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Research article

Putrescine overproduction negatively impacts the oxidative state of poplar cells in culture☆

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ABSTRACT

While polyamines (PAs) have been suggested to protect cells against Reactive Oxygen Species (ROS), their catabolism is known to generate ROS. We compared the activities of several enzymes and cellular metabolites involved in the ROS scavenging pathways in two isogenic cell lines of poplar (*Populus nigra* × *maximowiczii*) differing in their PA contents. Whereas the control cell line was transformed with *β-glucuronidase* (*GUS*), the other, called HP (High Putrescine), was transformed with a mouse *ornithine decarboxylase* (m*ODC*) gene. The expression of m*ODC* resulted in several-fold increased production of putrescine as well its enhanced catabolism. The two cell lines followed a similar trend of growth over the seven-day culture cycle, but the HP cells had elevated levels of soluble proteins. Accumulation of H_2O_2 was higher in the HP cells than the control cells, and so were the activities of glutathione reductase and monodehydroascorbate reductase; the activity of ascorbate peroxidase was lower in the former. The contents of reduced glutathione and glutamate were significantly lower in the HP cells but proline was higher on some days of analysis. There was a small difference in mitochondrial activity between the two cell lines, and the HP cells showed increased membrane damage. In the HP cells, increased accumulation of Ca was concomitant with lower accumulation of K. We conclude that, while increased putrescine accumulation may have a protective role against ROS in plants, enhanced turnover of putrescine actually can make them vulnerable to increased oxidative damage.

1. Introduction

The relationship between polyamines (PAs) and Reactive Oxygen Species (ROS) is highly controversial; on the one hand, PAs have been suggested to protect the cells against ROS, on the other, their catabolism actually generates ROS [14,27,44,50]. Earlier studies in this field have relied heavily on correlative changes in ROS and PAs either in response to environmental factors or using exogenously supplied PAs and/or their inhibitors. The genetic manipulation of PA metabolism enables us to test this relationship in a way uniquely different from the pharmacological approaches, since PA metabolism can be altered without the pleiotropic effects of chemical inhibitors or the complex responses to an environmental factor. Although ROS are deemed important for their roles as second messengers in signal transduction cascades, their adverse effects on cells are equally well known [38,41 and references therein]. Also termed as 'active oxygen species' (AOS), they encompass superoxide and hydroxyl radicals, hydrogen peroxide (H_2O_2), and singlet oxygen. ROS are produced as byproducts of metabolic reactions in all parts of the cell, particularly in organelles like chloroplasts and mitochondria, at times when there is enhanced oxidation of glucose and fatty acids [2,12]. Each cellular compartment has its own mechanism to regulate the synthesis of these compounds so as to prevent 'oxidative damage', a term used to define the collective harmful effects of ROS [2].

As mentioned above, ROS play important roles in cell signaling, e.g. in root gravitropism [23], regulation of plant cell growth [13],

Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, reduced ascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; HP, high putrescine cell line; MDHAR, monodehydroascorbate reductase; ODC, ornithine decarboxylase; PA, polyamine; Put, putrescine; ROS, reactive oxygen species.

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defense reaction to pathogen attack via oxidative burst [2,55], cellular responses to abiotic stress, and in programmed cell death [32,33,38,41]. Oxidative stress can be induced by various factors, including drought [34], salinity [18], light [15], and aluminum [56]. The oxidative damage control system within plant cells is comprised of several antioxygenic enzymes and metabolites (Fig. 1) that aid in processing reactions to rapidly break down ROS and protect the cell from injury. These include glutathione reductase (GR – EC 1.6.4.2), ascorbate peroxidase (APX – EC 1.11.1.11), ascorbate oxidase (AO – EC 1.10.3.3), dehydroascorbate reductase (DHAR – EC 1.8.5.1), monodehydroascorbate reductase (MDHAR – EC 1.6.5.4), superoxide dismutase (SOD – EC 1.15.1.1), and catalase (CAT – EC 1.11.1.6). Although CAT converts H_2O_2 into H_2O and O_2, H_2O_2 can also be broken down by peroxidases, especially in the absence of CAT (e.g. in the chloroplasts). Peroxidases need a reductant like reduced glutathione (GSH), ascorbate (AsA), NADH or NADPH to reduce H_2O_2. Both GSH and AsA are known to act as reductants in the ROS scavenging pathway in plant cells [41]. The ascorbate–GSH cycle (Fig. 1) functions efficiently to bring about reactions that prevent oxidative stress. The reduction of H_2O_2 to H_2O and O_2 by APX generates MDHA, which gets (non-enzymatically) converted to DHA, which is then reduced to ascorbate by DHAR, using GSH as the reductant. Oxidized glutathione (GSSG), produced as a result is reduced to GSH by GR. While APX is believed to be present in the chloroplast, the cytoplasm as well as in the mitochondria, catalase is localized to the peroxisomes [32]. Glutathione reductase is also present in the cytoplasm, chloroplasts and mitochondria [1], and DHAR and MDHAR have been reported mostly in the chloroplasts [7].

Polyamines (putrescine – Put, spermidine, and spermine) are low molecular weight polycations that are obligate requirements for cell growth and sustenance [8]. Earlier work from our laboratory [3,4,43] has shown that manipulating a single step in the PA metabolic pathway to enhance the production of Put leads to coordinated changes in the activities of key enzymes involved in this and related pathways. There are several places where PAs have been suggested to interact with ROS generation or amelioration. While PAs prevent the synthesis of ROS as well as scavenge the hydroxyl radicals [27], through their catabolism they also generate large quantities of H_2O_2. In order to test the hypothesis that enhanced metabolism of PAs may actually be detrimental to the oxidative state of plant cells due to the increased catabolism of PAs; we have used a high Put (HP) producing transgenic poplar cell line in which increased catabolism of Put keeps pace with its increased biosynthesis [4]. This cell line overproduces Put due to the transgenic expression of a mouse *ornithine decarboxylase* (m*ODC*) gene. We demonstrate here that manipulation of Put metabolism affects the metabolism of related compounds like proline (Pro), whose cellular concentration has been known to increase under oxidative stress; and glutamate (Glu), a precursor to the synthesis of Pro, Put, γ-aminobutyric acid (GABA) and GSH. All these metabolites are known scavengers of ROS [27]. The altered metabolism of these compounds in turn influences the oxidative state of the cells.

