* genome, which differed from the *Pm*-δOAT by 15 bp over the 1,261 bp length of the cloned fragment. The expression of *OAT* (Fig. 4c) increased significantly in response to fresh medium in both cell lines; the response was faster and greater in the control cells than the HP cells. The *OAT* transcripts almost tripled by the third day of culture but declined to base level by day 5. However, changes were less pronounced in HP cells; on day 7, the two cell lines had similar relative amounts of transcripts of this gene.

Polyamine biosynthetic genes

The details of expression data for a single Pm*ADC*, the m*ODC* transgene, three Pm*SAMDC*s, and two Pm*SPDS* genes have been published earlier (Page et al. 2007); these data are not duplicated here but are included in the cluster analysis for comparison of the two cell lines.

Hierarchical clustering

Clustering of the data presented here combined with those published earlier (Page et al. 2007) showed that expression of most of the genes of the Orn/Arg and PA biosynthetic pathways in the control cells peaked within 24 h of transfer to fresh medium except four genes whose expression peaked on day 3 of the culture cycle (Fig. 5a, c). For HP cells, while expression of all genes except OAT also increased within 24 h of transfer to fresh medium, the peak of expression was delayed by 1 or 2 days as compared to the control cells (Fig. 5b, d). The transgenic m*ODC* expression rose to a peak on day 3, fell to its lowest level on day 5 and recovered by day 7 to levels similar to those of day 0. Examination of the outliers in the control cells (Fig. 5c, red lines) and the HP cells (Fig. 5d, blue lines) confirmed that expression levels of NAGS, ADC, OAT, SPD1, SAM2 and SAM3 did not display different expression profiles in control and HP lines. The clustering of expression with time and the differences between the two cell lines become more obvious when the combined data are plotted irrespective of the cell line (Fig. 6a, b). Two main clusters with somewhat different characteristics became apparent: one contained mainly genes from control cells, while genes from HP cells predominantly populated the other cluster. The first cluster (blue lines) displayed an initial sharp increase in expression at 24 h following transfer to fresh medium, followed by steady decline throughout the rest of the culture period (Fig. 6b). The expression of genes in the second cluster showed more gradual increases on transfer to fresh medium and typically peaked on day 3 (Fig. 6b, red lines). Thereafter (days 3–5), transcripts of genes in this cluster declined sharply, becoming comparable to those in the control cells, and showed only a minor change between days 5 and 7. Although as a general rule, gene expression peaked in the control cells on day 1 and in the HP cells on day 3, there were a few exceptions. On close examination of the clustering of gene expression profiles in control cells (Fig. 5a, c), it becomes evident that *NAGS*, *OAT*, *ADC* and *SPDS*1 showed patterns more similar to the bulk of the genes in the HP cells, while in the HP cells (Fig. 5b, d), *SAMDC*2 and *SAMDC*3 showed expression patterns more similar to most of the genes in control cells. Hierarchical clustering also revealed that in HP cells, m*ODC* and *OAT* clustered away from bulk of the other genes (Fig 5b, d).

Discussion

Data analysis methods

Microarray studies have been conducted in poplar (*P. trichocarpa*) to examine changes during development of wood (Schrader et al. 2004; Andersson-Gunneråas et al. 2006; Park et al. 2008), in response to wounding, infection and herbivory (Christopher et al. 2004; Azaiez et al. 2009; Duplessis et al. 2009); elevated CO_2 (Taylor et al. 2005), treatment with safener (Rishi et al. 2004), drought (Street et al. 2006), and in relation to cell death (Moreau et al. 2005). In a microarray comparison of the transcriptome of HP and the control cells used here, we observed significant differences in the expression of several hundred genes (Page et al., unpublished). However, apparent differences in gene expression of the PA and the related metabolic pathways were either small and/or not discernible due to the lack of precise characterization of spots on the poplar microarray chips. It is apparent from our earlier studies (Bhatnagar et al. 2001, 2002; Page et al. 2007; Mohapatra et al. 2009, 2010) that: (1) there is

