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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 NH_4NO_3 and KNO_3 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 NH_4^+ and NO_3^- 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 NH_4NO_3, more tolerance to KNO_3, 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 NH_4^+/NO_3^- 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.

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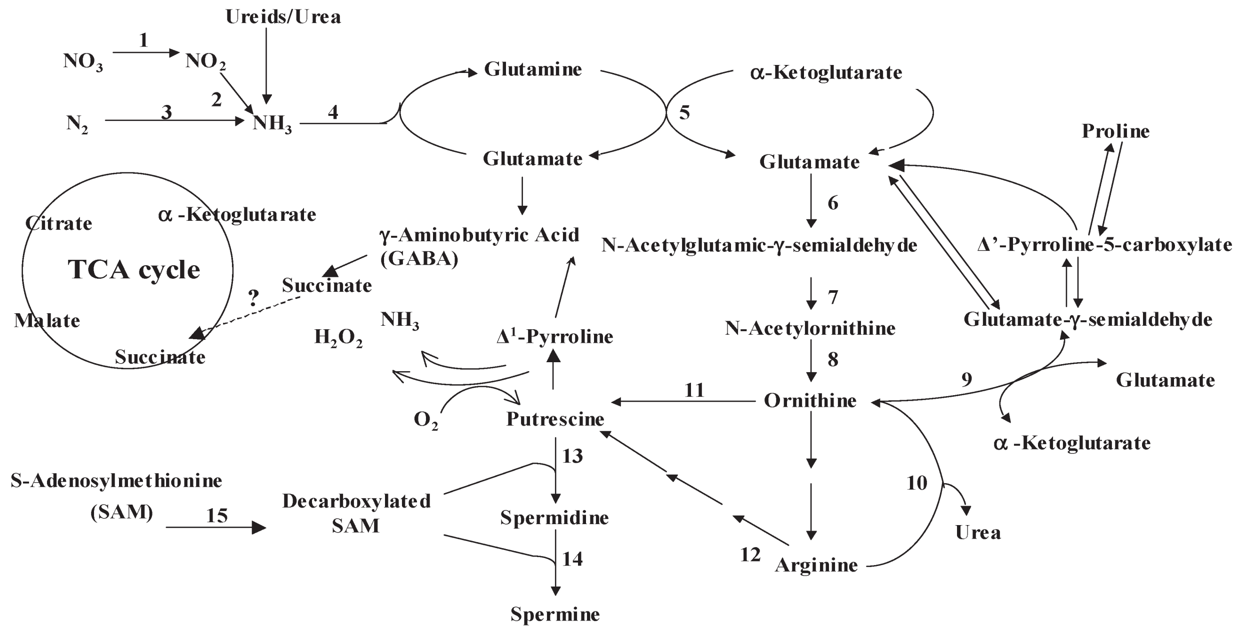

Figure 1. Polyamine and nitrogen metabolism in plants. The enzymes are: 1. nitrate reductase, 2. nitrite reductase, 3. nitrogenase, 4. glutamine synthetase, 5. glutamate synthase, 6. glutamate reductase, 7. acetylglutamic-γ-semialdehyde transaminase, 8. acetylornithinase, 9. ornithine aminotransferase, 10. arginase, 11. ornithine decarboxylase, 12. arginine decarboxylase, 13. spermidine synthase, 14. spermine synthase, 15. *S*-adenosylmethionine decarboxylase.

Although many cells and tissues in culture can utilize either NH_4^+ or NO_3^- 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 NH_4^+. 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 cells (2E) of poplar (*Populus nigra* × *maximoviczii*) 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 NH_4NO_3 or 5, 10, 20, 40, 60 or 80 mM KNO_3 was added in addition to the 20.6 mM NH_4NO_3 and 18.8 mM KNO_3 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 flasks were maintained under the conditions described for the stock cultures. 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.

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For experiments involving treatments with N concentrations lower than the amount present in MS medium, 3-day-old cells were washed with NH_4NO_3-free medium or total N-free medium by centrifugation, resuspended in NH_4NO_3-free medium or N-free medium, and NH_4NO_3 or NH_4Cl was then added. Treatments included: unwashed controls, no NH_4NO_3, 20.61 mM NH_4NO_3 (concentration in MS medium), 4.12 mM NH_4NO_3, 20.61 mM NH_4Cl and 0 mM NH_4NO_3 or KNO_3. The experiments were repeated twice with three replicates each time.