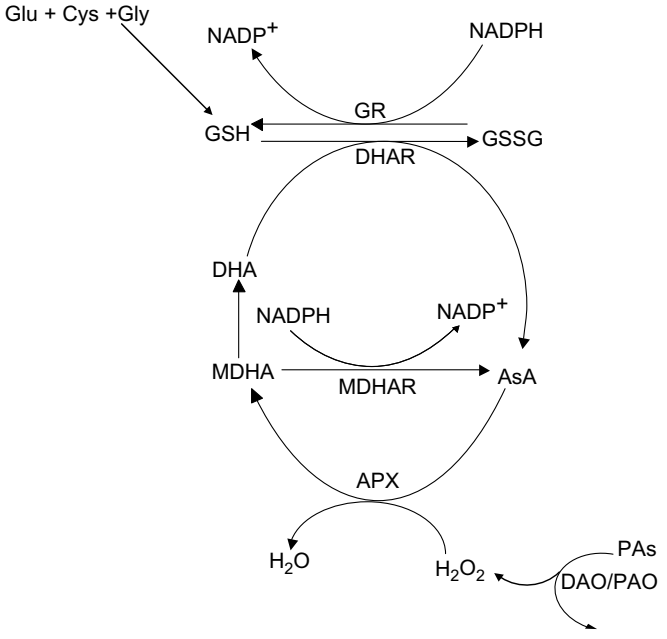

Fig. 1. The ascorbate–glutathione cycle showing the important enzymes of the ROS related pathway. Modified from Noctor and Foyer [41].

2. Results

The cellular PA contents over the 7-day culture period in the two cell lines used here have been published earlier [43]. During the present study, the HP cells had 3–5-fold higher amounts of Put as compared to control cells on different days of analysis; their spermidine contents were slightly higher than the control cells, and spermine contents were comparable (data not presented).

2.1. Total cell mass and soluble protein

On transfer of a 7 mL aliquot of seven-day old cell suspensions into fresh medium, the progression of growth in the two cell lines was similar (Fig. 2A). Between days 3 and 5, the HP cells showed slightly faster growth, but the differences were rather small and mostly insignificant. Cellular contents of total soluble (buffer extractable) protein (g^{-1} FW) were, however, significantly higher in the HP than in the control cells on days 1 through 4 (Fig. 2B). Starting with similar amounts of soluble protein on day 0 (which is the same as day 7), the HP cells showed a rapid and significant increase in protein content with a peak on day 2, followed by a decline during the next 3 days. On the contrary, in the control cells, there was only a small fluctuation in protein content over the 7-day culture cycle. For the last 3 days of culture, protein contents g^{-1} FW were similar in the two cell lines.

2.2. Glutathione reductase (GR)

The activity of GR remained low during the entire period of growth in the control cells (Fig. 2C) but increased several-folds (when calculated as units.g^{-1} FW) following transfer to fresh medium. During the entire 7-d culture period, the GR activity was significantly higher in the HP cells than the control cells whether calculated as units g^{-1} FW or as specific activity (units mg^{-1} protein) (Fig. 2C, D). The peak of enzyme activity (g^{-1} FW) was seen on day 3 following transfer of cells to fresh medium, and the lowest activity calculated either way was seen around days 5–7. The difference in trend of changes in GR activity over time between the two calculations was due to significant differences in their soluble protein content.

2.3. Ascorbate peroxidase (APX)

Significant differences in the activity of APX (units g^{-1} FW) were seen between the HP and the control cells on some days of the 7-day culture cycle; although both cell lines exhibited similar trends of changes in this enzyme (Fig. 3A). In both cell lines, two to three-fold increases were seen between days 1 and 4 after transfer to fresh medium. This was followed by a decline in enzyme activity;

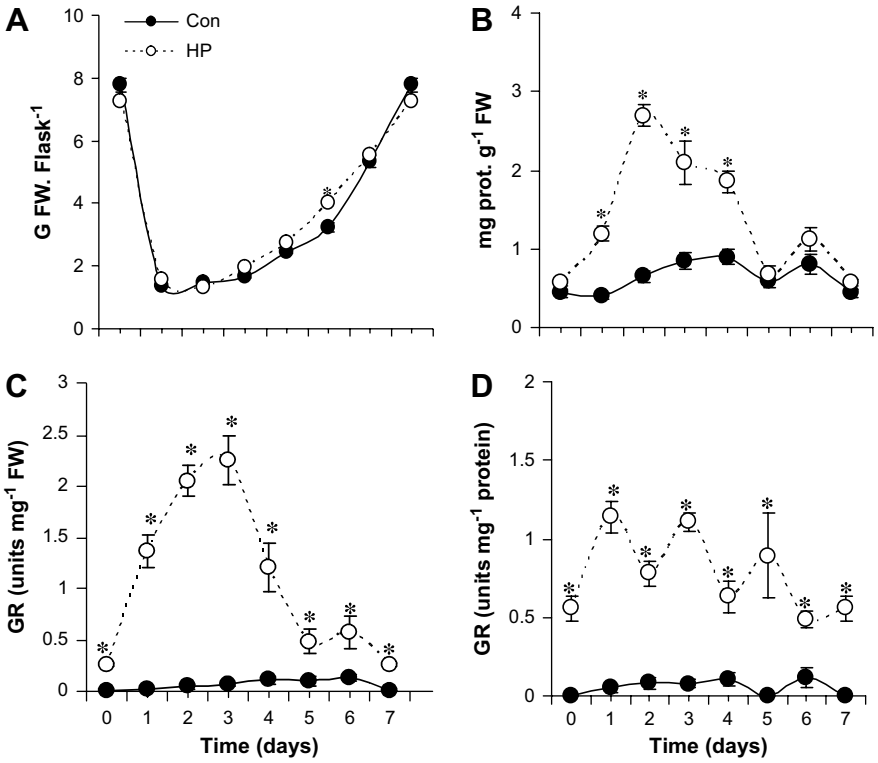

Fig. 2. Changes in fresh weight of cells per flask (A), the soluble protein content (B), and the activity of GR (C, D) in the control and the HP cells on different days of culture. Data are mean ± SE of six replicates from two experiments. An asterisk indicates significant difference ($P < 0.05$) between the two cell lines on a given day. Since the cells were subcultured on day 7, it is the same cells that were used for 0 day analysis; therefore the data for these two days are identical in Fig. 2B and in Figs. 3–7.

