

Transgenic manipulation of a single polyamine in poplar cells affects the accumulation of all amino acids

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Abstract The polyamine metabolic pathway is intricately connected to metabolism of several amino acids. While ornithine and arginine are direct precursors of putrescine, they themselves are synthesized from glutamate in multiple steps involving several enzymes. Additionally, glutamate is an amino group donor for several other amino acids and acts as a substrate for biosynthesis of proline and γ -aminobutyric acid, metabolites that play important roles in plant development and stress response. Suspension cultures of poplar (*Populus nigra* × *maximowiczii*), transformed with a constitutively expressing mouse ornithine decarboxylase gene, were used to study the effect of up-regulation of putrescine biosynthesis (and concomitantly its enhanced catabolism) on cellular contents of various protein and non-protein amino acids. It was observed that up-regulation of putrescine metabolism affected the steady

state concentrations of most amino acids in the cells. While there was a decrease in the cellular contents of glutamine, glutamate, ornithine, arginine, histidine, serine, glycine, cysteine, phenylalanine, tryptophan, aspartate, lysine, leucine and methionine, an increase was seen in the contents of alanine, threonine, valine, isoleucine and γ -aminobutyric acid. An overall increase in percent cellular nitrogen and carbon content was also observed in high putrescine metabolizing cells compared to control cells. It is concluded that genetic manipulation of putrescine biosynthesis affecting ornithine consumption caused a major change in the entire ornithine biosynthetic pathway and had pleiotropic effects on other amino acids and total cellular carbon and nitrogen, as well. We suggest that ornithine plays a key role in regulating this pathway.

Keywords Genetic manipulation · Polyamines · Ornithine decarboxylase · Poplar

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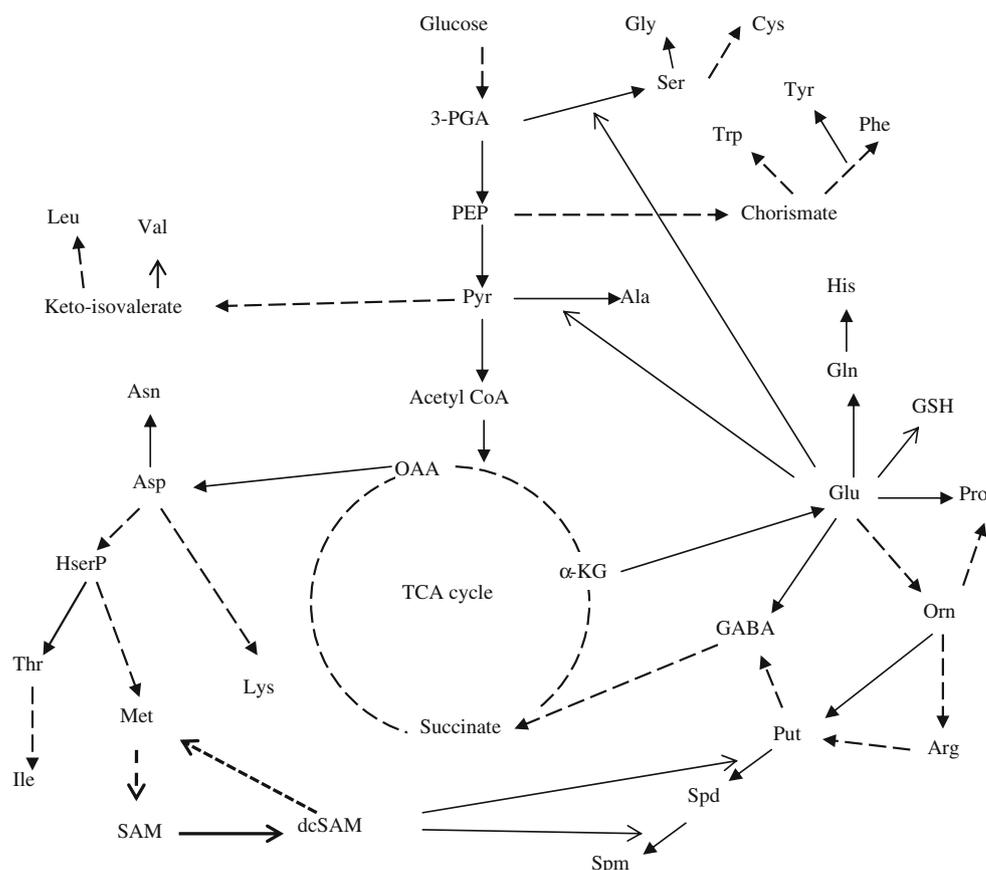
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Introduction

The importance of glutamate (Glu) as a key intermediate in cellular N metabolism is well known (Forde and Lea 2007). It occupies a central position in N assimilation (Coruzzi and Last 2000; Mifflin and Habash 2002; Weber and Flüge 2002; Foyer et al. 2003) and serves as a substrate for synthesis of numerous important metabolites, thus playing a vital role in C and N balance in the cell (Fig. 1). It acts as a direct precursor to the biosynthesis of key amino acids such as Gln and Asp through aminotransferase and transaminase reactions (Coruzzi and Last 2000; Ferrario-Méryl et al. 2000; Forde and Lea 2007), and a precursor to the biosynthesis of Pro (Kocsy et al. 2005; Simon-Sarkadi et al. 2005) and Orn; the latter in turn is involved in the

Fig. 1 Role of Glu as a precursor of various metabolites in plants and interaction of pathways involved in the biosynthesis of amino acids in plants (modified from Singh 1999a; Coruzzi and Last 2000; Forde and Lea 2007)



biosynthesis of Arg and the diamine putrescine (Put). Putrescine produces two other common polyamines (PAs), spermidine (Spd) and spermine (Spm). Direct decarboxylation of Glu by GAD (Glu decarboxylase, EC 4.1.1.15; Bouché and Fromm 2004) and the catabolism of Put together produce the non-protein amino acid γ -aminobutyric acid (GABA), an important metabolite in stress response (Mazzucotelli et al. 2006) and cell signaling (Bouché and Fromm 2004; Fait et al. 2008). By entering the tricarboxylic acid (TCA) cycle via succinate, GABA recycles N and C components of both Glu and Put (Bouché and Fromm 2004).

