Physiological responses of wild type and putrescine-overproducing transgenic cells of poplar to variations in the form and concentration of nitrogen in the medium

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Summary We determined: (a) the physiological consequences of overproduction of putrescine in transgenic poplar (Populus nigra × maximoviczii) cells expressing an ornithine decarboxylase transgene; and (b) effects of variation in nitrogen (N) concentration of the medium on cellular polyamine concentration in transgenic and non-transgenic cells. Cells grown in the presence of supplemental (to the normal concentrations of N sources in the growth medium) and reduced amounts of NH4NO3 and KNO3 were used to study effects on membrane permeability, mitochondrial respiratory activity, protein accumulation, growth rates and changes in cellular polyamine concentration. The N concentration of the MS medium was not a limiting factor for continued overproduction of putrescine in transgenic cells. However, continued supplies of NH4+ and NO3− were required to maintain homeostatic amounts of putrescine in both cell lines. The presence of high amounts of putrescine in transgenic cells had significant effects on the physiological parameters measured. Compared with non-transgenic cells, transgenic cells had greater plasma membrane permeability, less tolerance to NH4NO3, more tolerance to KNO3, and accumulated higher amounts of soluble protein.

Keywords: ammonia, cell viability, mitochondrial activity, nitrate, ornithine decarboxylase, polyamines, spermidine, spermine.

Introduction Genetic manipulation of the polyamine (PA) biosynthetic pathway has been the target of numerous studies in recent years (Andersen et al. 1998, Capell et al. 1998, Kumar and Minocha 1998, Bhatnagar et al. 2001, 2002, Mehta et al. 2002). Polyamines constitute a major group of nitrogen-rich metabolites that can accumulate in millimolar quantities in plant cells (Minocha and Minocha 1995, Cohen 1998). The diverse roles of PAs in plants include storing excess N, reducing NH4+/NO3− toxicity, as well as specific roles in interacting with DNA, RNA and other macromolecules to stabilize them and to regulate their transcription or translation (Slocum and Flores 1991, Cohen 1998). In response to physical or chemical stress, plant cells often accumulate large amounts of proline (Pro), putrescine (Put) and γ-aminobutyric acid (GABA), all of which are derived from glutamate (Glu) (Minocha et al. 1992, 1996, 1997, 2000, Bouchereau et al. 1999, Shelp et al. 1999, Wargo et al. 2002). Pathways for PA and ethylene biosynthesis share a common precursor, S-adenosylmethionine (SAM), suggesting that PA metabolism may compete with ethylene metabolism in plants (Minocha 1988). Cellular PAs can be both up- and down-regulated by transgenic manipulation (Capell et al. 1998, Kumar and Minocha 1998, Bhatnagar et al. 2001, 2002); however, the effects of enhanced PA synthesis on the cellular physiology of the host plant or the transgenic cells have been studied in only a few cases (Roy and Wu 2001, Mehta et al. 2002). Because PAs can sequester relatively large amounts of reduced N in the cell, and increased N demand in response to up-regulation of PA biosynthesis in transgenic cells results in increased N uptake by the cells (Bhatnagar et al. 2001), we postulate that up- and down-regulation of PA biosynthesis depends on N availability.

The smallest of the three common PAs, Put, is synthesized from either arginine (Arg) or ornithine (Orn) by reactions catalyzed by Arg decarboxylase (ADC) and Orn decarboxylase (ODC), respectively (Cohen 1998, Figure 1). Sequential additions of aminopropyl groups derived from decarboxylated SAM to Put lead to the synthesis of spermidine (Spd) and spermine (Spm). The precursors of Orn and Arg are synthesized from glutamine (Gln) and Glu (Figure 1). Glutamate also serves as a precursor of proline (Pro); Arg can be converted back to Orn via the urea cycle; and Pro can be converted into Glu by the Pro shunt (Coruzzi and Last 2000). Despite these relationships, few studies have analyzed PA metabolism in response to either excess or limiting amounts of N.
Although many cells and tissues in culture can utilize either NH₄⁺ or NO₃⁻ as the sole N source, others require a particular N form (Halperin and Wetherell 1965, Kirby et al. 1987). In either case, the primary entry point for N into the metabolic pathway is NH₄⁺. Because PA biosynthesis depends on the availability of reduced N, the increased production of PAs in odc-transgenic cells must depend on an exogenous N supply.