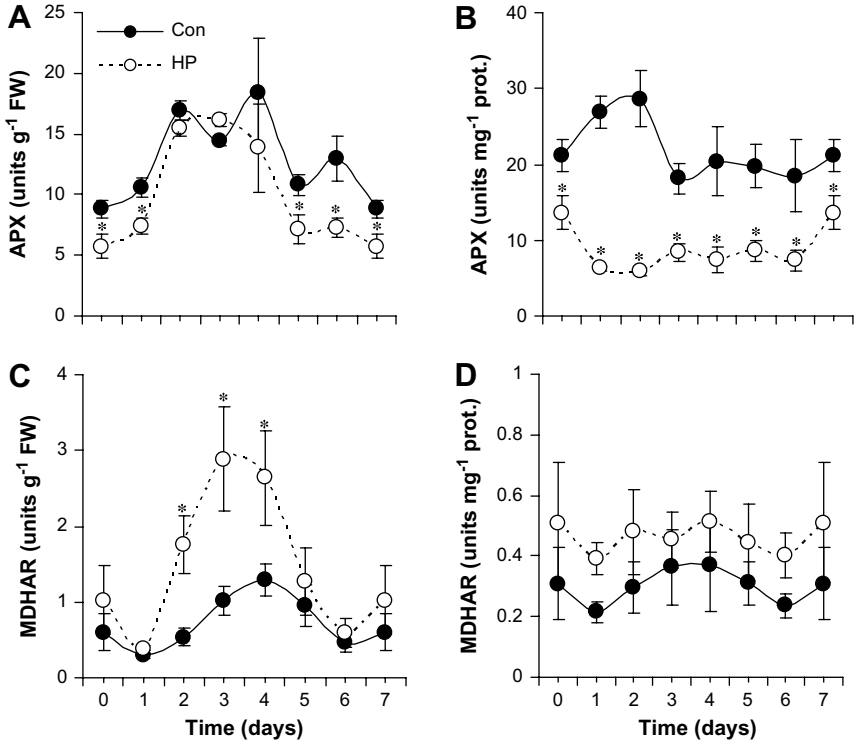

Fig. 3. Changes in the activity of APX (A, B) and MDHAR (C, D) in the control and the HP cells on different days of culture. Data are mean ± SE of six replicates from two experiments. An asterisk indicates significant difference ($P < 0.05$) between the two cell lines on a given day.

with the HP cells maintaining somewhat lower APX activity than the control cells, particularly during the last 3–4 days of culture. When adjusted to differences in protein contents of the two cell lines, statistically significant differences in specific activity of APX were seen for all 7 days of the culture cycle; the specific activity of APX always was lower in the HP cells than the control cells (Fig. 3B).

2.4. Monodehydroascorbate reductase (MDHAR)

As with GR, the activity of MDHAR was also significantly higher in HP cells than the control cells on days 2, 3 and 4 (Fig. 3C). In both cell lines the peak of enzyme activity (units g^{-1} FW) was seen around midweek. When MDHAR activity (g^{-1} FW) was normalized to cellular protein content and presented as specific activity (Fig. 3D), differences between the two cell lines over the entire week of study were smaller; still the HP cells had higher activity than the control cells. This was obviously due to the peak of MDHAR activity in HP cells coinciding with the peak of their soluble protein contents. While enzyme activity on FW basis showed a clear fresh medium stimulation, it was offset by parallel changes in protein content of HP cells.

2.5. Accumulation of H_2O_2 and glutathione

Production of H_2O_2 was significantly higher in the HP cells than the control cells on most days of the week (Fig. 4A). While there was a decline in H_2O_2 production in the HP cells at 24 h after transfer to fresh medium, no such decline was seen in the control cells. Other than this, a similar pattern of fluctuations in H_2O_2 accumulation was seen in both cell lines over the 7-d culture cycle; both showing a gradual increase in H_2O_2 during the first few days, followed by lower amounts on days 6 and 7.

Cellular contents of GSH remained significantly lower in the HP cells than in the control cells on each of the 7 days of growth cycle (Fig. 4B); the differences were two to three-folds. There was little effect of transfer to fresh medium, and the GSH content of the cells did not change appreciably with time over the 7-d culture cycle.

2.6. Amino acids

Biosynthesis of GSH uses the amino acids Glu, Cys and Gly [42]; the first two combine to form γ-glutamylcysteine (γ-EC) in an ATP dependent reaction catalyzed by γ-EC synthetase (EC 6.3.2.2). Following this, γ-EC combines with Gly in another ATP dependent reaction catalyzed by the enzyme GSH synthetase (EC 6.3.2.3) to produce GSH. An increase in cellular content of Pro, another amino acid whose synthesis depends on Glu, is a known indicator of oxidative stress in plants and also plays a role in ROS detoxification [25]. We measured the cellular contents of the three amino acids relevant to this pathway over the entire 7-day culture cycle in the two cell lines; the results are presented in Fig. 5.