Amino acid metabolism is intricately linked to glycolysis and the TCA cycle, in that, these two pathways supply the carbon skeletons required for their biosynthesis (Fig. 1). In addition, PAs, which are obligatory requirements for cell survival and growth in all organisms (reviewed in Kusano et al. 2007), are also derived from amino acids Arg and/or Orn. Although genetic manipulations of cellular PAs or individual amino acids have been reported in several plants (Bhatnagar et al. 2001, 2002; Gallardo et al. 2003; Kocsy et al. 2005; Simon-Sarkadi et al. 2005), only a few studies have involved a comprehensive analysis of the effects of such manipulations on the entire set of related metabolites (Mattoo et al. 2006). Such analyses are quite important

because the targeted change(s) will most likely affect homeostasis of all metabolites whose biosyntheses share common substrates and/or precursors. In cases where the target is to achieve improved nutritional properties of a plant, it is even more important to demonstrate that the intended change does not have unintended consequences of adversely affecting other important nutrients. This is in contrast to the situation where genetic manipulation involves either a non-enzymatic protein (e.g., expression of a storage protein gene to enhance protein content, expression of *Bt* or *Cry* genes to impart insect tolerance, expression of a viral coat protein for virus resistance) or a terminal reaction in a pathway leading to a secondary metabolite (e.g., flower color). The present study demonstrates that genetic manipulation of a metabolite whose substrates are part of a network of interacting pathways causes changes that go beyond the immediate pathway.

The genetic manipulation approach has been used often to alter PA metabolism in plants to study their effects on development and/or responses to stress (Kumar and Minocha 1998; Capell et al. 2004; Liu et al. 2007). We have previously reported on the consequences of Put overproduction on several aspects of PA metabolism resulting from transgenic expression of a mouse Orn decarboxylase (mODC—EC1.1.1.1.) in poplar cells (Bhatnagar et al. 2001; Bhatnagar et al. 2002; Quan et al.

2002; Page et al. 2007; Mohapatra et al. 2009). The Put overproducing (“High Put” or HP) cell line with a constitutively expressed *mODC* gene under the control of a 2x35S CaMV promoter was compared with a control cell line expressing a β -glucuronidase (*GUS*) gene under the same promoter. A key finding relevant to the present report was that Put in the control cells was produced primarily from Arg, while in the HP cells it was produced from both Arg and Orn. The former pathway in the HP cells remained unaffected though the latter became a dominant source of Put (Bhatnagar et al. 2001). It was further demonstrated that Put was catabolized rapidly via its enhanced oxidation (Bhatnagar et al. 2002). Since steady state concentrations of Orn are extremely low in poplar cells, it was postulated that increased utilization of Orn in the HP cells would cause a considerable increase in its biosynthesis as compared to the control cells. Consequently, it would result in greater utilization of Glu, the primary source of Orn, and will consequently have a major effect on other amino acids, particularly those whose biosynthesis depends on Glu. In order to test this hypothesis, we analyzed the steady state cellular contents of all protein amino acids along with two non-protein amino acids, GABA and Orn, in the two cell lines on each of the 7 days of the culture period. To further check if increased PA accumulation affected total N and C contents of cells, they were also analyzed. The results clearly show that genetic manipulation of a single step in the complex pathway of Glu to PA metabolism has far-reaching effects not only on amino acids that are derived directly from Glu, but also on most others.

Materials and methods

Cell growth and harvest

The HP (High Put) and the control cell lines of *Populus nigra* \times *maximowiczii* used here have been previously described (Bhatnagar et al. 2001, 2002; Page et al. 2007; Mohapatra et al. 2009). The former (*a.k.a.* 2E—Bhatnagar et al. 2001, 2002) constitutively expresses a mouse *ODC* gene, while the latter expresses the *GUS* gene under the control of an identical 2x35S promoter; both cell lines also express *NPTII* selectable marker gene. The HP cell line was chosen for more detailed metabolic work after screening several transgenic lines (Bhatnagar et al. 2001). The two cell lines used here were maintained as described in Bhatnagar et al. (2002) and Mohapatra et al. (2009). Suspensions were subcultured weekly by adding 7 ml of 7-day-old suspension to 50 ml fresh medium in 125 ml Erlenmeyer flasks, in the presence of 100 mg l⁻¹ kanamycin; the antibiotic was absent for at least 2 weeks before experimentation.

Amino acid analysis

Following vacuum filtration, 200 \pm 20 mg (FW) of cells were mixed with 4 \times volume (i.e., 800 \pm 80 μ l) of 5% (v/v) perchloric acid (PCA) and frozen at -20°C . Prior to dansylation, the freeze–thaw cycle was repeated three times and dansyl-PAs and amino acids were separated and quantified by HPLC, as described in Minocha et al. (1994). Dansyl-PAs were partitioned into toluene fraction (used for PA analysis) and aqueous fraction (used for quantification of amino acids). Asparagine and Tyr could not be analyzed by this method, since they eluted under a single large peak with the solvent front.

Total percentage of carbon and nitrogen

For the total percentage of carbon and nitrogen, cells (about 1 g FW) were harvested, dried at 70 $^{\circ}\text{C}$ and analyzed by a CHNS analyzer (Thermo Scientific CE Elantech Flash EA1112 Soil). National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) samples 1515 (Apple Std) and 1547 (Peach Std) were used for procedure verification.

Statistical analysis

All PA and amino acid analyses were performed using cells collected from three replicate flasks for each cell line at the same time every day for 7 days. Two experiments were conducted in 2005 and additional two in 2007 with the same cell lines to check the stability of these lines and the repeatability of the results over time. The results of the two experiments from 2005 ($n = 6$) are presented here in Figs. 1, 2, 3, 4, 5, 6, 7; however data for Gln and Ala are taken from the 2007 experiments. This is because in 2005, Ala could not be quantified, since it was used to terminate the dansylation reaction and Gln showed a poor peak resolution. In 2007, the procedure was changed slightly to use Asn (instead of Ala) to terminate the dansylation reaction, which allowed quantification of Ala and Gln. This change, however, affected the quantification of Asn and Ile in 2007. Of the 16 amino acids that were reliably quantified in all four experiments, the results were highly repeatable and thus the combined results are presented in Figs. 8 and 9, and Supplementary Tables 1 and 2. High degree of repeatability after 2 years of growth in culture shows the stability of the two cell lines over this time period.

Pooled data for each day were analyzed as series of one-way analysis of variance to determine whether statistically significant differences exist between the two cell lines for a particular day. When *F* values were significant, differences were tested using Tukey’s multiple comparisons test (Systat version 10.2—SYSTAT Inc., Evanston, IL, USA);