This study is a continuation of our efforts to understand the impact of genetically manipulating a single reaction on the regulation of the PA biosynthetic pathway, and on pathways that interact with intermediates of the PA pathway (Figure 1). We report on the physiological responses of non-transgenic (NT) and transgenic (2E) cells of poplar (Populus nigra × maximowiczii) that overproduce Put (because of the constitutive expression of a mouse odc gene) to variations in the form and concentration of N in the medium. Specifically, we determined: (1) how increased PA metabolism affects membrane permeability, mitochondrial activity and protein accumulation; and (2) how PAs respond to variation in N supply.

Materials and methods

Transformation and cell culture

The plasmid pCW122-odc, which contains a truncated mouse odc cDNA regulated by a 2× 35S CaMV promoter along with the nptII gene under the control of a single 35S CaMV promoter, was biolistically bombarded into poplar (Populus nigra × maximowiczii) cells grown in suspension culture (Bhatnagar et al. 2001, 2002). Liquid and solid cultures of both the non-transgenic (NT) and the transgenic (2E) lines were maintained in Murashige and Skoog (1962) medium containing B5 vitamins (Gamborg et al. 1968), 2% (w/v) sucrose and 0.5 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D). Suspension cultures were subcultured every 7 days by transferring 7 ml of cell suspension to 50 ml of fresh medium in 125-ml Erlenmeyer flasks. Cell suspension cultures were shaken on a gyratory shaker at 160 rpm at 25 ± 2 °C (Bhatnagar et al. 2001, 2002).

Experimental treatments

Aliquots of 7-day-old cell suspensions (NT or 2E) were centrifuged at 2000 g for 2 min to obtain packed cell volumes of 20 or 25 ml. Cells were resuspended in 350 ml of fresh medium from which 10-ml aliquots were transferred to 50-ml flasks to which 5, 10, 20, 40, or 60 mM NH₄NO₃ or 5, 10, 20, 40, 60 or 80 mM KNO₃ was added in addition to the 20.6 mM NH₄NO₃ and 18.8 mM KNO₃ already present in the MS medium. In some experiments (where specified), these additions were made to 3-day-old cultures maintained in 50-ml flasks. The cells were collected on Miracloth (Calbiochem, La Jolla, CA) by vacuum filtration and washed twice with an equal volume of fresh growth medium. After recording the total pellet fresh mass (FW), cells were subdivided into fractions for: (a) Evan’s Blue retention assay for membrane integrity; (b) MTT reduction assay for mitochondrial activity; (c) determination of soluble protein content; and (d) analysis of PAs. Experiments were repeated at least three times with 3–4 replicates each time.
For experiments involving treatments with N concentrations lower than the amount present in MS medium, 3-day-old cells were washed with NH$_4$NO$_3$-free medium or total N-free medium by centrifugation, resuspended in NH$_4$NO$_3$-free medium or N-free medium, and NH$_4$NO$_3$ or NH$_4$Cl was then added. Treatments included: unwashed controls, no NH$_4$NO$_3$, 20.61 mM NH$_4$NO$_3$ (concentration in MS medium), 4.12 mM mixed with 1.0 ml of medium containing 0.25 µg ml$^{-1}$ of MTT to quantify mitochondrial activity. Briefly, 100 mg FW of cells was used with minor modifications (Minocha et al. 2001) to quantify mitochondrial activity. The procedure of Mosmann (1983) for mammalian cells as modified by Ikegawa et al. (1998, 2000, Minocha et al. 2001). For this assay, 100 mg (FW) of cells was suspended in 0.05% (w/v in water) perchloric acid (PCA), and the samples were frozen (–20 °C) and thawed (3–4 h at room temperature) three times and centrifuged at 13,000 g for 10 min. A 100-µl aliquot of supernatant was danylized and quantified by high performance liquid chromatography as described by Minocha et al. (1990, 1994).