Cellular contents of Glu were significantly lower in the HP cells than the control cells during the entire week of culture (Fig. 5A). In both cell lines, Glu increased significantly within a day after transfer to fresh medium but declined by day 2. After 2 or 3 d, Glu content was stable for the rest of the week. Cellular contents of Cys (plus cystine) were also lower in the HP cells than the control cells on most days (Fig. 5B); their contents in HP cells declined by more than 75% within 24 after transfer to fresh medium but did less so in the control cells. Thereafter, Cys content generally increased in both cell lines from days 2 through 5, followed by a sharp decline between days 5 and 7; more so in the control than the HP cells. A somewhat similar pattern was seen for Gly (Fig. 5C) where in both cell lines, following an initial lowering of its content from day 0 through day 3, there was a steady increase for the next three days. Significant differences in Gly contents were seen between the two cell lines towards later half of the week.

Proline was higher in the HP cells than in the control cells on the first two days of the week immediately following transfer to fresh medium (Fig. 5D); thereafter, Pro in HP cells declined and reached concentrations comparable to those in the control cells. Proline remained mostly unchanged with time in the control cells following transfer to fresh medium. While in control cells Pro and Glu were present in equimolar concentrations on all days except day 1, in the HP cells Pro was almost twice as much as Glu on any given day.

2.7. Accumulation of Ca and K

Fig. 6A and B shows a comparison of the accumulation of acid extractable Ca and K, respectively, in the control and the HP cells over the 7-d culture period. The accumulation of Ca was almost twice in the HP cells as compared to the control cells for three out of 7 days of culture period, and was significantly higher on other days as well. On the other hand, an opposite response was seen for the accumulation of K in these cells in that it was significantly lower in the HP cells as compared to the control cells for the entire culture period. A distinct and significant fresh medium effect on the uptake of K was seen in both cell lines within 24–48 h of transfer, with a gradual decline in the same thereafter.

2.8. Mitochondrial activity and membrane function

Colorless MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] interacts with the mitochondrial electron

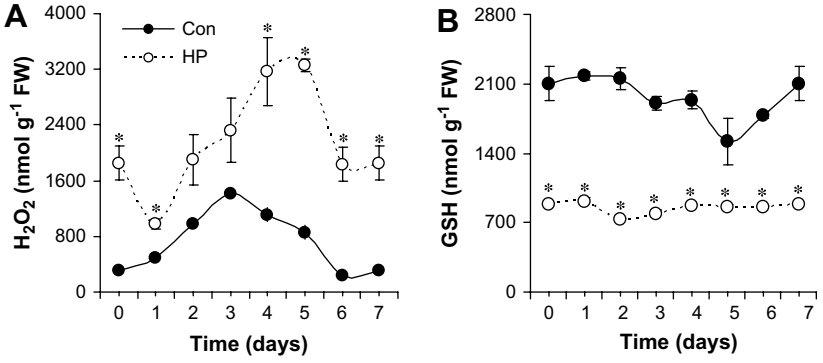

Fig. 4. Changes in the amount of H_2O_2 (A) and GSH (B) in the control and the HP cells on different days of culture. Data are mean ± SE of six replicates from two experiments. An asterisk indicates significant difference ($P < 0.05$) between the two cell lines on a given day.

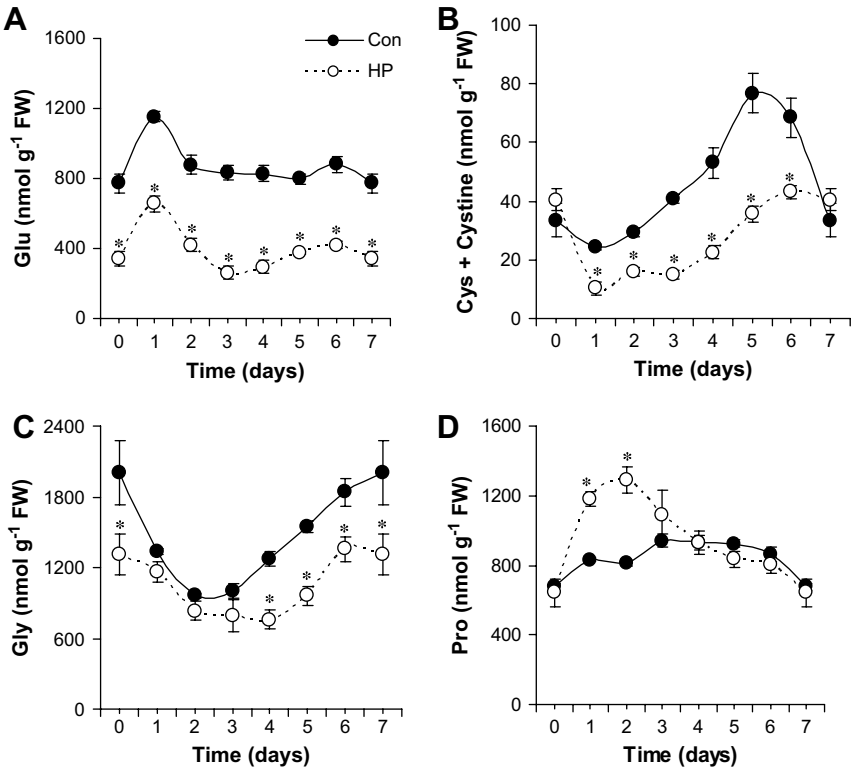

Fig. 5. Changes in the amounts of Glu (A), Cys (B), Gly (C) and Pro (D) in the control and the HP cells on different days of culture. Data are mean ± SE of six replicates from two experiments. An asterisk indicates significant difference ($P < 0.05$) between the two cell lines on a given day.

transport chain and gets reduced to form a blue colored product called formazan [31,35]. Thus, when the overall mitochondrial oxido-reductase activity is higher, the intensity of blue color is proportionately higher. As shown in Fig. 7A, the mitochondrial activity was somewhat lower in the HP cells on the three days that analyses were done; however the differences were not statistically significant.

Evans Blue retention is a reliable method for assessment of membrane damage in cells because it can enter the cells only through a damaged (depolarized) plasma membrane [31,35]. Hence, higher the number of membrane-compromised cells, greater is the absorbance of the supernatant obtained after allowing the cells to absorb the dye and then releasing it with SDS. Evans Blue retention was significantly higher in the HP cells than the control cells on all three days of analysis (Fig. 7B), indicating a significantly higher number of membrane-compromised HP cells in the culture. But the membrane integrity of HP cells appears to improve with time between days 3 and 5, as seen by lower dye retention in the cells. Evans Blue retention in the control cells remained practically unchanged between days 3 and 5.