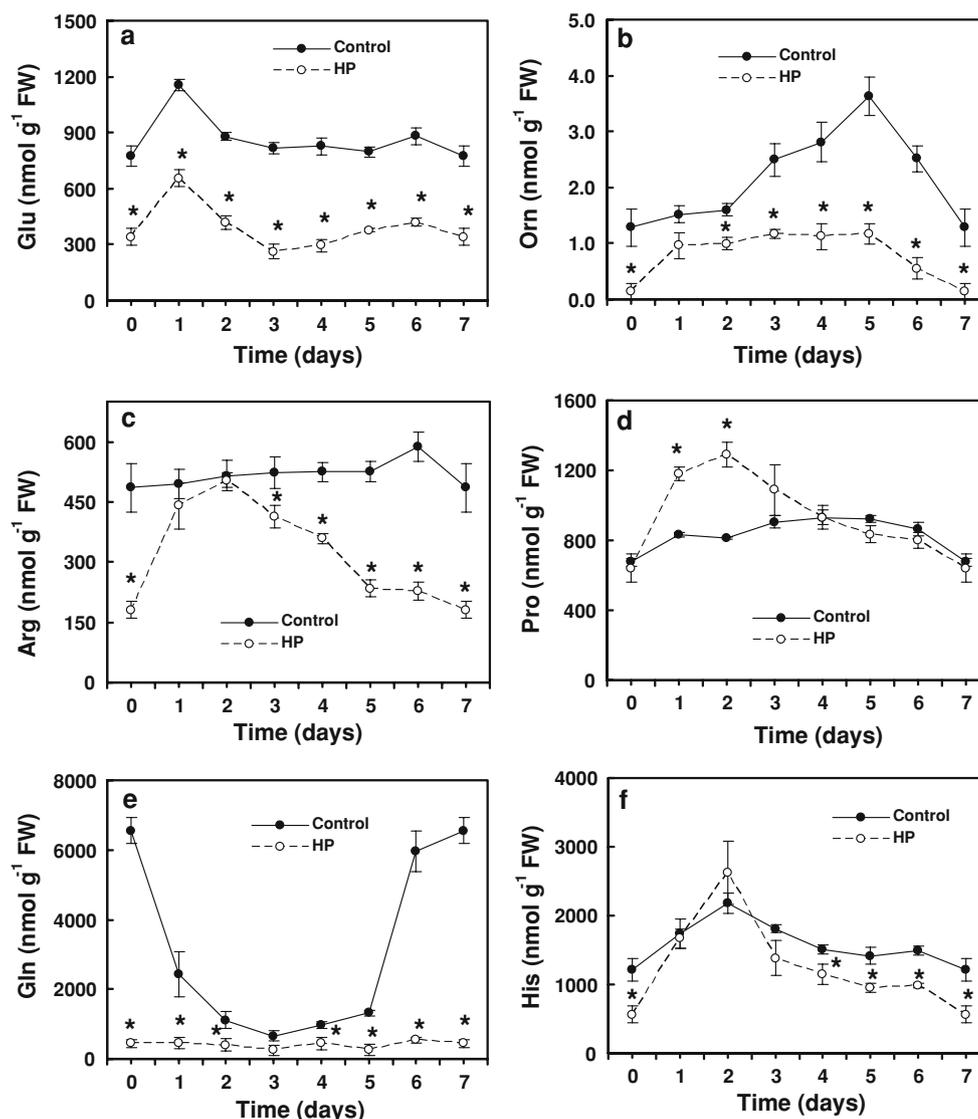


Fig. 2 Cellular contents of **a** glutamic acid, **b** ornithine, **c** arginine, **d** proline, **e** glutamine and **f** histidine in control and HP cells over the 7-day culture cycle. Data are mean (\pm) SE of six replicates from two experiments for all amino acids. An *asterisk* indicates significant

difference ($P \leq 0.05$) between control and HP cells on a given day. Glu and Pro data have been published in Mohapatra et al. (2009) and are presented here to facilitate discussion in relation to other amino acids

P value of ≤ 0.05 was used unless otherwise specified. The data presented here for 0 and 7 days are the same, since the beginning of the 1-week culture cycle was the end of the previous week's cycle. It was essential to repeat these data on the graphs to highlight the fresh medium effects between day 0 and 1 after subculture.

Results

Polyamines and soluble proteins

Cellular concentrations of PCA-soluble PAs in the HP and the control cells have been reported earlier (Bhatnagar

et al. 2002; Page et al. 2007). During the period of the present study, Put in HP cells was fourfold to fivefold higher than the control cells on most days of analysis, and Spm was similar in the two cell lines; of the three PAs, it was present in the least amount. As reported earlier, Spd was higher in the HP cells on some days, but not others. It should be pointed out that poplar cells contain $< 1\%$ of the total amount of PAs in the conjugated or bound forms (Bhatnagar et al. 2002). Soluble protein content in HP cells was almost twice that of control cells for the first 2–3 days of culture (Mohapatra et al. 2009). The growth rates (measured as the yield of fresh mass of cells per flask on any given day) of the two cell lines were quite comparable during the 7-day culture period (Mohapatra et al. 2009).

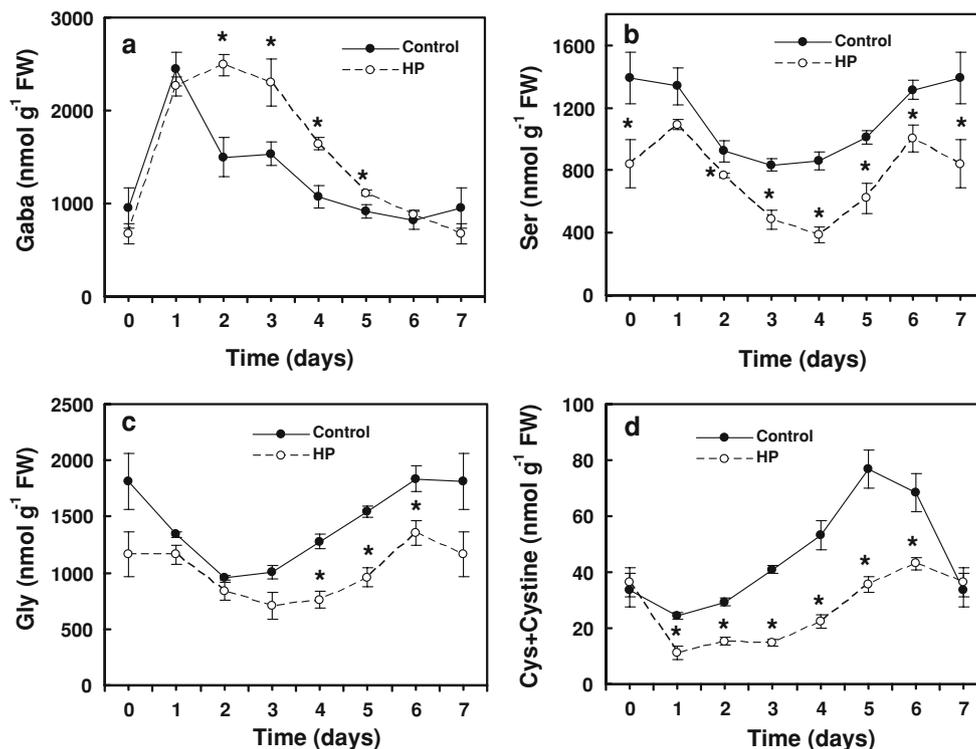


Fig. 3 Cellular contents of **a** GABA, **b** serine, **c** glycine and **d** Cys + cystine in the control and the HP cells over the 7-day culture cycle. Data are mean (\pm) SE of six replicates from two experiments.