Quantitation of plasma membrane integrity (cell viability)

Evan’s Blue is a non-permeating dye that can enter the cells only through damaged plasma membranes (Ikegawa et al. 1998, 2000, Minocha et al. 2001). For this assay, 100 mg (FW) of cells was suspended in 0.05% (w/v in water) Evan’s Blue solution and incubated for 15 min at room temperature. Cells were collected by centrifugation at 16,000 g for 5 min and washed with distilled water until no more dye was eluted. The trapped dye was then released by adding 1.0 ml of 1% (w/v) SDS and ultrasonication in a water bath for 5 min and then by centrifugation for 5 min. A 100-µl aliquot of supernatant (13,000 g for 10 min) was determined spectrophotometrically.

Quantitation of mitochondrial activity

The procedure of Mosmann (1983) for mammalian cells as modified by Ikegawa et al. (1998, 2000) for plant cells was used with minor modifications (Minocha et al. 2001) to quantify mitochondrial activity. Briefly, 100 mg FW of cells was mixed with 1.0 ml of medium containing 0.25 µg ml$^{-1}$ of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Following a 1-h incubation (gyratory shaker, 100 rpm, room temperature), the cells were harvested by centrifugation (13,000 g, 5 min) and resuspended in 1 ml of acetic-acproanol (0.04 M HCl in isopropanol) by vortexing, centrifuged at 400 g for 2 min, and the supernatant analyzed spectrophotometrically at 590 nm for the formation of formazan.

Soluble protein determination

Soluble protein in cell extracts prepared by freeze-thawing cells (3×) in Tris-HCl (50 mM, pH 8.4) buffer was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with BSA as standard (Bradford 1976).

Statistical analysis

For each treatment, there were 3–4 replicates and each experiment was repeated at least 2–3 times. Unless stated otherwise, data from the 2–3 experiments were pooled. Because all treatments with additional NH$_4$NO$_3$ or KNO$_3$ were not given on the same day, different experiments involving different concentrations of these compounds had their own controls, the values for controls in each set of treatments are shown next to the data from the treatments. Data for each variable were evaluated by one-way analysis of variance (ANOVA). When F values for one-way ANOVA were significant (P < 0.05), treatment means were tested with Tukey’s multiple comparisons test. The ANOVA and Tukey’s tests were performed with SYSTAT for Windows, version 7.01 (SYSTAT, Evanston, IL).

Results

Fresh mass and dry mass

Percent dry mass was similar for both cell lines (6.03 + 0.07% for NT and 6.09 + 0.09% for 2E; n = 15) on any given day. Therefore, all parameters were compared on a FW basis. The addition of extra NH$_4$NO$_3$ to the standard MS medium generally had a negative effect on FW of the NT and 2E cell lines, with a more than a 50% reduction in FW occurring in response to the highest concentrations tested (Figures 2A and 2B). The increase of 5 to 10 mM KNO$_3$ caused a small but significant (P < 0.05) increase in FW of 2E cells, but had no effect on FW of NT cells (Figures 2C and 2D). The FW of 2E cells increased in response to the 20 and 40 mM KNO$_3$ treatments, whereas the treatments had no effect on FW of NT cells. The 60 and 80 mM KNO$_3$ treatments caused significant decreases in FW of both cell lines. The positive effects of the 20 and 40 mM KNO$_3$ treatments on the FW of 2E cells was probably unrelated to the presence of additional K$^+$ ions because 20 mM KCI almost completely inhibited growth of both cell types (data not shown).