3. Discussion

The postulated association of PAs with oxidative stress has a long history [47] and has also received considerable attention in recent years [39,44,50]; the relationship being somewhat contradictory and controversial. On the one hand PAs are postulated to protect the cells against ROS, but on the other hand, they potentially are the

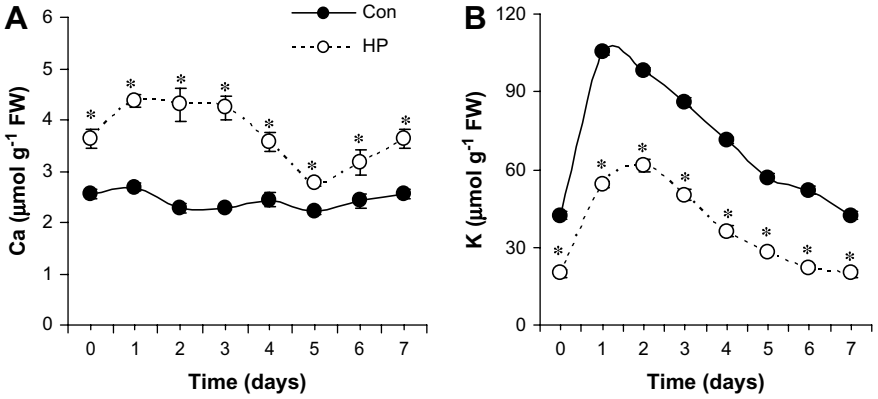

Fig. 6. Changes in the cellular contents of Ca (A) and K (B) in the control and the HP cells on different days of culture. Data are mean ± SE of six replicates from two experiments. An asterisk indicates significant difference ($P < 0.05$) between the two cell lines on a given day.

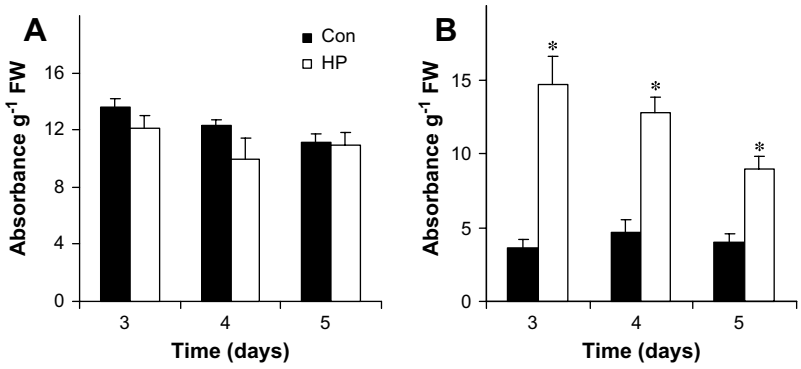

Fig. 7. Mitochondrial activity as measured by MTT reduction (A) and cell viability as measured by Evans blue retention (B) in the control and HP cells on different days of culture. Data are mean ± SE of 24 replicates from 8 experiments in (A) and 9 replicates from 3 experiments in (B). An asterisk indicates significant difference ($P < 0.05$) between the two cell lines on a given day.

source of H_2O_2 as byproduct of their catabolism. H_2O_2 is an oxidant that is particularly notorious for its role in lipid peroxidation and consequent damage to membranes [17]. Papadakis and Roubelakis-Angelakis [44] showed that all three major PAs could suppress ROS generation in tobacco cell cultures, presumably by inhibiting the microsomal membrane NADPH oxidase. They also found that exogenous Put ameliorated the toxic effects of H_2O_2 generated via increased PA catabolism. Nayyar and Chander [39] had earlier observed an increase in PAs as a response to cold and water stress in chickpea (*Cicer arietinum* L.), along with an enhanced accumulation of ROS scavenging metabolites like AsA and GSH. We wanted to test if a constitutive increase in Put accumulation and a concomitant increase in its catabolism in poplar cells may have made them prone to enhanced oxidative stress. Therefore, we examined the activities of several enzymes related to oxidative stress amelioration pathway, and the production of H_2O_2, GSH and other related metabolites in poplar cells whose PA content had been altered through genetic manipulation.

Biochemical indicators of greater ROS generation in plant cells include a higher activity of ROS scavenging enzymes, such as GR, MDHAR, APX, and CAT [25]. The redox state of a cell is further indicated by pools of antioxidants like GSH, AsA, Pro, and the production of H_2O_2 in the cells. The presence of large quantities of these antioxidants is responsible for the regulation of redox homeostasis in the cells [14]. Increased accumulations of reduced metabolites like AsA and GSH point to lower oxidative stress and thus, better cell health. From the data presented here, it is apparent that not only is the accumulation of H_2O_2 much higher in the HP cells than in the control cells; the activities of antioxidative enzymes GR and MDHAR are also higher in these cells. On the other hand, cellular content of the ROS scavenger GSH is lower in the HP cells than the control cells on all days of culture. These data, coupled with observed increase in membrane damage in the HP cells, possibly as a consequence of enhanced lipid peroxidation (from increased H_2O_2 generation), point to a state of higher oxidative stress in them as compared to the control cells.

In general, overall metabolism of HP cells is much higher during the first two days after transfer to fresh medium as exemplified by rapid increase in protein biosynthesis (Fig. 2B), accumulation of Pro (Fig. 5D), increased expression of several PA biosynthetic enzymes, and the accumulation of PAs [43]; most of them showing a decline afterwards. The activities of GR and MDHAR also increased in these cells from days 1–3. These data correlate well with the Evans Blue retention data to possibly suggest that these cells experience the highest level of oxidative stress between days 2 and 4, a period coincident with their heightened metabolism. This is followed by a decline in soluble protein content in the HP cells. We believe that this decrease in soluble proteins is due to increased proteolysis.