Analysis of free polyamines

One hundred mg (FW) of cells was added to 400 µl of 5% (v/v) 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 dansylated 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 ultrasonicating the cells for 1 h at room temperature or by freezing and thawing the cells twice. The methods yielded comparable results. Absorbance at 600 nm of the 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 acid-propanol (0.04 M HCI 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_4NO_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_4NO_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 addition 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 KCl 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_4NO_3 treatments, but increased slightly in the 40 mM NH_4NO_3 treatment (Figure 3A). Protein concentration of 2E cells was unaffected by the 5 and 10 mM NH_4NO_3 treatments, but there was a small significant increase in soluble protein concentration in the 20, 40 and 60 mM NH_4NO_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-

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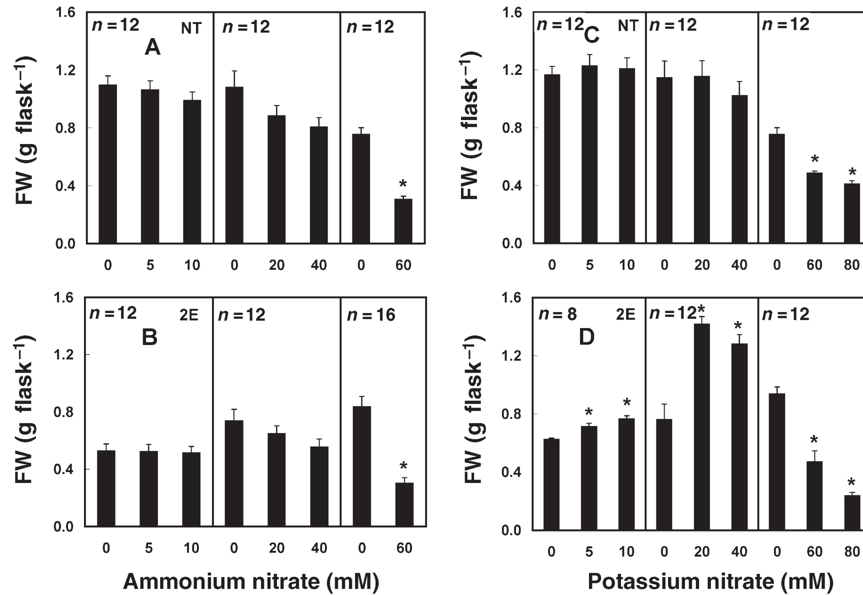

Figure 2. Effects of NH_4NO_3 and KNO_3 supplementation (added to the N present in MS medium) on the fresh mass (FW) of non-transgenic (NT) and transgenic (2E) cells. A = NH_4NO_3, NT; B = NH_4NO_3, 2E; C = KNO_3, NT; and D = KNO_3, 2E. Supplementation with 5 and 10 mM NH_4NO_3 and KNO_3 was given to 3-day-old cultures for 48 h. For other concentrations, additional NH_4NO_3 or KNO_3 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 $P < 0.05$.

der 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 NH_4NO_3 did not significantly affect dye retention in the NT cells (Figure 4A), whereas NT cells treated with 20–40 mM NH_4NO_3 retained slightly greater amounts of dye. In the 60 mM NH_4NO_3 treatment, NT cells retained significantly smaller amounts of dye than control cells. For 2E cells, low concentrations of NH_4NO_3 had a negligible effect on dye retention, but significant increases in dye retention were seen in the 60 mM NH_4NO_3 treatment (Figure 4B). Additional KNO_3 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 KNO_3 treatments (Figure 4D). However, the 80 mM KNO_3 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 NH_4NO_3 and KNO_3 on mitochondrial activ-