An *asterisk* indicates significant difference ($P \leq 0.05$) between control and HP cells on a given day. As in Fig. 2, Gly and Cys data have been published in Mohapatra et al. (2009)

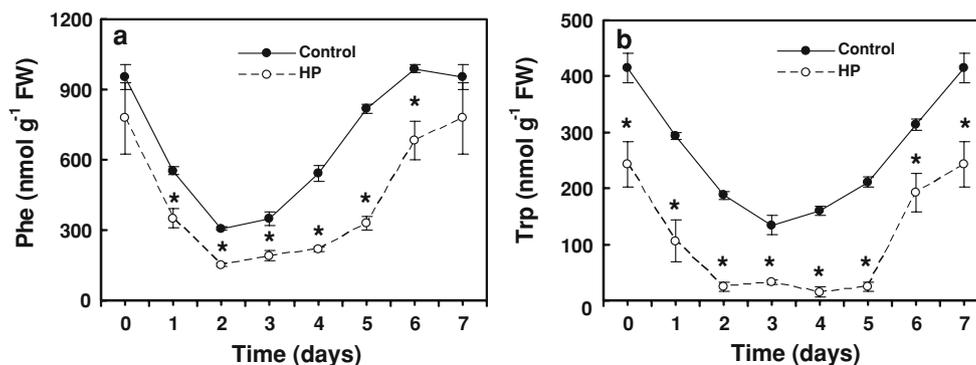


Fig. 4 Cellular contents of **a** phenylalanine and **b** tryptophan in control and HP cells over the 7-day culture cycle. Data are mean (\pm) SE of six replicates from two experiments. An *asterisk* indicates significant difference ($P \leq 0.05$) between control and HP cells on a given day

Amino acids derived from α -ketoglutarate (the glutamate family)

The data for Glu showed that HP cells had significantly lower (almost half) amounts of Glu than the control cells on all 7 days of the week (Fig. 2a; Mohapatra et al., 2009). Within 24 h of transfer to fresh medium, there was approximately a doubling of cellular Glu in both cell lines; this was followed by a parallel decline in both cell lines within the next day. Thereafter, both cell lines showed only minor changes in Glu with the time of culture. Cellular

Orn, a substrate used by transgenic mODC, was present in very low amounts at all times in both cell lines (< 1% that of Glu and < 0.03% of total amino acids on any given day). Furthermore its concentration was significantly lower in HP cells than in the control cells on most days of analysis (Fig. 2b). In the control cells, Arg content remained mostly unchanged throughout the week. However, in the HP cells, its concentration increased on transfer to fresh medium until day 2 and declined thereafter (Fig. 2c). The content of Arg was generally lower than Glu in both cell lines.

Fig. 5 Cellular contents of **a** leucine, **b** valine, **c** isoleucine and **d** alanine in control and HP cells over the 7-day culture cycle. Data are mean (\pm) SE of six replicates from two experiments. An *asterisk* indicates significant difference ($P \leq 0.05$) between control and HP cells on a given day

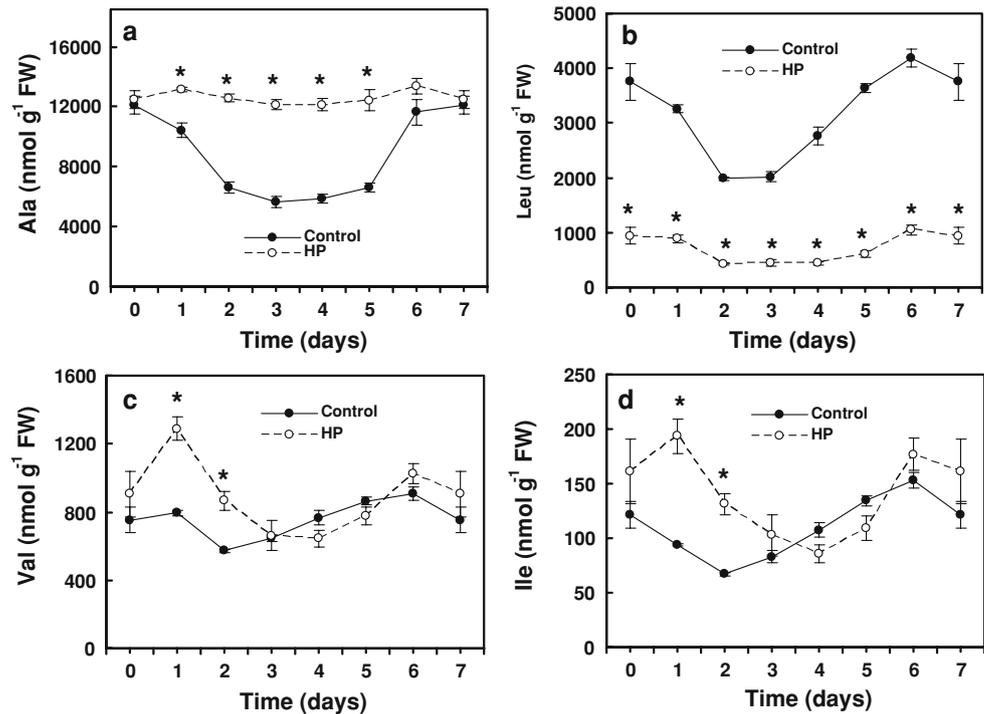
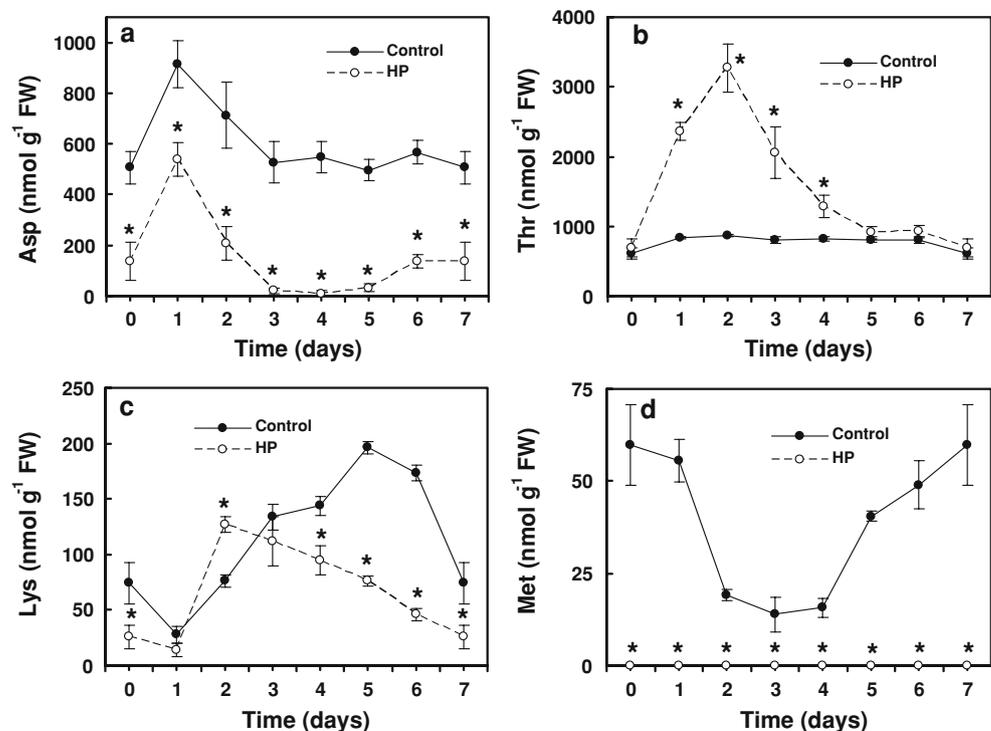