The absorbance of Evans Blue by the HP cells showed a decrease between days 3 and 5, indicating a recovery in cell health with time. This happens even though there is greater accumulation of H_2O_2 on day 5 than on day 3 in the HP cells. These patterns are not seen in the control cells on different days of the week; they maintain a rather steady state of these metabolites, and also of total soluble proteins. While it is premature to suggest a mechanistic model correlating changes in Put with the rapid changes in protein content of HP cells, it can be argued, however, that a major difference between the two cell lines is that on transfer to fresh medium, the HP cells undergo a flurry of metabolic activity as a result of increased activity of ODC (due to transcription as well as translation of the m*ODC* gene – [43]). Consequently, increased Put production is accompanied by its increased catabolism, and hence, increased production of H_2O_2, resulting in a situation where a protector molecule (i.e. Put) actually becomes a contributor to oxidative stress in these cells; consequently, there is enhanced protein degradation.

Many ROS are generated in the mitochondrial electron transport chain under situations of enhanced utilization of glucose; the enzyme NAD(P)H oxidase plays a key role in this. The excessive amounts of electron donors, mainly $FADH_2$ and NADH generated by the tricarboxylic acid cycle, provide electrons which eventually react with molecular oxygen in the mitochondrial respiratory chain generating superoxide that can subsequently be degraded to H_2O_2. Although AsA has a direct role in scavenging ROS, it is the cascade of reactions following oxidation of AsA that reduces GSSG to GSH (Fig. 1). While GR uses NADPH to reduce GSSG to GSH, various free radicals and oxidants are able to oxidize GSH back to GSSG [42]. Also, as a response to high oxidative stress, greater recycling of AsA in the HP cells might be responsible for the lower amounts of GSH in these cells. This causes a change in the normal redox state of the cells, leading to decline in cell health in the HP cells as opposed to the control cells.

Glutathione and AsA are important metabolic indicators of the oxidative state of a cell. As mentioned above, while APX activity (g^{-1}FW) is significantly lower in the HP cells at least on some days of the week (Fig. 3A, B), both GR (Fig. 2C, D) and MDHAR (Fig. 3C, D) activities are higher in them on almost all days of the week. This shows that the rate of H_2O_2 scavenging via APX is probably lower in the HP cells than in the control cells. But, AsA recycling from MDHA is higher in HP cells, potentially resulting in higher GSH metabolism. Hence the question arises: "*what happens to the excess AsA in HP cells if it is not being consumed to scavenge H_2O_2 via APX*"?

Pignocchi et al. [46] have argued that in plant cells, although AsA is localized mostly in the cytoplasm, a portion of it is transported to the apoplast where the first line of defense against antioxidants is generated. This AsA is then oxidized in the apoplast to MDHA by AO; the unstable MDHA being rapidly converted into DHA, which is then converted back to AsA via DHAR. Thus, in the HP cells, the higher MDHAR activity could be a consequence of high AO activity. Furthermore, it is possible that, although in the control as well as HP cells a similar amount of AsA is being oxidized by APX to produce MDHA, there is higher MDHA production in the apoplast of HP cells by AO, thus providing additional substrate for MDHAR activity, ultimately resulting in higher recycling of AsA as explained below.

Ascorbic acid (AsA) plays a multitude of roles in plant cells. Not only is it a major contributor to scavenging of ROS, it also acts as a cofactor in the hydroxylation of prolyl and lysil residues by peptidyl-prolyl and -lysil hydroxylases, playing a role in cell wall synthesis and in cell division [9]. Smirnoff [52] and Conklin [9] have proposed a role of AsA and AO in the regulation of cell expansion as well. As discussed above, the cell wall can generate MDHA via AO and reduce it back to AsA by a plasma membrane-bound NADPH-requiring cytochrome b [19]. Smirnoff [52] further suggested that AsA and MDHA in the cell wall play a role in cell expansion by helping regulate the cross-linking of cell wall proteins and polysaccharides, lignification, and Ca levels. The fact that MDHAR activity is higher in the HP cells on days 2 and 3, a period when cell division is in the logarithmic phase, supports the idea that increased MDHAR activity is a consequence of increased AO activity in these cells. It has been shown in tobacco cells that during cell elongation, AsA and the apoplastic activity of AO increase [24].

As discussed above, GSH biosynthesis involves condensation of Glu with Cys and Gly [42]. As shown in Fig. 5, the cellular contents of Glu, Cys and Gly are all lower in the HP cells than the control cells on all days of the week. The lowering of Glu is perhaps a consequence of its increased utilization in the production of Put in HP cells. Glu is the precursor of both Orn and Arg, and their consumption is enhanced several-folds in the HP cells [3,4]. Another point to note is the rather low proportion of cellular Cys as compared to Glu and Gly. The lower cellular content of GSH in the HP cells (Fig. 4B) could then result from either reduced cellular Glu or Gly; but more likely, it may be due to limited availability of Cys in these cells. Lower Cys and Gly in the HP cells, particularly during the first few days of growth, may be the consequence of increased protein synthesis following their transfer to fresh medium. Higher GR activity in the HP cells points towards a greater requirement of the cells to recycle GSH from GSSG, probably in an attempt to scavenge the higher quantities of ROS being generated in them. To further complicate this interaction, it has been shown that stress-induced alterations in Pro may also influence the amount of GSH, and hence, the activity of GR, since Glu is a precursor of Pro as well [25].The HP cells during the first few days do have higher amounts of Pro, the reason for which is not obvious.