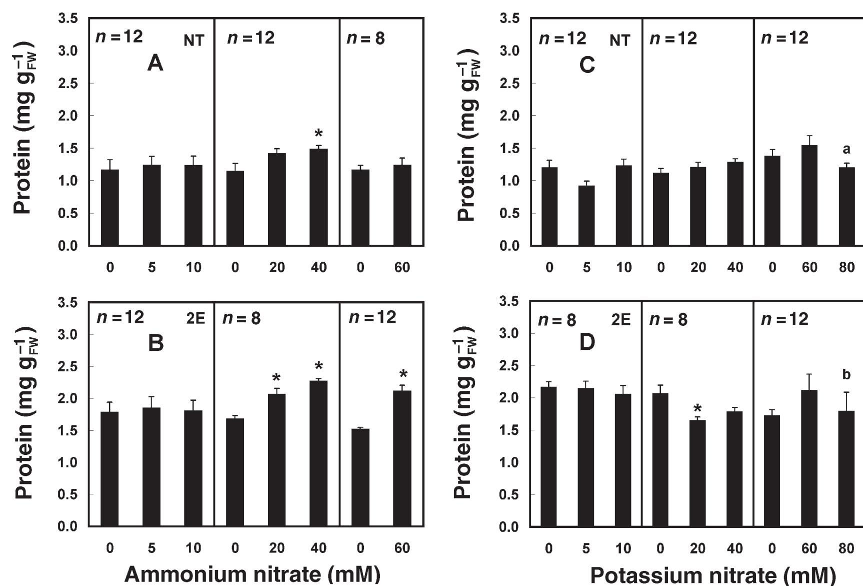

Figure 3. Effects of NH_4NO_3 and KNO_3 supplementation (added to the N present in MS medium) on cellular soluble protein concentrations of non-transgenic (NT) and transgenic (2E) cells. A = NH_4NO_3, NT; B = NH_4NO_3, 2E; C = KNO_3, NT; and D = KNO_3, 2E. Supplementation with 5 and 10 mM NH_4NO_3 and KNO_3 was given to 3-day-old cultures for 48 h. For other concentrations, additional NH_4NO_3 or KNO_3 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 KNO_3.

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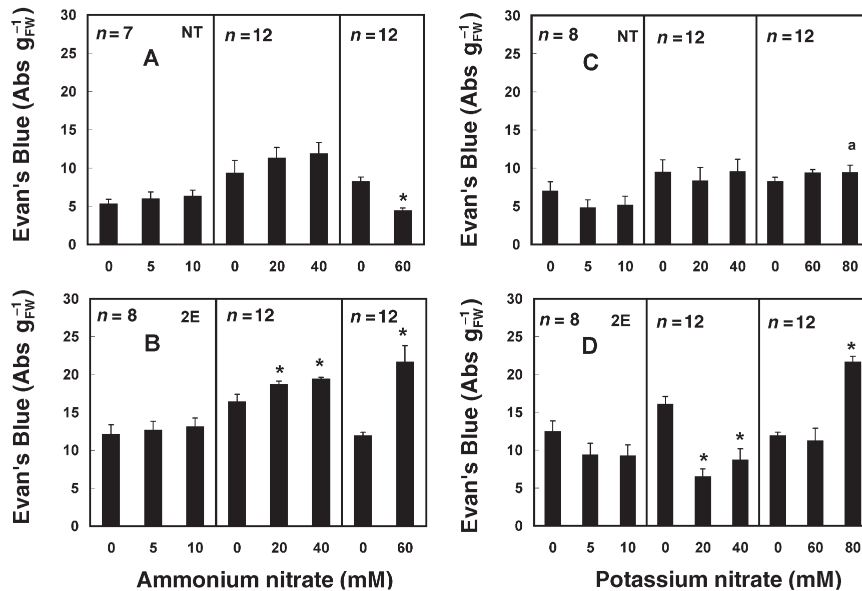

Figure 4. Effects of NH_4NO_3 and KNO_3 supplementation (added to the N in MS medium) on membrane integrity (Evan's Blue retention) of non-transgenic (NT) and transgenic (2E) cells. A = NH_4NO_3, NT; B = NH_4NO_3, 2E; C = KNO_3, NT; and D = KNO_3, 2E. Supplementation with 5 and 10 mM NH_4NO_3 and KNO_3 was given to 3-day-old cultures for 48 h. For other concentrations, additional NH_4NO_3 or KNO_3 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 $P < 0.05$. In panel C, the letter a denotes $n = 8$ for 80 mM.