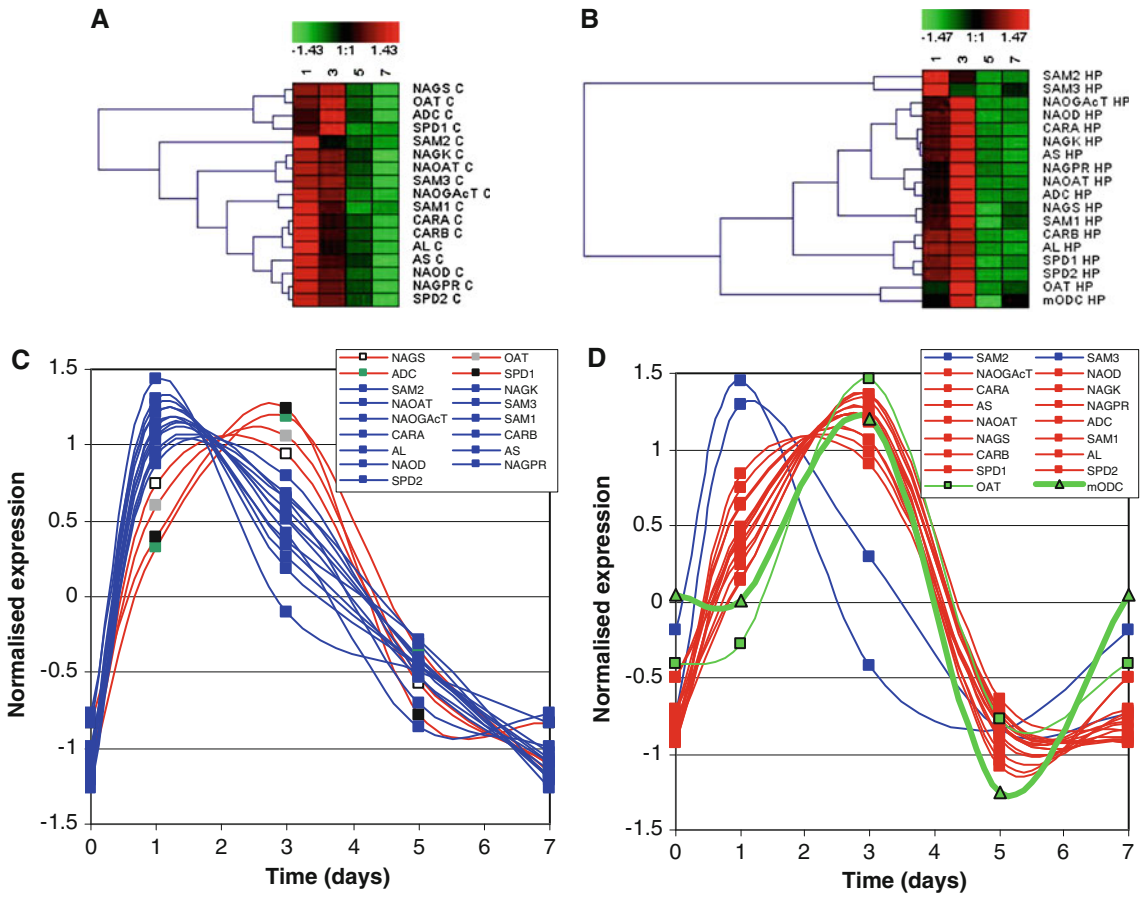

Fig. 5 **a**, **b** Dendrograms and heat maps of all genes shown in Fig. 1 and those from Page et al. (2007) in control and HP cells, respectively. **c**, **d** Expression profiles of all genes shown in Fig. 1 and those from Page et al. (2007) from control and HP cells, respectively, showing main clusters by *color*

several-fold increase in Put production from Orn due to the transgenic mODC with little impact on its biosynthesis from Arg, and (2) the concentrations of Orn, the substrate of ODC (and indirectly that of ADC, since it is a precursor of Arg), in the cells are extremely low relative to the amounts of Put being produced from it. Since both Arg and Orn are produced primarily from Glu in plants, it is logical to hypothesize that the production of Orn from Glu must increase in proportion to its use in Put and Arg production. This increase would have to be several-fold, as steady-state cellular contents of Orn in these cells are extremely low (Mohapatra et al. 2010). While various steps in the pathway for the production of Orn/Arg from Glu are known (Slocum 2005), little is known about the regulation of these steps at the level of transcription of the enzyme proteins or their enzyme activities (cf. Morris 2007; Kalamaki et al. 2009a). Enzyme assays for most of these steps in plants have not been optimized either. Hence, as a first step toward understanding the regulation of this pathway, we compared relative transcript abundance of genes encoding enzymes of the entire pathway (Fig. 1) in control and HP cells of poplar. Using information from the EST and the cDNA sequence databases for Arabidopsis and the genomic sequence of *P. trichocarpa*, we cloned homologous sequences for each gene and used them to design specific RT-qPCR primers for the cell lines used here. The data presented in Table 1 is the first attempt at the annotation of these genes in any plant other than Arabidopsis.