Protein concentration

On the basis of FW, the total buffer-extractable (soluble) protein concentration of the cell lines varied only slightly in the different experiments (Figures 3A–D). In general, untreated 2E cells had higher soluble protein concentration than untreated NT cells. Protein concentration of NT cells remained unchanged in the 5 to 20 mM NH$_4$NO$_3$ treatments, but increased slightly in the 40 mM NH$_4$NO$_3$ treatment (Figure 3A). Protein concentration of 2E cells was unaffected by the 5 and 10 mM NH$_4$NO$_3$ treatments, but there was a small significant increase in soluble protein concentration in the 20, 40 and 60 mM NH$_4$NO$_3$ treatments (Figure 3B). The KNO$_3$ treatments had no effect on protein concentrations in NT and 2E cells except for a small decrease in 2E cells in the 20 mM KNO$_3$ treatment (Figures 3C and 3D).

Cell viability (membrane function)

Evan’s Blue dye is absorbed and retained by cells that are partly or fully impaired in their membrane function, e.g., un-
nder conditions of membrane depolarization (Ikegawa et al. 1998, 2000). The retention of greater amounts of the dye in this assay shows greater membrane damage or a higher proportion of dead cells in the population. We determined that the retention of dye in a mixed population of live and dead (killed) cells in different ratios was proportional to the amount of dead cells (data not shown). Because PAs have been implicated in stabilizing membrane function, we tested the effects of increased N availability in the medium on cell membrane permeability in NT and 2E cells. Addition of 5 to 40 mM NH₄NO₃ did not significantly affect dye retention in the NT cells (Figure 4A), whereas NT cells treated with 20–40 mM NH₄NO₃ retained slightly greater amounts of dye. In the 60 mM NH₄NO₃ treatment, NT cells retained significantly smaller amounts of dye than control cells. For 2E cells, low concentrations of NH₄NO₃ had a negligible effect on dye retention, but significant increases in dye retention were seen in the 60 mM NH₄NO₃ treatment (Figure 4B). Additional KNO₃ in the medium had no significant effect on dye retention in NT cells at any concentration (Figure 4C), but a significant reduction in dye retention was observed in 2E cells in the 20 and 40 mM KNO₃ treatments (Figure 4D). However, the 80 mM KNO₃ treatment increased membrane damage. In general, NT cells had lower dye retention than 2E cells on any given day.

Mitochondrial activity
The reduction of MTT to formazan has been used to measure mitochondrial respiratory activity in cells (Mosmann 1983, Ikegawa et al. 1998, 2000). We studied the effects of different concentrations of NH₄NO₃ and KNO₃ on mitochondrial activ-

Figure 2. Effects of NH₄NO₃ and KNO₃ supplementation (added to the N present in MS medium) on the fresh mass (FW) of non-transgenic (NT) and transgenic (2E) cells. A = NH₄NO₃, NT; B = NH₄NO₃, 2E; C = KNO₃, NT; and D = KNO₃, 2E. Supplementation with 5 and 10 mM NH₄NO₃ and KNO₃ was given to 3-day-old cultures for 48 h. For other concentrations, additional NH₄NO₃ or KNO₃ was present for 5 days from the time of subculture. Data are means ± SE of indicated number of replicates (n) that were pooled from repeat experiments. Asterisks indicate significant differences from respective controls at P < 0.05.

Figure 3. Effects of NH₄NO₃ and KNO₃ supplementation (added to the N present in MS medium) on cellular soluble protein concentrations of non-transgenic (NT) and transgenic (2E) cells. A = NH₄NO₃, NT; B = NH₄NO₃, 2E; C = KNO₃, NT; and D = KNO₃, 2E. Supplementation with 5 and 10 mM NH₄NO₃ and KNO₃ was given to 3-day-old cultures for 48 h. For other concentrations, additional NH₄NO₃ or KNO₃ was present for 5 days from the time of subculture. Data are means ± SE of the indicated number of replicates (n) that were pooled from repeat experiments. Asterisks indicate significant differences from respective controls at P < 0.05. In panel C, the letter a denotes n = 8 and in panel D, the letter b denotes n = 4 for 80 mM KNO₃.
ity of NT and 2E cells after 48 h and 5 days of treatment. The 5
to 20 mM NH₄NO₃ treatments had no effect on mitochondrial
activity of NT cells but the 40 and 60 mM NH₄NO₃ treatments
significantly increased MTT reduction (Figure 5A). In 2E
cells, the 60 mM NH₄NO₃ treatment had no effect on mito-
chondrial activity, whereas the 10 to 40 mM NH₄NO₃ treat-
ments caused small increases in mitochondrial activity (Fig-
ure 5B). The KNO₃ treatments caused small increases in MTT
reduction in both cell lines (Figures 5C and 5D). Overall, mi-
tochondrial activity was similar in the control cultures of both
cell lines.