ity of NT and 2E cells after 48 h and 5 days of treatment. The 5 to 20 mM NH_4NO_3 treatments had no effect on mitochondrial activity of NT cells but the 40 and 60 mM NH_4NO_3 treatments significantly increased MTT reduction (Figure 5A). In 2E cells, the 60 mM NH_4NO_3 treatment had no effect on mitochondrial activity, whereas the 10 to 40 mM NH_4NO_3 treatments caused small increases in mitochondrial activity (Figure 5B). The KNO_3 treatments caused small increases in MTT reduction in both cell lines (Figures 5C and 5D). Overall, mitochondrial 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 the NT cells (Figures 6A–D) (cf. Bhatnagar et al. 2001, 2002). Treatment with increasing concentrations of NH_4NO_3 and KNO_3, in general, increasingly reduced Put concentrations in both cell types (Figures 6A–D). At 60 mM NH_4NO_3, a 60–70% reduction in Put was observed in both cell lines. The 60–80 mM KNO_3 treatments reduced Put concentrations by more than 70% in both cell lines (Figures 6C and 6D).

The NH_4NO_3 and KNO_3 treatments either had no effect or increased Spd concentrations. For 2E cells, the 10 to 60 mM NH_4NO_3 treatments significantly increased Spd concentrations, whereas NT cells showed a significant increase at 40 mM and a decrease at 60 mM NH_4NO_3 (Figures 7A and 7B). Significant increases in Spd concentration were observed in the 20 to 60 mM KNO_3 treatments in 2E cells and in the 20

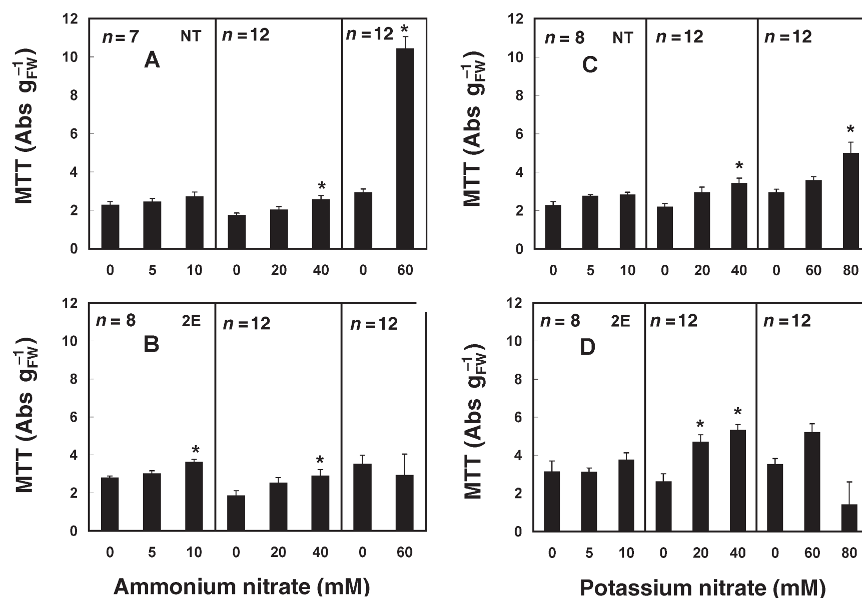

Figure 5. Effects of NH_4NO_3 and KNO_3 supplementation (added to the N in MS medium) on the mitochondrial activity (MTT conversion to formazan) of non-transgenic (NT) and transgenic (2E) cells. A = NH_4NO_3, NT; B = NH_4NO_3, 2E; C = KNO_3, NT; and D = KNO_3, 2E. Supplementation with 5 and 10 mM NH_4NO_3 and KNO_3 was given to 3-day-old cultures for 48 h. For other concentrations, additional NH_4NO_3 or KNO_3 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 $P < 0.05$.