We have presented our data in two formats: (1) changes in relative expression of individual gene(s) involved in Arg/Orn metabolism, and (2) examination of the trend of quantitative changes in groups of genes with time of growth in fresh medium during the 7-day growth cycle. The intent was to examine the level of coordination of gene expression for various enzymes of the entire Glu to Orn/Arg pathway, and then to PA biosynthetic pathway, in order to shed light on the regulation of various steps at the transcript level (Chatterjee and Yuan 2006; Seki et al. 2007).

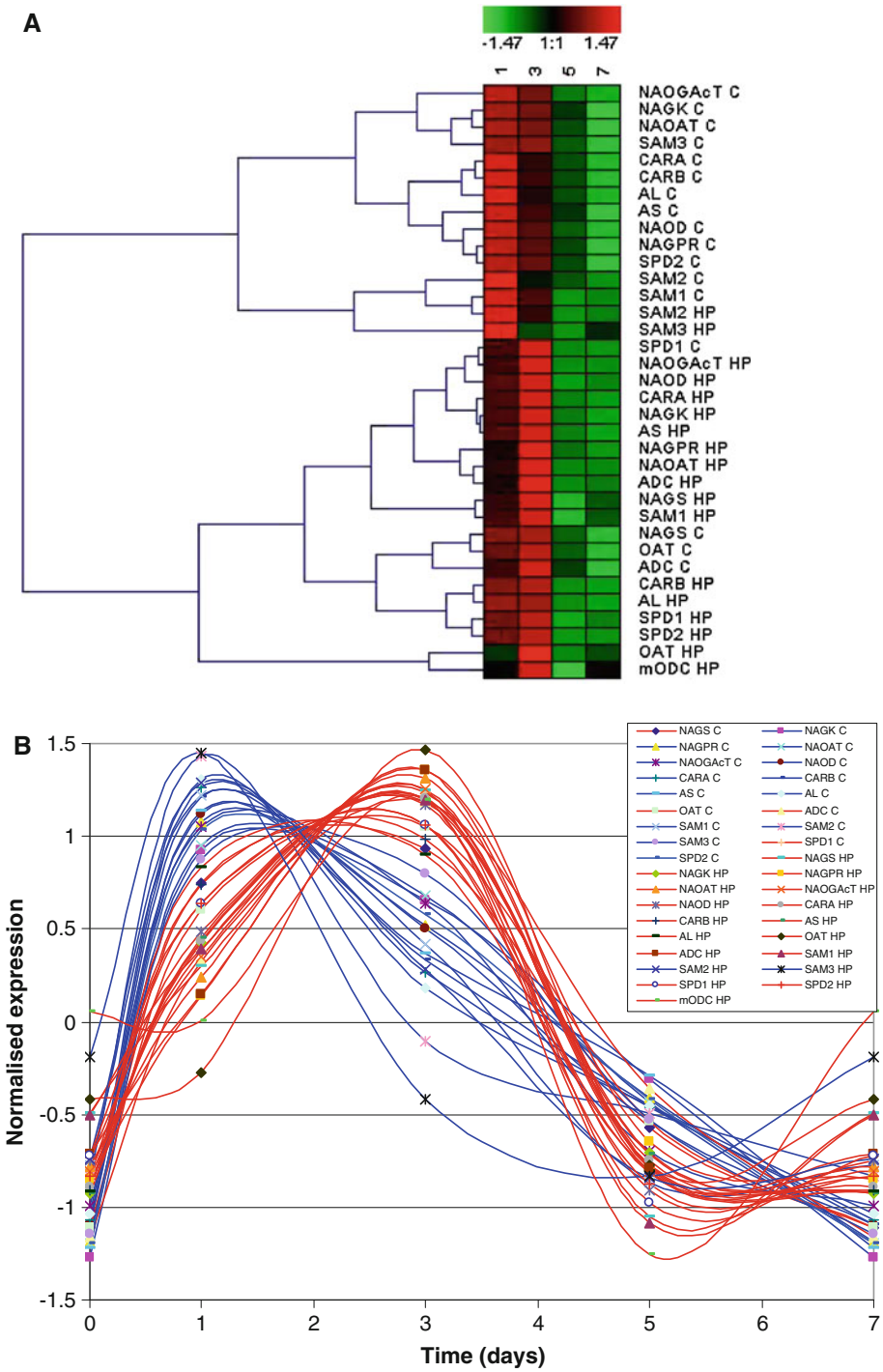

Fig. 6 **a** Dendrogram showing clustering pattern; **b** normalized expression of all genes shown in Fig. 1 and those from Page et al. (2007) over the 7-day culture period. *Red* and *blue* colors are used to show clustering of genes into two main expression patterns; *blue lines* mostly represent genes in control cells and *red lines* those from HP cells. The *letter C* after the gene name = control cells, *HP* high Put cells

Poplar genes involved in Orn/Arg biosynthesis are highly conserved

The gene sequences encoding enzymes involved in the biosynthesis of Orn/Arg from Glu were all identified in the *P. trichocarpa* database based on comparisons with their counterparts in Arabidopsis cDNA and annotated genome databases and as described in Slocum (2005). The sequences of all cloned partial cDNAs of *P. nigra* × *maximowiczii* showed ≥99% sequence identity with those from *P. trichocarpa*. Moreover, the number of cloned cDNAs for each gene in *Pm* matched very well with those in Arabidopsis and also recognizable in the *Pt* database. A similar homology was observed between cloned sequences

of the PA biosynthetic genes whose expression was reported earlier (Page et al. 2007), indicating that genomes of *P. trichocarpa* and the hybrid poplar (*P. nigra* × *maximowiczii*) are highly conserved.

It is well established that in branched pathways that involve multi-directional uses of a single substrate (e.g., Glu), regulation of the pathway often occurs at the branch point by the first enzyme of the sub-pathway. This regulation may occur at transcriptional, post-transcriptional, translational, post-translational, and enzymatic levels via various