Cellular polyamines

Although the cellular Put concentration in the two cell lines
varied among experiments conducted on different days, the 2E
cells always had several-fold higher concentrations of Put than
Treatment with increasing concentrations of NH₄NO₃ and
KNO₃, in general, increasingly reduced Put concentrations in
both cell types (Figures 6A–D). At 60 mM NH₄NO₃, a
60–70% reduction in Put was observed in both cell lines. The
60–80 mM KNO₃ treatments reduced Put concentrations by
more than 70% in both cell lines (Figures 6C and 6D).

The NH₄NO₃ and KNO₃ treatments either had no effect or
increased Spd concentrations. For 2E cells, the 10 to 60 mM
NH₄NO₃ treatments significantly increased Spd concentra-
tions, whereas NT cells showed a significant increase at
40 mM and a decrease at 60 mM NH₄NO₃ (Figures 7A and
7B). Significant increases in Spd concentration were observed
in the 20 to 60 mM KNO₃ treatments in 2E cells and in the 20
and 40 mM KNO₃ treatments in NT cells (Figures 7C and 7D). Cellular Spd concentrations were higher in 2E cells than in NT cells (cf. Bhatnagar et al. 2001).

Spermine constituted less than 3% of total soluble PAs in NT cells and less than 1% in 2E cells. The presence of additional NH₄NO₃ had little effect on Spm concentration in NT cells, except at 40 and 60 mM where a significant increase was seen in some experiments (Figure 8A). Small but significant increases in Spm concentration were seen in the 2E cells at and above 10 mM additional NH₄NO₃ (Figure 8B). Small increases in Spm concentrations in both cell lines were observed in the 10 to 60 mM KNO₃ treatments (Figures 8C and 8D).

**Effects of reduction in nitrogen availability**

Three-day-old cells were washed with NH₄NO₃-free medium and incubated in fresh medium: (a) without NH₄NO₃; (b) with two different concentrations of NH₄NO₃; or (c) with an equimolar (with respect to NH₄) concentration of NH₄Cl. Unwashed cells were used as controls. Cells were collected at 24 and 72 h after the experimental treatments. In an alternate setup, the cells were washed with N-free medium (i.e., no NH₄NO₃ or KNO₃) and allowed to grow without N for 96 h. Samples were collected at different times for PA analysis.

Putrescine concentration was lower in washed cells than in unwashed cells regardless of whether NH₄NO₃ was present in the medium (Figure 9A). The absence of NH₄NO₃ from the medium caused a rapid reduction in Put concentration of washed NT and 2E cells. At 24 h, the reduction in Put concentration was greater in NT cells, whereas the reduction in the absolute amount of Put was greater in 2E cells. The effect of

Figure 6. Effects of NH₄NO₃ and KNO₃ supplementation (added to the N in MS medium) on the concentrations of cellular putrescine of non-transgenic (NT) and transgenic (2E) cells. A = NH₄NO₃, NT; B = NH₄NO₃, 2E; C = KNO₃, NT; D = KNO₃, 2E. Supplementation with 5 and 10 mM NH₄NO₃ and KNO₃ was given to 3-day-old cultures for 48 h. For other concentrations, additional NH₄NO₃ or KNO₃ was present for 5 days from the time of subculture. Data are means ± SE of indicated number of replicates (n) that were pooled from repeat experiments. Asterisks indicate significant differences from respective controls at P < 0.05; ND = not determined.