Fig. 6 Cellular contents of **a** aspartate, **b** threonine, **c** lysine, **d** methionine in control and HP cells over the 7-day culture cycle. Data are mean (\pm) SE of six replicates from two experiments (three replicates only for Asp). An *asterisk* indicates significant difference ($P \leq 0.05$) between control and HP cells on a given day



Cellular Pro in the HP cells almost doubled soon after transfer to fresh medium (days 1 and 2), but was comparable to that in control cells on other days of culture (Fig. 2d). Proline concentration was often higher than Arg, but similar to Glu in control cells at most of the times.

Glutamine was higher in control cells as compared to the HP cells, both at the beginning and toward the end of the culture cycle; the differences being severalfold on days 1, 6 and 7 (Fig. 2e). In these cells, Gln showed a sharp decline within 24 h on transfer to fresh medium and then a gradual

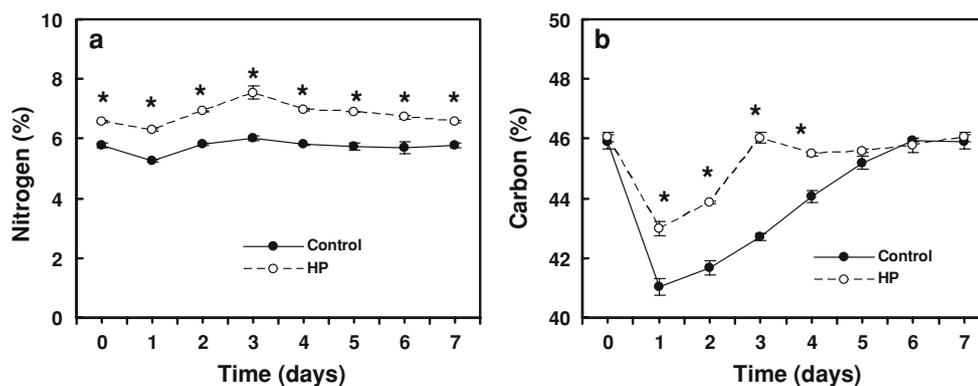


Fig. 7 Percent C and N content in the control and the HP cells over the 7-day culture cycle. Data are mean (\pm) SE of six replicates. An *asterisk* indicates significant difference ($P \leq 0.05$) between control and HP cells on a given day

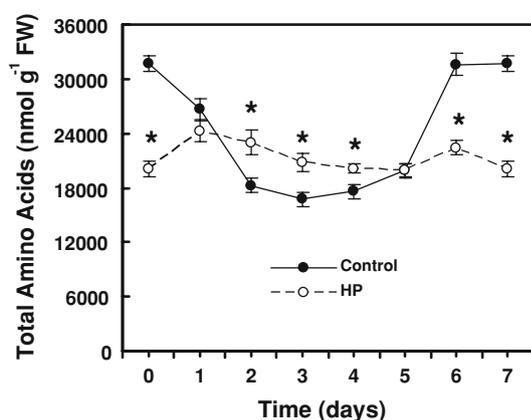


Fig. 8 Total soluble amino acids content in the control and the HP cells over the 7-day culture cycle. Data are mean (\pm) SE of 12 replicates from four experiments. An *asterisk* indicates significant difference ($P \leq 0.05$) between control and HP cells on a given day

increase during the latter half of the growth cycle (i.e., days 5–7). The Gln content of HP cells remained rather low and unchanged during the entire 7 days culture period. Finally His, which is the most basic of this group of amino acids, showed small but significant differences between the two cell lines on several days of analysis (Fig. 2f). Both cell lines showed increase in His content on transfer to fresh medium, with a peak around day 2.

γ -Aminobutyric acid is a non-protein amino acid that can be produced directly from Glu by GAD as well as from the catabolism of Put by diamine oxidase (Fig. 1). Through reactions of the GABA shunt, it serves as an intermediate in recycling C of Glu and Put. A distinct fresh medium effect was seen for GABA content in both cell lines (Fig. 3a), resulting in almost threefold increase within the first day. Thereafter, the content of GABA decreased sharply in the control cells ($\sim 50\%$ reduction from day 1 to 2), but slowly in the HP cells. In the latter, the content of

GABA was higher for at least 4 days during the 7-day culture period.

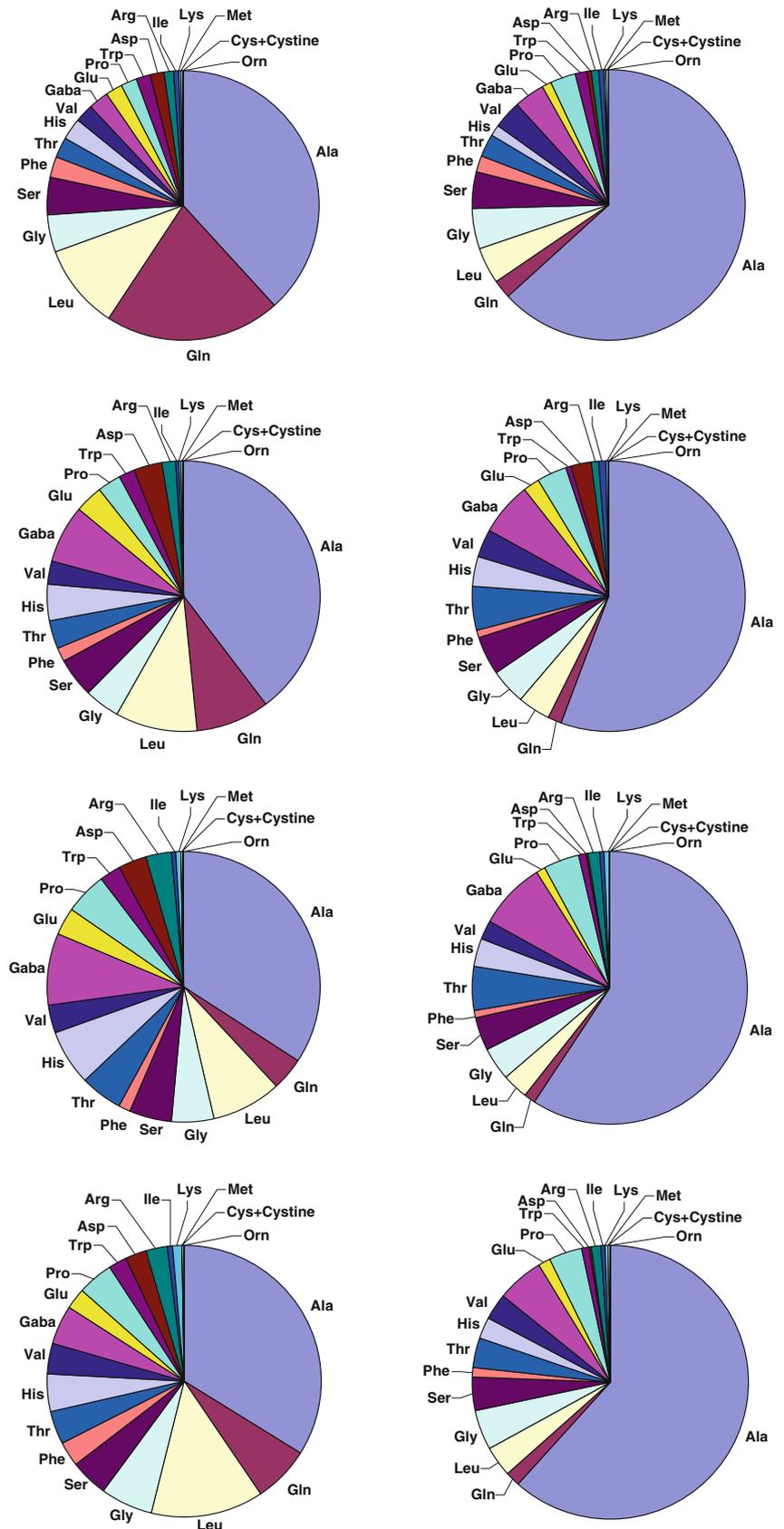
Amino acids derived from 3-phosphoglycerate