mechanisms involving feedback inhibition, metabolite sensing, and substrate limitation. The two enzymes NAGS and NAGK at the beginning of the pathway presumably regulate Orn and Arg biosynthesis in bacteria, fungi, and also in plants (Kalamaki et al. 2009a); however, in animals the regulation of cellular Orn is affected more by arginase since most of the Arg comes from dietary intake (Caldovic and Tuchman 2003; Morris 2007). Nevertheless, animals do synthesize Orn/Arg from Glu using a pathway similar to that found in other organisms (Fig. 1). While *NAGS* genes of bacteria and yeast show little evolutionary relationship with each other, the human and mouse genes are highly homologous with the fungal genes (Caldovic and Tuchman 2003). According to Slocum (2005), the N-terminus of the two Arabidopsis *NAGS* gene sequences are more like those in bacteria (and unlike those in fungi and animals), while the sequence representing the kinase domain of these enzymes does not resemble either prokaryotes or eukaryotes. The poplar *NAGK* shows a high degree of sequence identity with that of Arabidopsis, indicating that it is more plant-like than animal-like. Kalamaki et al. (2009a) found a 66–69% amino acid similarity between a cloned tomato *NAGS* and the two Arabidopsis *NAGS* genes. They further showed that overexpression of the tomato gene in Arabidopsis resulted in a significant increase in Orn and citrulline accumulation in the leaves but had little effect on Arg accumulation.

Overall, *NAGK*s of all organisms show relatively high sequence homology (Ramon-Maiques et al. 2002). It is known that Arg inhibits plant NAGK activity, whereas NAG activates it (McKay and Shargool 1981). Like *NAGK*, there is only one known copy each of *NAGPR, NAOAT, NAOGAcT*, OAT and *AL* in Arabidopsis; this is consistent with what we observed in *Pm* and also what we found in the *Pt* genome. On the other hand, while only one each of *NAOD* and *AS* have been reported in Arabidopsis (Slocum 2005); there were two highly homologous sequences in *Pm*, which matched closely with similar sequences in *Pt*. A similar discrepancy was seen for *CARA*, the small subunit of CPS. Two copies of this gene were identified in *Pm*; both were ≥99% identical to their homologues in *Pt*.