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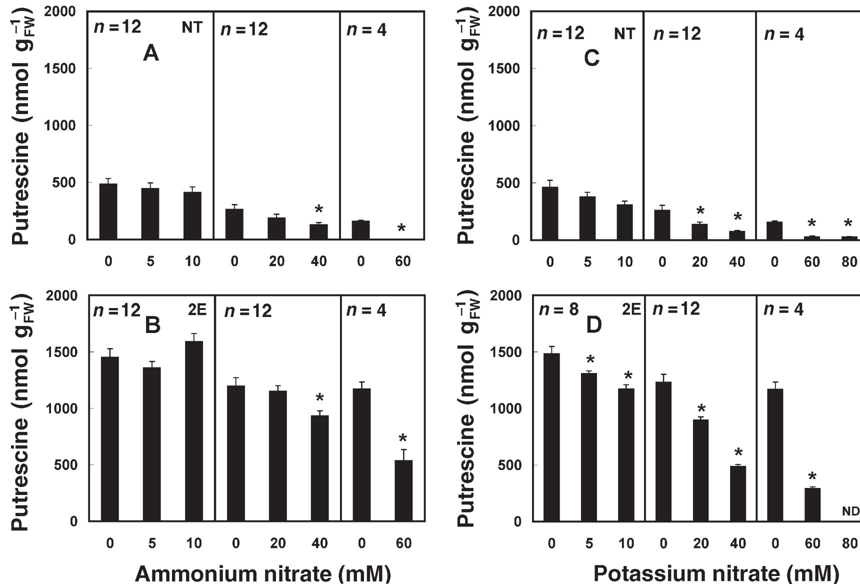

Figure 6. Effects of NH_4NO_3 and KNO_3 supplementation (added to the N in MS medium) on the concentrations of cellular putrescine of non-transgenic (NT) and transgenic (2E) cells. A = NH_4NO_3, NT; B = NH_4NO_3, 2E; C = KNO_3, NT; D = KNO_3, 2E. Supplementation with 5 and 10 mM NH_4NO_3 and KNO_3 was given to 3-day-old cultures for 48 h. For other concentrations, additional NH_4NO_3 or KNO_3 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.

and 40 mM KNO_3 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 NH_4NO_3 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 NH_4NO_3 (Figure 8B). Small increases in Spm concentrations in both cell lines were observed in the 10 to 60 mM KNO_3 treatments (Figures 8C and 8D).

Effects of reduction in nitrogen availability

Three-day-old cells were washed with NH_4NO_3-free medium and incubated in fresh medium: (a) without NH_4NO_3; (b) with two different concentrations of NH_4NO_3; or (c) with an equimolar (with respect to NH_4) concentration of NH_4Cl. 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 NH_4NO_3 or KNO_3) 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 NH_4NO_3 was present in the medium (Figure 9A). The absence of NH_4NO_3 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

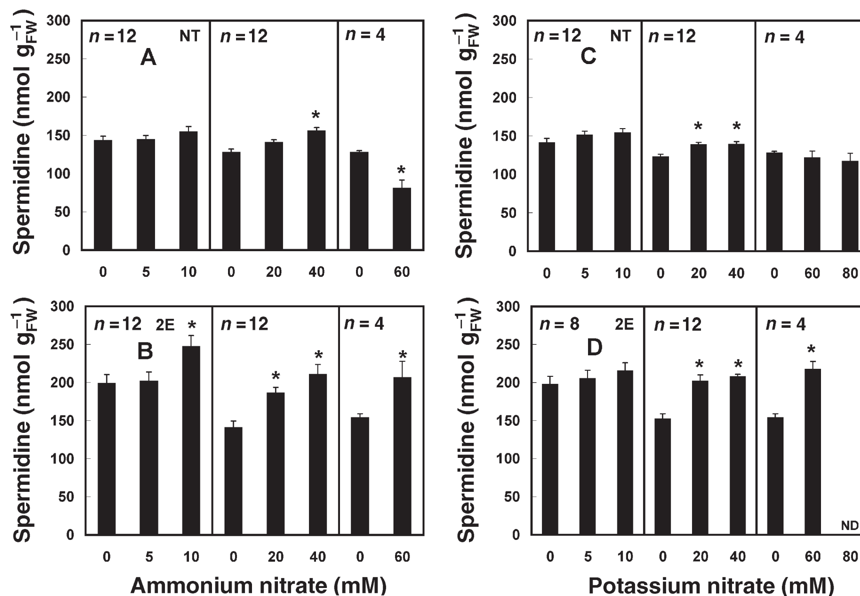

Figure 7. Effects of NH_4NO_3 and KNO_3 supplementation (added to the N in MS medium) on the concentrations of cellular spermidine of non-transgenic (NT) and transgenic (2E) cells. A = NH_4NO_3, NT; B = NH_4NO_3, 2E; C = KNO_3, NT; and D = KNO_3, 2E. Supplementation with 5 and 10 mM NH_4NO_3 and KNO_3 was given to 3-day-old cultures for 48 h. For other concentrations, additional NH_4NO_3 or KNO_3 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.