Figure 7. Effects of NH₄NO₃ and KNO₃ supplementation (added to the N in MS medium) on the concentrations of cellular spermidine of non-transgenic (NT) and transgenic (2E) cells. A = NH₄NO₃, NT; B = NH₄NO₃, 2E; C = KNO₃, NT; and D = KNO₃, 2E. Supplementation with 5 and 10 mM NH₄NO₃ and KNO₃ was given to 3-day-old cultures for 48 h. For other concentrations, additional NH₄NO₃ or KNO₃ was present for 5 days from the time of subculture. Data are means ± SE of indicated number of replicates (n) that were pooled from repeat experiments. Asterisks indicate significant differences from respective controls at P < 0.05; ND = not determined.
NH₄NO₃ removal was more pronounced at 72 h of treatment than at 24 h. The addition of NH₄NO₃ to washed cells resulted in significant (but not complete) recovery of Put concentration in both cell lines; 4.12 mM NH₄NO₃ in the medium being as effective as the normal NH₄NO₃ concentration in MS medium (20.6 mM). The response of the two cell types was similar. Recovery of Put concentration was detectable at 24 and 72 h following addition of NH₄NO₃ and was dependent on the presence of NO₃⁻ as a counter ion with NH₄⁺. Substitution of NH₄NO₃ with NH₄Cl resulted in a 90% or greater reduction in Put concentration in both cell lines. This reduction may be the result of Cl toxicity and not the absence of NO₃⁻ because, in the KNO₃ experiments, the 20 and 40 mM KNO₃ treatments increased the FW of 2E cells, whereas addition of 20 mM KCl completely inhibited the growth of both cell lines (data not presented).

Spermidine concentrations were generally higher in washed cells than in the unwashed cells at 20.61 mM NH₄NO₃ concentration (Figure 9B). The absence of NH₄NO₃ from the medium significantly reduced Spd concentrations at 24 and 72 h in both cell lines. In all cases, almost complete recovery of Spd concentrations occurred following the addition of 4.12 mM NH₄NO₃. As with Put, the presence of NH₄Cl caused a significantly greater loss of Spd than the absence of NH₄NO₃, and the effect increased with time.

In both cell lines, there was no change in Spm concentration at 24 h and an increase at 72 h following washing and removal of NH₄NO₃ from the medium (Figure 9C). Increases in Spm concentration occurred in response to re-addition of either 4.12 or 20.61 mM NH₄NO₃. The presence of NH₄Cl significantly reduced Spm concentrations of both cell lines. At both 24 and 72 h after addition of 20.61 mM NH₄NO₃, washed cells had significantly higher Spm concentrations than unwashed control cells in medium containing 20.61 mM NH₄NO₃.

On removal of N from the medium, there was a time-dependent decrease in Put concentration in NT cells, starting as early as 12 h after removal of N and continuing for up to 96 h (Figure 10A). In 2E cells, the decrease in Put concentration was not seen until 24 or 48 h following removal of N. The NT cells lost 80–90% of their Put concentration by 96 h, whereas the maximum decline in Put concentration in 2E cells was only 50–60%. There was a much smaller reduction in Spd concentration compared with Put concentration in NT cells incubated in N-free medium, the reduction becoming visible within 4–12 h and continuing up to 96 h (Figure 10B). The greatest reduction at any time was 60%. Similar trends in Spd concentration were seen in 2E cells, which maintained higher concentrations of Spd than the NT cells. Changes in Spm concentration followed a similar pattern to that of Spd; Spm concentrations decreased with time in both cell lines, and the maximum decline was about 50% at 48 h (Figure 10C). The concentration of Spm was either similar in the two cell lines or was slightly higher in NT cells than in 2E cells.