Serine and Gly, which are readily interconvertible, are derived largely from 3-phosphoglycerate (Fig. 1), although Gly can also be produced by direct transamination of glyoxalate (Bourguignon et al. 1999). Both pathways use Glu as the donor of the amide group. Ser and Gly were found in a similar concentration range in a given cell line; both showed parallel trend of changes in the two cell lines during the week. There was a decline in their cellular contents on transfer to fresh medium, which was followed by recovery during the latter half of the week (Fig. 3b, c). Both amino acids were significantly lower in HP cells on days 4–6 and Ser was lower even on days 2 and 7. The content of Ser increased transiently on transfer to fresh medium in HP cells (Fig. 3b), but not in the control cells. Cys (including Cystine), which is derived from Ser, was present only in very low amounts (cf. Ser or Gly; $< 0.2\%$ of total amino acids in both cell lines) on any given day (Fig. 3d); both cell lines showed a sharp decline in its content on day 1, which was followed by a gradual rise until day 5 or 6. The HP cells had almost half the contents of Cys as compared to the control cells on any day of analysis.

Aromatic amino acids

Of the three amino acids in this group, two (Tyr and Phe) use Glu as the amide group donor, while Trp takes its amino group from Ser (Siehl 1999). Their carbon skeleton comes from PEP via chorismate (Fig. 1). The contents of Phe and Trp varied parallel to each other in the two cell lines during the week (Fig. 4), although Phe (Fig. 4a) was higher than Trp (Fig. 4b) on any given day of analysis.

Fig. 9 Pie charts representing relative contents of different amino acids as a percent of total soluble amino acid pool in the control and HP cells on different days of the 7-day culture period. Data are mean of 12 replicates from four experiments. For more details, see Supplementary data



Both showed a sharp (> twofold) decline in the two cell lines on transfer to fresh medium and an increase during the latter half of the week. Differences between the two cell lines were significant for both on almost all days of culture; both being lower in the HP cells versus the control cells. Tyrosine was not resolved by the HPLC method used here; therefore, data for this amino acid are not available.

Alanine and branched-chain amino acids

Leucine, Val and Ile constitute a group of branched-chain amino acids, the former two being derived from pyruvate (Pyr) and the third from oxaloacetate (OAA). However, the enzymes involved in Val and Ile biosynthesis perform parallel reactions using different substrates (Fig. 1). On the other hand, Ala can also be derived from Pyr by direct transamination using Glu or GABA (Rhodes et al. 1999); therefore, its discussion is included here. In control cells, Ala showed a greater fluctuation on different days than in the HP cells; it was always significantly higher in the latter as compared to the former on most days of the week (Fig. 5a). Control cells showed a significant reduction in Ala in response to fresh medium; no such change was seen in the HP cells.

Of the three amino acids in this group, Leu was predominant in both cell lines (Fig. 9; Supplementary Tables 1, 2), followed by either Val or Ile on most of the days of analyses. It was often severalfold higher than the next abundant amino acid of this group (Fig. 9; Supplementary Tables 1, 2). The contents of Leu were at least threefold lower in the HP than the control cells on most days of the week (Fig. 5b). As with Ala, in control cells, Leu content declined to nearly 50% during the first 2 days of culture in the fresh medium; this was followed by a consistent increase during the next 3 days. In HP cells, changes in Leu with time paralleled those in Ala, but the relative concentrations of these two amino acids in the two cell lines were opposite and severalfold different.

Valine (Fig. 5c) and Ile (Fig. 5d) showed almost identical pattern of changes with time in the two cell lines with a transient increase for both in the HP cells on transfer to fresh medium. In control cells, both declined initially, but increased in concentration after 2 days. Valine in both cell lines was fivefold to sevenfold higher than Ile on any given day.

Amino acids of the aspartate family

Six amino acids including Ile (discussed above under branched-chain amino acids) that constitute the Asp family utilize OAA as the carbon skeleton (Fig. 1); five of them could be quantified here. Each showed somewhat different pattern of changes during the week, a different response to

the presence of high Put in HP cells, and different response on transfer to fresh medium (Fig. 6). The two dominant amino acids of this group in poplar cells were Thr and Asp, while Met was the least abundant. Cellular Asp almost doubled within a day of transfer to fresh medium in both cell lines and declined soon thereafter; its concentration was always significantly lower in the HP versus the control cells on any given day (Fig. 6a). Threonine did not change much during the entire period of culture in the control cells, but showed a rapid (fourfold) increase in HP cells for the first 2 days in fresh medium (Fig. 6b). Following that, Thr declined to its original level (of day 0) by day 5 and stayed unchanged for the next 2 days.

Lysine declined in both cell lines during the first 24 h of culture in the fresh medium; thereafter, its concentration increased transiently in the HP cells, but more consistently in the control cells (Fig. 6c). Cellular Lys concentrations were significantly lower in HP than in the control cells from day 4 onwards. Met was the lowest in amount among the amino acids of this group, and also low overall (< 0.25% of the total amino acids in control and < 0.06% in HP cells), being close to detection limits in the HP cells on most days of analysis. Its content was significantly higher in control as compared to HP cells on all days of culture.