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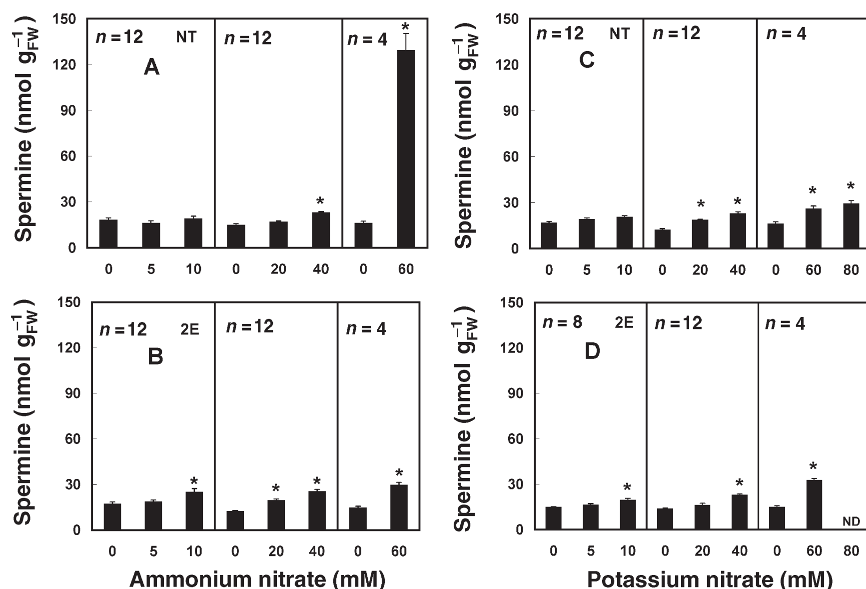

Figure 8. Effects of NH_4NO_3 and KNO_3 supplementation (added to the N in the MS medium) on the concentrations of cellular spermine of non-transgenic (NT) and transgenic (2E) cells. A = NH_4NO_3, NT; B = NH_4NO_3, 2E; C = KNO_3, NT; and D = KNO_3, 2E. Supplementation with 5 and 10 mM NH_4NO_3 and KNO_3 was given to 3-day-old cultures for 48 h. For other concentrations, additional NH_4NO_3 or KNO_3 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.

NH_4NO_3 removal was more pronounced at 72 h of treatment than at 24 h. The addition of NH_4NO_3 to washed cells resulted in significant (but not complete) recovery of Put concentration in both cell lines; 4.12 mM NH_4NO_3 in the medium being as effective as the normal NH_4NO_3 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 NH_4NO_3 and was dependent on the presence of NO_3^- as a counter ion with NH_4^+. Substitution of NH_4NO_3 with NH_4Cl 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 NO_3^- because, in the KNO_3 experiments, the 20 and 40 mM KNO_3 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 NH_4NO_3 concentration (Figure 9B). The absence of NH_4NO_3 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 NH_4NO_3. As with Put, the presence of NH_4Cl caused a significantly greater loss of Spd than the absence of NH_4NO_3, 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 NH_4NO_3 from the medium (Figure 9C). Increases in Spm concentration occurred in response to re-addition of either 4.12 or 20.61 mM NH_4NO_3. The presence of NH_4Cl significantly reduced Spm concentrations of both cell lines. At both 24 and 72 h after addition of 20.61 mM NH_4NO_3, washed cells had significantly higher Spm concentrations than unwashed control cells in medium containing 20.61 mM NH_4NO_3.