The observed increase in cellular Ca and a decrease in K are additional indicators of heightened oxidative state of the HP cells. Foreman et al. [13] demonstrated that an inwardly rectifying Ca^{2+} channel was activated by $OH^{\bullet}$ radicals generated via the membrane-associated NADPH oxidase; this activation did not respond to treatments with H_2O_2 or Cu^{2+} or AsA. The consequence of this activation was increased Ca accumulation in root cells in the elongation zone. Pei et al. [45] had earlier shown that the Ca^{2+} channel could actually be activated by H_2O_2 in the guard cells of *Vicia faba*. A connection between the ROS and the signal transduction pathways involving cytosolic Ca has also been suggested [5]. Demidchik et al. [11] observed that there was a simultaneous activation of the Ca_{in} and K_{out} channels in response to increased ROS; they postulated that the activation of these channels was regulatory in nature and not due to a loss of membrane permeability. A similar activation of Ca and K channels by ROS has been demonstrated in animal cells [26]. Thus it can be argued that an increase in Ca and a decrease in K accumulation in the HP cells observed here concomitant with an apparent loss of membrane integrity may not merely be the harmful effects of increased ROS activity; rather these effects may be mediated by independent mechanisms. Moreover, since $OH^{\bullet}$ itself is not membrane permeable [11], it is possible that either these radicals are being produced in the apoplast or it is the H_2O_2 produced by PA oxidation that is responsible for this response [45].

It can further be suggested that while the Ca influx and K efflux may be due to the activation of specific channels, the loss of membrane integrity as seen by Evans blue retention in HP cells may be due to the harmful effects of ROS on lipid peroxidation in the membrane, which is a well known phenomenon in both animal and plant cells [53]. Stark [53] has further pointed out that increased accumulation of Ca may be due to depolarization of the membrane potential, and may actually be the cause of cell death.

While an interaction of ROS with plasma membrane Ca^{2+} channels seems widely documented, only a few reports have explored a strong and specific interaction of ROS with voltage-sensitive outward rectifying K^+ efflux channels [10,11,49]. This interaction leads to an efflux of K from the cells. Our data on lower K accumulation in the HP cells vs. the control cells are consistent with this argument. A further effect of ROS that would non-specifically influence the cellular ionic imbalance as well as result in increased permeability to the Evans blue dye would be through membrane lipid peroxidation [10,26,53 and references therein].

Bhatnagar et al. [4] compared the rates of Put catabolism in the two cell lines of poplar used here. Using [U-^{14}C] Put incorporation in 3-d-old cultures, they found that by 8 h after transfer to label-free medium, about 2 μmol Put g^{-1} FW of cells was being catabolized by the HP cells. The catabolism of Put generates H_2O_2. We found that on day 3, the HP cells had about 2 μmol H_2O_2 g^{-1} FW of cells; this number increased to as much as ~3 μmol g^{-1} FW by day 6, but declined back to ~2 μmol g^{-1} FW on day 7 (Fig. 4A). While Put catabolism is by no means the only source of H_2O_2 in these cells, nor is the amount of H_2O_2 accumulated only due to its synthesis (for review see [40]); nevertheless, the difference between the HP and the control cells correlates well with the loss of Put in the two types of cells. This further suggests that the overall catabolism of H_2O_2 may also be higher in the HP cells than the control cells while the former maintain a higher threshold of H_2O_2 in them.

Based on the vast amount of literature pointing to contrasting roles of ROS in plants (i.e. a key function in signal transduction, opposite effects on Ca^{2+} and K^+ transport, and harmful effects on membrane peroxidation), it is not surprising that a somewhat contradictory effect of enhanced Put accumulation is seen in poplar cells. While the past discussion on PAs has generally emphasized their positive roles in ROS scavenging and increasing plant tolerance to a variety of abiotic stress responses [14,43,50], we demonstrate here that an overproduction of PAs could actually be detrimental to the cells, particularly if it is accompanied by their enhanced catabolism. We show here that Put overproduction changes the oxidative state of poplar cells in culture; the HP cells exhibiting several of the biochemical effects of increased ROS (Fig. 8), including enhanced activity of ROS scavenging enzymes GR and MDHAR, a reduction in GSH, reduced accumulation of K and a boost in accumulation of Ca, and increased membrane damage. While exogenously supplied PAs in moderate to high quantities may act as protectants against oxidative stress, and in turn other forms of abiotic stress, enhancement of their production within the cell may actually

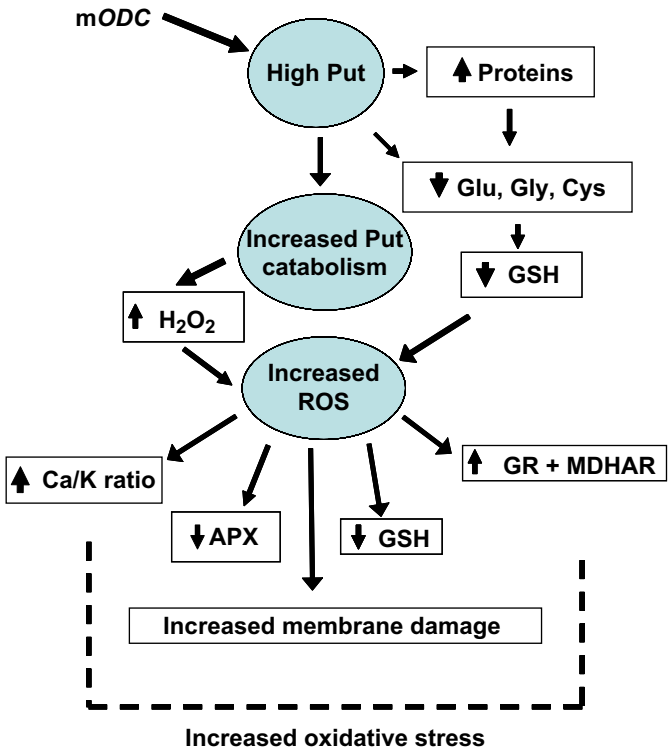

Fig. 8. Summary of biochemical changes related to enhanced putrescine biosynthesis via transgene expression and some aspects of the oxidative stress machinery in plant cells.

become detrimental due to a concomitant increase in their catabolism which results in increased H_2O_2 production. It is the balance of the two contrasting phenomena that will determine the overall health of cells.