Genes of the entire Orn/Arg pathway show coordinated expression

It has been suggested that NAGS, NAGK and possibly NAGPR may constitute a metabolon working as a complex to coordinate this part of the pathway in fungi (Abadjieva et al. 2001; Pauwels et al. 2003); however, nothing is known about such a complex in plants (Slocum 2005; Kalamaki et al. 2009a, b). While reviewing the limited expression data in the literature, Slocum (2005) suggested that in Arabidopsis the relative expression of *NAOGAcT* was greater than that of *NAGS*, which is in contradiction to what we experimentally observed in poplar; here, *NAOGAcT* was the least expressed (in relative terms) gene of the group. This may reflect the contribution of the two different sets of reactions in the biosynthesis of NAG; i.e., due to high rate of cell division, perhaps poplar cells do not recycle the acetyl moiety from acetyl-Orn and primarily use the linear pathway (refer to Fig. 1) for Orn/Arg biosynthesis. This argument is consistent with Slocum's suggestion that the linear pathway is preferred for Orn/Arg biosynthesis under conditions of high demand for Arg; in the transgenic poplar cells, it may be for the high demand for Orn. Slocum further suggested that not only are NAGS, NAGK and NAOGAcT activities subject to inhibition by high cellular contents of Orn and Arg, the expression of their genes may also be inhibited by these products. It would then imply that reduced levels of Orn and Arg should promote the expression of these genes; this is however not what our results show. The HP cells have significantly lower concentrations of both these amino acids as compared to the control cells (Mohapatra et al. 2010), yet there is only a small or no effect on transcripts of any of these genes during the entire 7-day growth cycle. Also, the relative expression of most other genes is similar in the two cell lines or sometimes even lower in HP cells than in control cells. *Argininosuccinate lyase* is the only gene whose expression is higher in the HP cells on most days of analysis. Since Orn synthesis (hence the flux of Glu through the entire pathway) in the HP cells must be several-fold higher than in the control cells, it can be argued that enhanced gene expression is not involved in the regulation of most of these steps.

The results presented here and those published earlier (Page et al. 2007) reveal high degree of coordination in the expression of several genes for enzymes that regulate the biosynthesis of Orn, Arg, GABA, and the higher PAs. Relative expression of 17 of these genes clusters into two major groups in these cells, which differ only in terms of temporal pattern of their expression during the 7-day culture cycle. In control cells, 13 of the 17 genes show peak of expression early in the culture cycle (24–48 h after transfer to fresh medium), while the remainder show a broad peak

around day 3. All genes show the lowest expression around day 7, by which time the cells have reached a stationary phase of growth, and face reduced nutrition in the medium. Slocum (2005), in his concluding remarks, alluded to the possibility of a 'coordinated regulation of Orn pathway enzyme activities at the level of gene expression', but proposed no hypothesis regarding regulation of this coordination. Here we provide experimental evidence for this argument and also a hypothesis to test it. Since the only direct effect of transgenic ODC in HP cells is on Orn utilization, and Orn is the least abundant amino acid in these cells, we propose that this part of the metabolic pathway probably uses Orn as a sensor for its regulation. This idea is consistent with the suggestions of Morris (2006, 2007) that in animals, Orn instead of ODC may act as a trigger/regulatory molecule for PA biosynthesis, and of Slocum (2005) for the regulation of Arg biosynthesis in Arabidopsis. A similar high degree of coordination in the expression of several genes involved in starch biosynthesis has been demonstrated by Smith et al. (2004) and Grennan (2006).