Percentage of cellular carbon and nitrogen

Percentages of both C and N were significantly higher in HP cells than in the control cells on most days of culture (Fig. 7). The C percentage content decreased slightly within 24 h of transfer to fresh medium, recovering to its highest levels by day 3 in HP cells and day 5 in the control cells. Percentage of C content was similar in the two cell lines thereafter. Percentage of N was significantly higher in HP cells on all 7 days of the culture period. With time of culture, a small decrease in the percentage of N was seen in both cell lines on transfer to the fresh medium.

Relative changes in total amino acids

Changes in total amounts of PCA-soluble amino acids in the two cell lines on different days of the 7-day culture period are shown in Fig. 8. During the mid-week, a time when the cultures are in log phase of growth (in terms of cell division), the total amino acid pool in HP cells was significantly greater than that in the control cells. On transfer to fresh medium, the total soluble amino acids in control cells declined by almost half during the first 2 days; in the HP cells, on the other hand, there was a small increase. During the last 2 days of culture, a reverse phenomenon was seen in the two cell lines; i.e., control cells showed a sharp increase and HP cells showed a small decrease.

Figure 9 shows a summary of the relative abundance of various amino acids as percentage of total PCA-soluble amino acid pool in the two cell lines on four different days during the week-long culture period; for numerical values (as percentage of total) for each amino acid on all 7 days, see Supplementary Tables 1 and 2. It is apparent from the results that different amino acids varied differently in their relative abundance, as well as with time of culture, in the two cell lines. While Ala remained the predominant amino acid in both cell lines on all days of culture, its relative abundance did change in response to high Put production in the HP cells. Among the amino acids that were present in high relative concentrations and showed major changes with time in the two cell lines are Gln, Leu, His and GABA in control cells and GABA and Thr in the HP cells. Among the least abundant amino acids in both cell lines on most days were Met, Cys and Orn. It should be pointed out that data for both the total soluble amino acids and their relative abundance are derived from four separate experiments done 2 years apart.

Discussion

Ornithine plays a key role in the regulation of Pro, GABA and polyamine biosynthesis

Similarities between documented (and postulated) roles of Pro, Put and GABA, particularly under conditions of abiotic stress, are remarkable. While no specific mechanism has been documented, all three often respond to a variety of stress treatments in a similar manner (Aziz and Larher 1995; Aziz et al. 1998; Houdusse et al. 2005; Simon-Sarkadi et al. 2005, 2006; Sharma and Dietz 2006; Seki et al. 2007). They are all relatively rich in N, but differ in the charges they carry under physiological conditions, which may call for different roles for these metabolites of the interacting sub-pathways shown in Fig. 1. Since they all are derived from Glu, it is possible that a common signal transduction mechanism (molecule) triggers all three sub-pathways in a coordinated manner. While the mechanism remains unknown, we believe that Orn plays a major role in this signal transduction, perhaps acting both as a sensory and a regulatory molecule. If that was the case, the most likely site of regulation will be the initial reactions that direct Glu into the three interacting pathways (Fig. 1); i.e., the steps involving NAGS (Slocum 2005), P5CS (Roosens et al. 2002; Székely et al. 2008) and GAD (Bouché and Fromm 2004).

Ornithine is not only the target substrate of the transgenic mODC in HP cells used here, but also an intermediate in the formation of Arg (Slocum 2005), and presumably Pro (Roosens et al. 2002), both of which are

produced in relatively large quantities. Since its steady state cellular concentration is extremely low, compared to most other amino acids in both cell lines, it can be safely argued that the reactions that produce Orn must keep pace with its utilization in Put, Arg, and Pro production. While the regulation of Orn biosynthesis is not well understood, at least a dozen enzymatic steps are involved in the sub-pathways leading to Orn, Arg and Pro biosynthesis in plants (Slocum 2005). A logical hypothesis for the involvement of Orn in signal transduction regulating this entire set of sub-pathways would entail a sensing mechanism for monitoring its content in the cells, which would trigger the entire pathway starting with Glu. Morris (2007) has implicated Arg as a sensor molecule to regulate its own cellular content in animals. It should however be pointed out that in animals, nutritional Arg is the starting point for biosynthesis of some of the same metabolites (Orn, Pro, Glu and PAs) as Glu is in plants, where Arg is mostly a terminal product for use in protein synthesis, PA biosynthesis and storage of excess N. Whereas Arg with its multiple roles in the production of urea, PAs, Pro, GABA and nitric oxide may be a sensory molecule in animals, Orn, with a parallel set of roles in the production of these compounds from Glu, may play a similar role in plants. Two mechanisms suggested by Morris (2007) for Arg sensing are via the ratio of charged to uncharged tRNA^{Arg} and via G-Protein-coupled Receptor 6A. Whereas the former is not a possibility for Orn (since it is a non-protein amino acid), parallels to the latter mechanism for regulation by Orn are also not known at present.

Glutamate is the precursor of a large number of nitrogenous compounds, in plants in addition to being a common constituent of proteins and the direct source of N for most other amino acids (Fig. 1; Singh 1999a; Forde and Lea 2007). Thus, its cellular contents are subject to numerous regulatory signals, both on the production side and the consumption side (Forde and Lea 2007). How quickly do plant cells respond to changes in the demand for Glu in various metabolic pathways, is not understood. In the present case, since Glu is the primary source of Orn whose consumption is increased severalfold by the mODC, it can be argued that its flux through this part of the pathway must also keep pace with its consumption; this, however, requires experimental confirmation.

Morandini (2009) has discussed various mechanisms of regulating flux rates in related pathways, which share common substrates. One mechanism to modulate biosynthesis of a cellular metabolite without changes in relative amounts of the enzymes (or their transcripts) is via increased demand of the product. Zhu and Galili (2003) demonstrated, for example, that lysine content in *Arabidopsis* was increased severalfold by manipulating its degradation. The decrease in Glu as well as Gln in the HP

cells is an indication of their increased utilization in this set of reactions. Our quantitative analysis of transcripts of all genes involved in Orn/Arg biosynthesis (Page A, Minocha R and Minocha SC to be published elsewhere) show only minor differences between the two cell lines, which further supports the importance of biochemical regulation of this pathway through increased demand of the product.

Other amino acids respond to reduction in Glu

Once the demand for Orn production from Glu in response to its increased utilization by mODC and for continued production of Arg have been met, the HP cells then must adjust to the reduction in Glu availability. The resultant decreases in cellular contents of Gln, His, Arg, Ser, Gly, Phe, Trp, Asp, Lys, Leu, Cys and Met (Fig. 9; Supplementary Tables 1, 2) are reflection of such an adjustment. It is interesting to note that steady state concentrations of each amino acid varied independently with the time of culture, particularly with respect to change in response to the fresh medium. Lower Gln content in the HP cells is consistent with its use in replenishing Glu being used in Orn/Arg production. Likewise, a lower content of Arg in HP cells is probably due to its reduced production from Orn and its continued utilization by ADC. We have shown earlier (Bhatnagar et al. 2001) that Arg is the primary source of PAs in control cells and its utilization for Put accumulation in HP cells is not affected by the presence of transgenic mODC. It is also noteworthy that two amino acids, Ala and GABA, whose cellular concentrations were higher in HP cells, are both related to Put catabolism, which increases concomitant with its increased production (Bhatnagar et al. 2002).