Discussion

Based on changes in FW, membrane permeability and mitochondrial activity, it appears that 2E cells are less tolerant to excess amounts of NH₄NO₃ and KNO₃, and accumulate larger amounts of total protein than NT cells. We also found that the total N concentration of the MS medium is not limiting for the production of additional Put and Spd by the ODC pathway in 2E cells; however, a continuous supply of N in the form of both NH₄⁺ and NO₃⁻ is required to maintain homeostatic concentrations of Put in each cell line. Fluctuations in Put concentration in response to variation in N availability of the medium were not accompanied by similar changes in Spd and Spm concentrations. Poplar cells did not sequester excess N in PAs.

Previously we showed that the rate of biosynthesis of Put and Spd in 2E cells is several-fold higher than in NT cells, that Orn is synthesized largely via the Gln/Glu pathway and not from Arg, that availability of Arg to ADC in transgenic cells is
unaffected by the increased utilization of Orn, and that the rates of Put biosynthesis and catabolism are proportional (Bhatnagar 2002, Bhatnagar et al. 2001, 2002). Based on the pathway for Orn and Arg biosynthesis, we hypothesize that, in response to an increased utilization of Orn for Put production in E2 cells, there is a compensatory stimulation of the regulatory step(s) in the pathway, resulting in increased uptake of NH$_4^+$ or NO$_3^−$ and sustained and continuous production of Orn.

The hypothesis that a continuous and sustained production of Orn is coordinated with its utilization for Put biosynthesis in NT cells is supported by the observation that Put concentration decreased within 12 h after removal of N from the medium. By 24 and 48 h, the Put concentration of the cells had declined by more than 60 and 80%, respectively. Although a similar effect was expected for 2E cells, the decline in Put concentration in these cells was smaller and slower than in NT cells even at 48 and 96 h after the removal of N from the medium (Figure 10). Because the rates of Put catabolism and biosynthesis are directly proportional (Bhatnagar et al. 2001, 2002) in these cells, a reduction in N supply, which reduced Put biosynthesis, possibly also reduced its catabolism, leading to a higher homeostatic threshold of Put in the 2E cells. Concomitant with the decline in Put concentration, there were decreases in Spd and Spm concentrations in both cell lines when N was removed.

Figure 9. Effects of different treatments with NH$_4$NO$_3$ and NH$_4$Cl to 3-day-old cells on the concentrations of cellular putrescine (A), spermidine (B) and spermine (C). Normal strength MS medium contains 20.6 mM NH$_4$NO$_3$. Data presented are means + SE of six replicates from two experiments. Asterisks indicate significant differences ($P < 0.05$) from washed control (treatment number 2) for the same cell line at a given time period.

Figure 10. Cellular concentrations of putrescine (A), spermidine (B) and spermine (C) in the non-transgenic (NT) and transgenic (2E) cells after transfer of cells to N-free medium. Three-day-old cells were transferred to N-free medium for the indicated time periods. Data presented are means + SE of six replicates from two experiments. Asterisks indicate significant differences ($P < 0.05$) between control and treatment for the same cell line at a given time period.
The decrease in Spd concentration was much slower than the decrease in Put concentration. This observation is consistent with the longer calculated half-life of Spd than Put in poplar cells (Bhatnagar 2002, Bhatnagar et al. 2002). The finding that removal of NH$_4$NO$_3$ alone from the medium (while keeping a normal concentration of KNO$_3$ in the MS medium) resulted in a decline in Put concentration at 72 h (Figure 9) comparable with that observed at 48 h after removal of all N sources (Figure 10) is consistent with the preferential use of NH$_4^+$ by plant cells in general (Forde and Clarkson 1999).