Orn/Arg homeostasis is regulated via change in flux

The absence of major changes in transcripts of the genes for Orn/Arg metabolic enzymes is not surprising; rather, it is in line with the well-known cases of feedback regulation at the enzyme/substrate levels for many cellular metabolites, including amino acids. Several reviews are available on the regulation of Arg metabolism in animals (Pauwels et al. 2003; Caldovic and Tuchman 2003; Morris 2006, 2007); little is however known about the metabolism of Arg, and particularly that of Orn, in plants. While parallels do exist in the interacting pathways of Arg, Orn, Pro and PA metabolism in animals and plants (Caldovic and Tuchman 2003; Slocum 2005; Kalamaki et al. 2009a, b), it is important to draw a contrast between the two in relation to their regulatory mechanisms. Arg does play similar roles in both plants and animals with respect to its utilization into protein synthesis, Orn (and maybe Pro) production (during seed germination), and the production of PAs and NO (nitric oxide). However, in most cases the sources of Arg as well as its catabolism are different in animals and plants. In the former, two enzymes that dominate Arg metabolism are arginase and NO synthase, which regulate the production of Orn and NO, respectively. This is because Arg is a semi-essential amino acid in animals that is supplied mostly through nutrition. Although it has been argued that ODC is the primary regulatory enzyme for PA production in animals, the limited supply of Orn (from Arg) has been repeatedly demonstrated as a key factor in PA accumulation in several types of animal cells; the regulation being provided by arginase (Morris 2006, 2007). In most plant tissues in contrast, the primary source of both Orn and Arg is Glu, and the biosynthesis of Put is controlled by combined actions of ODC and/or ADC. Kalamaki et al. (2009a) showed that while the biosynthesis of Orn can be enhanced by over-expression of *NAGS* (the first step in the Glu-to-Orn pathway); however, its conversion into Arg involves independent regulation. In their transgenic Arabidopsis plants, which accumulated up to ninefold higher amounts of Orn, neither citrulline nor Arg concentrations were enhanced much. Polyamines were not studied in these plants; however, in the absence of ODC in Arabidopsis, one should not expect a significant change in PAs here.

While discussing the central role of Arg in mammalian cells in biosynthesis of Orn, PAs, Pro and NO, Morris (2006) raised a key question with respect to its homeostasis; that is, "What mechanisms exist for sensing Arg concentration in the cells?" He argued that it was possibly through a combination of: (1) sensing changes in the ratio of charged: uncharged $tRNAs^{Arg}$, and (2) Arg acting as a ligand for G-protein-coupled receptor 6A (GPRC6A), which can also sense other amino acids like Orn and Lys (Wellendorph et al. 2005). A similar question can be raised for the role of Orn in plants since it is perhaps more central to the biosynthesis of these metabolites than Arg. While the former mechanism for Orn sensing is not realistic, the latter is a good candidate for sensing cellular Orn concentration in plants. It must be pointed out that only the cytoplasmic Orn is available to ODC and to GPRC6A; and its transport from mitochondria is regulated by specific mitochondrial Orn transporters, e.g., ORNT1 and ORNT2 (Camacho et al. 2003). Similar mitochondrial Orn transporters have been identified in Arabidopsis (Hoyos et al. 2003), but the regulation of their expression has not been studied.

At the metabolic level, some additional questions raised by the present study are: (1) Is there a competition between ODC and OTC for Orn in the production of Put and Arg, respectively? (2) Is there a competition between NAGS, P5CS and GAD for Glu in the production of Orn/Arg, Pro and GABA? All these metabolites respond in a similar way to high Put production (in the HP cells) and also in response to abiotic stress (Minocha et al. 1997, 2000; Bouché and Fromm 2004; Kasukabe et al. 2004; Alcazar et al. 2006; Seki et al. 2007; Kusano et al. 2007); however, little is known about the co-regulation of these pathways. Based on the microarray (Page et al., data not presented here) and RT-qPCR data, it can be argued that most of this regulation in poplar cells is post-transcriptional, even post-translational; i.e., at the biochemical level involving substrate concentrations, co-factors and other factors that affect enzyme activities. A similar conclusion was drawn by Schafleitner et al. (2007) with regard to regulation of Pro accumulation in relation to drought tolerance in Andean potato.

Conclusions

The observed pattern of changes in the expression of genes encoding various enzymes of Orn/Arg biosynthesis in high and low Put producing poplar cells supports the idea of their coordinated expression, and points to the possibility that these genes constitute a well-coordinated pathway which perhaps responds to a common metabolite sensing mechanism for its regulation. The key question is: "To what metabolic change(s) does the entire pathway respond?" Whereas the role of Arg in regulating its own biosynthesis through feedback inhibition of NAGS/NAGK complex and indirectly that of CP via NAG stimulation of CPS is well known, not much has been said about the role of Orn. In the present study, it is Orn whose overconsumption (by transgenic mODC) was targeted. Consequently, the flux of metabolites from Glu to Orn must be enhanced several-folds concomitant with a reduction in its (Orn) cellular content (see also, Mayer and Michael 2003). Thus it can be argued that regulation of the pathway from Glu to Orn may respond to cellular Orn and not to Arg, and further that this regulation occurs mostly at the biochemical level with little change in transcripts of the enzymes.

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