The changes with time in amino acids within each group do not always follow parallel trends, showing independent regulation of each sub-pathway (Singh 1999a). For example, seven different enzymes, each with several isoforms and a family of genes encoding them (Azevedo et al. 2006; Curien et al. 2007), regulate Asp family amino acids. Though the primary source of amide group for all members of this family (except Asn) is Glu, and the key regulatory enzyme is Asp aminotransferase, different amino acids of this group show different trends of changes with time in the two cell lines. While Asp, Lys and Met were lower in HP cells throughout the growth period, Thr and Ile were higher in these cells during first few days. This is consistent with the suggestion of Curien et al. (2007) that even though all seven enzymes regulating this set of reactions are allosterically controlled and show common features in their structural and functional organization; yet they are regulated differently.

Alanine is a product of reductive amination of Pyr, catalyzed by Ala aminotransferase (AT, EC 2.7.6.1), where

the amino group is donated by Glu or by GABA (Coruzzi and Last 2000). On several days of the week, Ala was apparently the most abundant amino acid in both control and the HP cells and was significantly higher in the latter (Supplementary Tables 1, 2). According to de Sousa and Sodek (2003), Ala is a major product of anaerobic metabolism in plants, due to the induction of AT under these conditions. Since the poplar cells used in our study were grown in liquid medium, perhaps under limited availability of oxygen, it is not surprising that Ala is the most abundant amino acid in these cells on most days of the week. Alanine naturally occurs in two forms (α and β), which were not distinguished by the HPLC method used here. While α -Ala is synthesized directly from Pyr (Coruzzi and Last 2000), β -Ala in plants is produced by degradation of Spd, Spm, propionate and uracil (Terano and Suzuki 1978; Raman and Rathinasabapathi 2004). Our metabolomic analysis (which could distinguish between α and β Ala) of the two cell lines also shows a major increase in β -Ala in the HP cells (Minocha SC et al. data to be presented elsewhere).

The profile of changes in Val and Ile accumulation in both cell lines was almost identical (Fig. 5b, c), though the two were parts of different sub-pathways, and Val content in both cell lines was fivefold to sixfold higher than Ile at any given time. This is consistent with the arguments (1) that the two sub-pathways utilize the same four enzymes which work on different substrates (Singh 1999b), and (2) that Val biosynthesis is less subject to feedback inhibition by Ile than is Ile biosynthesis (Halgand et al. 2002).

The two sulfur containing amino acids, Met and Cys are also synthesized in two separate pathways; the latter acts as the primary source of S for the former (Hoefgen and Hesse 2007). Both amino acids were present in relatively small amounts (less than 0.3% of the total soluble amino acid pool) and were lesser in the HP cells than in the control cells.

Total cellular C and N respond to increased N utilization in the amino acid and polyamine pathways

Genetic manipulation of the biosynthesis of a single amino acid causing a change in the cellular content of other amino acids has been previously reported. Simon-Sarkadi et al. (2005, 2006) showed that genetic manipulation of Pro biosynthesis in soybean altered the concentrations of several other amino acids, whether or not they were directly related to Pro metabolism. Increase in Pro concentration in response to simultaneous drought and heat stress was accompanied by concomitant increase in Glu, but a reduction in Arg and GABA; there also was an increase in amino acids of the Asp family in the Pro-overproducing transformants. It was concluded that

reduction in Arg was due to increased utilization of Orn in Pro production, and decline in GABA was the result of redirecting Glu for Pro biosynthesis. These observations indicated a possible competition for Glu among these sub-pathways. The data presented here do not point to the existence of any competition between Pro, Arg and Put biosynthesis from Glu; however, a reduction in GABA production via GAD cannot be ruled out. Unfortunately, no data on PA contents were provided by Simon-Sarkadi et al.

Zhu and Galili (2003) found that increased accumulation of Lys in *Arabidopsis*, achieved by reduction in its degradation, was also accompanied by changes in several other amino acids. In HP poplar cells, increased consumption of Glu for Orn production and increased utilization of the latter by mODC may be directly responsible for decreases in steady state levels of Orn, Arg and His, as well as the precursors Glu and Gln; the increase in GABA on the other hand is perhaps due to increased catabolism of Put (Bhatnagar et al. 2002). This is further substantiated by the observed increase in succinate in the HP cells (Minocha SC et al., metabolomic analysis to be published elsewhere). Moreover, since the observed reduction in Glu molar concentration in HP cells is smaller than its utilization in the production of Put via both ODC and ADC, it can further be argued that the biosynthesis of Glu (i.e., total N assimilation) had to be enhanced more than just to meet the deficit due to Orn/Arg production in these cells. This would necessitate additional N assimilation; the data presented in Fig. 7 lend further support to this argument.

Foyer et al. (2003) and Miller et al. (2007) have reviewed the regulation of total N acquisition in plants; they suggested that primary N assimilation is controlled (negatively) by the total N status of plants in a complex manner involving soluble protein content, as well as soluble amino acid pool. No mention is made in their discussion of the role of cellular PAs. Our results indicate that total N and C assimilation may be impacted positively by increased utilization of N and C in PA biosynthesis, though soluble protein content and the total amino acid pool may be higher, as is the situation in the HP cells. It is also possible that it is not the total amino acid pool, but the concentrations of certain specific amino acids (e.g., minor amino acids, see Foyer et al. 2003) that control N and C assimilation by the cells.

It must also be pointed out that the interpretations on metabolic regulation are often based on the assumption that all substrates are freely available to the enzymes within a cell; this assumption is made for the discussion presented here as well. In reality, however, subcellular compartmentation of metabolites is often an impediment to such free interactions.

Conclusions

Although pathways for the biosynthesis of various amino acids are regulated independently, each responding to a complex interaction of feedback inhibitory mechanisms and the availability of inducible and/or constitutive enzymes, perturbation in the biosynthesis or consumption of a single substrate like Orn, shows pleiotropic effects on the entire set of amino acid biosynthetic pathways. This is particularly intriguing since the steady state amounts of Orn in these cells are extremely low as compared to other amino acids. On the other hand, since both its major products (i.e., Arg and Put) are present in hundreds of folds higher concentrations, it can be safely argued that metabolic flux through Orn must be very high; it must also be further enhanced by its increased consumption by the mODC in HP cells. We conclude that steady state cellular Orn concentration may play an important role as a sensor in the signal transduction pathway that regulates its biosynthesis from Glu. This study also suggests the need for a thorough metabolic analysis of the transgenic plants, particularly when a key metabolite is the target of genetic manipulation.

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