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 NH_4NO_3 and KNO_3, 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 NH_4^+ and NO_3^- 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

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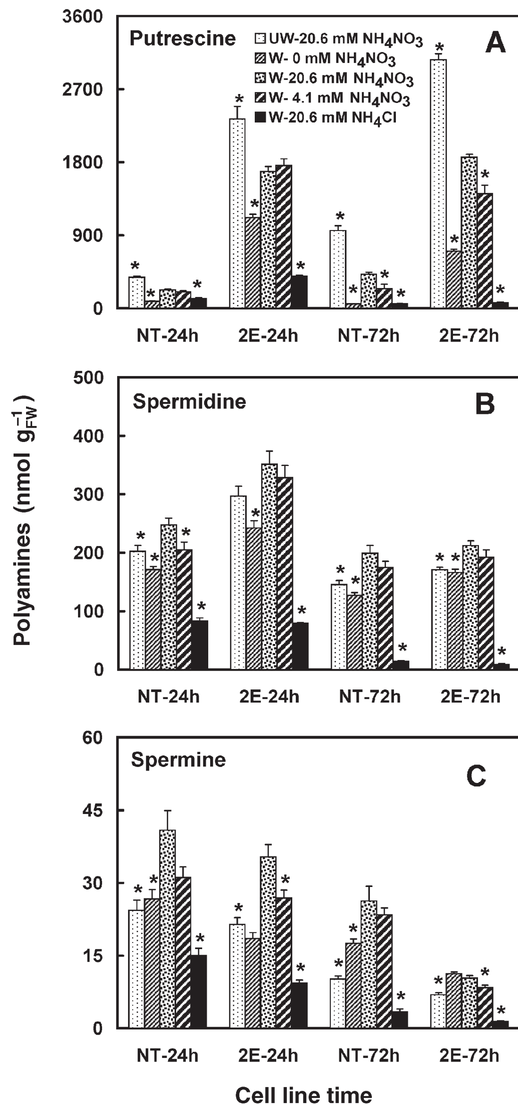

Figure 9. Effects of different treatments with NH_4NO_3 and NH_4Cl to 3-day-old cells on the concentrations of cellular putrescine (A), spermidine (B) and spermine (C). Normal strength MS medium contains 20.6 mM NH_4NO_3. Data presented are means + SE of six replicates from two experiments. Asterisks indicate significant differences ($P < 0.05$) from washed control (treatment number 2) for the same cell line at a given time period.

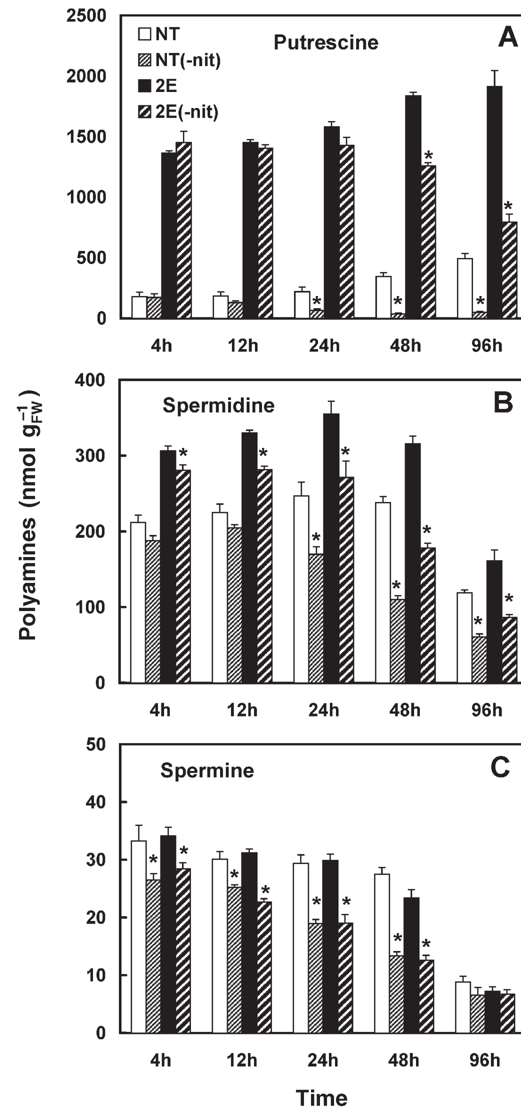

Figure 10. Cellular concentrations of putrescine (A), spermidine (B) and spermine (C) in the non-transgenic (NT) and transgenic (2E) cells after transfer of cells to N-free medium. Three-day-old cells were transferred to N-free medium for the indicated time periods. Data presented are means + SE of six 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.

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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 that 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

from the medium. 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_4NO_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_4NO_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_4NO_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_4NO_3 or KNO_3. On removal of NH_4NO_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_4NO_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_4NO_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_4NO_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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