The presence of additional NH$_4^+$ in the MS medium may affect cell physiology in several ways: (1) through osmotic effects; (2) through membrane depolarization as a result of increased NH$_4^+$ uptake; (3) as a source of extra N for amino acid and protein synthesis; and (4) through competitive interactions with cation uptake. The osmotic effects of NH$_4$NO$_3$ are probably not the primary cause of its effects in the present study because its uptake is proportional to its concentration in the medium (Forde and Clarkson 1999, Britto et al. 2000, Kronzucker et al. 2001). The toxic effects of NH$_4^+$ could result from increased NH$_4^+$ uptake followed by energy-dependent excess NH$_4^+$ efflux as suggested by Britto et al. (2001), and may account for the observed increase in mitochondrial activity in response to the 60 mM NH$_4^+$ treatment. Competition with uptake of other cations by nonselective cation channels is another possible explanation of the adverse effects of NH$_4^+$ (Forde and Clarkson 1999). However, the accumulation of major cations in these cells in the presence of elevated NH$_4^+$ show only minor effects on these cations (R. Minocha, S. Long and S.C. Minocha, unpublished data). It is also possible that the decrease in Put concentration in the presence of 60 mM NH$_4^+$ could be associated with increased loss of Put into the medium by the same mechanism that causes increased NH$_4^+$ efflux. However, this explanation cannot account for the decrease in Put concentration in response to increased KNO$_3$ availability.

The effects of increased NH$_4$NO$_3$ and KNO$_3$ availability on Spd concentrations in both cell lines differed from the effects on Put concentrations. In most cases, there was no significant effect of either treatment on Spd concentrations in NT cells, whereas the treatments caused a significant increase in Spd concentrations in 2E cells. Concentrations of Spm, which constituted only a minor fraction of total PAs, remained unchanged or increased in the presence of increased concentrations of NH$_4$NO$_3$ or KNO$_3$. On removal of NH$_4$NO$_3$ from the medium, changes in concentrations of Spd and Spm concentrations were much smaller than those of Put. These observations are consistent with the suggestion that the concentration of Spd is tightly regulated independently of changes in Put concentrations (Minocha and Minocha 1995, Bhatnagar et al. 2001, 2002). This suggestion is supported by the calculated half-life of Spd, which is much greater than that of Put (Bhatnagar 2002, Bhatnagar et al. 2002).

The increased concentration of soluble proteins in cells in response to additional NH$_4$NO$_3$, and in some cases to additional KNO$_3$, in the medium may reflect a cell response to increased availability of N in the medium. The effects of NH$_4$NO$_3$ and KNO$_3$ on mitochondrial activity in the two cell lines were similar, except at the highest concentrations.

A comparison of the effects of NH$_4$NO$_3$ and KNO$_3$ supplementation of the MS medium showed that both N forms had similar effects on: (1) FW of NT cells; (2) cellular protein concentration in both cell lines; (3) mitochondrial activity of both cell lines, except at the highest concentrations; and (4) Put, Spd and Spm concentrations in both cell lines. There were differences with respect to their effects on membrane permeability to Evan’s Blue and the KNO$_3$-induced increase in FW in 2E cells. Although increased NH$_4^+$ availability increased Evan’s Blue dye retention (decreased membrane integrity) in 2E cells, increased NO$_3^-$ availability either had little effect (NT cells) or caused a significant reduction in Evan’s Blue retention in 2E cells up to 60 mM. The similar physiological effects of NH$_4^+$ and NO$_3^-$ in these cell lines may reflect rapid conversion of nitrate N to ammonium N in the cells. On the other hand, the differences in the effects of NH$_4^+$ and NO$_3^-$ are consistent with the effects of excess NH$_4^+$, causing membrane depolarization. Uptake of excess NO$_3^-$ may increase the negative charges in the cells, thus increasing membrane polarization and counteracting the effects of NH$_4^+$, which is present in the MS medium at a relatively high concentration (20.6 mM). This may be the reason for increased growth (FW) in the 2E cells in the presence of extra KNO$_3$.

In summary, we conclude that: (1) in response to increased utilization of Orn by the transgenic ODC (Bhatnagar et al. 2001), there is a coordinated increase in the multiple steps leading to Orn biosynthesis, and (2) excess amounts of either NO$_3^-$ or NH$_4^+$ do not lead to sequestration of the excess N in the form of PAs. Our findings do not unequivocally exclude the possibility that PAs play an important role in sequestration of extra N in the whole plant, where in contrast to cells in suspension that are rapidly dividing, sequestration would occur in fully developed leaves and other storage tissues (Minocha et al. 2000).

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References