Two important points must be kept in mind when comparing the results of this study with the published literature on PAs and oxidative stress. The first is that some of the differences in results may relate to the use of cell cultures vs. whole plants in different studies. Although the regulation of cellular metabolism in cultured cells is largely reflective of that in the whole organism; nevertheless, differences related to nutrient status of the cells (in the culture medium) and the intercellular transport (in the whole organism) may be of consequence. Secondly, when dealing with transgenic cell lines that have been maintained in culture for several years, one must be able to demonstrate that the lines (genotypes) being compared are similar/identical in other ways than the (trans)gene in question, and they have been grown and treated in a similar/identical way prior to and during the study period. Our study meets all these criteria. The two cell lines being used here are isogenic, except for the presence of the m*ODC* vs. the *GUS* transgene in the HP and the control cells, respectively. Moreover, we use at least three additional parameters to test that the two lines are behaving consistently over time – (1) they are kanamycin resistant and grown under identical conditions in the presence of kanamycin until experimental analyses; (2) the HP line has consistently higher (4–6 folds on several days of the week) amounts of PAs in question; and (3) that the growth rates of the two lines are similar over the entire period of study. All these criteria are used as internal standards for comparison of other parameters. Thus it can be safely argued that the observed differences between the two lines should be related to the effects of the transgene.

4. Materials and methods

4.1. Cell growth and harvest

The high putrescine cell line (called HP) and the control cell line of *Populus nigra* × *maximowiczii* used here have been previously described [3,4,43]. The former (previously called 2E – [3,4]) expresses a mouse ornithine decarboxylase (m*ODC*) gene, while the latter expresses the *β-glucuronidase* (*GUS*) gene; both cell lines also express *NPTII* selectable marker gene. All three transgenes are controlled by 35S CAMV promoters. By using a transgenic control line the effect of the presence of kanamycin in the medium during maintenance of stock cultures gets neutralized. Cell lines were maintained in MS medium [36] containing B5 vitamins [16], 2% sucrose, 0.5 mg L^{-1} 2,4-D and 100 mg L^{-1} kanamycin; the antibiotic was absent for at least two weeks prior to experimentation. Liquid cultures were subcultured by adding 7 mL of 7-d-old suspension to 50 mL fresh medium in 125 mL Erlenmeyer flasks The collections done at this time are referred to as the zero day collection; which are the same as the 7-d collections of the previous week.

4.2. Glutathione reductase assay

The method of Schaedle and Bassham [48] as described by Jahnke et al. [21] was slightly modified to extract and assay this enzyme. For enzyme extraction, 100 mg (FW) of cells were collected in 200 μL of 50 mM potassium phosphate (K-Pi) buffer (pH 7.0) containing 0.2 mM diethylenetriamine pentaacetic acid (DTPA). After freezing (−20 °C) and thawing (on ice), the mixture was vortexed for 5 min and centrifuged (16,000 × g, 10 min). To 50 μL of the supernatant, 850 μL of K-Pi buffer (25 mM, pH 7.8 with 0.2 mM DTPA) was added. Then, 50 μL of 3 mM NADPH made in 3 mM NaOH were added. Change in absorbance (340 nm) was monitored for 30–50 s (U-2000 Spectrometer, Hitachi Instruments Inc., Schaumburg, IL). This was followed by addition of 50 μL of 10 mM GSSG and the rate of its reduction (to GSH) was monitored again by measuring the change in absorbance for 30 s. This rate of change of absorbance was subtracted from the one determined in the absence of GSSG. One unit of enzyme activity is 1 μmol NADPH oxidized min^{-1} using the millimolar extinction coefficient of NADPH ($\varepsilon_{340} = 6.2$; [22]).

4.3. Ascorbate peroxidase assay

The method of Nakano and Asada [37] as described by Jahnke et al. [21] was modified to assay this enzyme. Briefly, 50 μL of the supernatant (100 mg cells in 400 μL of 50 mM K-Pi buffer) was mixed with 850 μL K-Pi buffer, 25 μL of 10 mM ascorbic acid and 50 μL of 10 mM H_2O_2. The decrease in absorbance (290 nm) was monitored for 30 s. Enzyme activity is expressed as μmol AsA oxidized min^{-1} (millimolar extinction coefficient of AsA $\varepsilon_{290} = 2.8$; [22]).

4.4. Monodehydroascorbate reductase assay

Cell free extract (25 μL in 50 mM K-Pi buffer, pH 7.0) was mixed with 850 μL of K-Pi buffer and 50 μL 50 mM ascorbic acid and used as blank. Following the addition of 50 μL of 3 mM NADH (in 3.0 mM NaOH), absorbance (340 nm) was measured for 30 s (modified from Hossain et al. [20]). This was followed by the addition of 25 μL of AO (12 U mL^{-1}), and the slope of change in absorbance was recorded. Activity is expressed as μmol NADH oxidized min^{-1} (millimolar extinction coefficient of NADH $\varepsilon_{340} = 6.2$; [28]).

4.5. Measurement of H_2O_2

Cellular contents of H_2O_2 were measured by minor modification of the method of Ślesak et al. [51]. About 0.5 g (FW) of cells were mixed with 2.0 mL acetone, vortexed, and centrifuged (10,000 × g, 5 min). To 0.5 mL of the supernatant, 50 μL titanium reagent (20% titanium tetrachloride in concentrated HCl, v/v) was added. The peroxide-titanium complex was precipitated by adding 100 μL NH_4OH to the above mixture. Following centrifugation (10 min, 10, 000 × g), the supernatant was discarded and the precipitate was repeatedly washed with acetone. The precipitate was then dissolved in 1 mL of 2 N H_2SO_4 and absorbance of the resulting solution was measured at 415 nm using Spectronic® 20 Genesys™ spectrometer (Spectronic Instruments Inc., Rochester, NY). The absorbance was compared against a standard curve generated from known concentrations of H_2O_2.

4.6. Amino acids, polyamines, glutathione, inorganic ions, and soluble proteins

Following vacuum filtration, 200 ± 20 mg (FW) of cells were mixed with 4 × volume of 5% (v/v) PCA, and frozen (at −20 °C) and thawed (at room temperature) three times before dansylation and quantification of PAs [3] and amino acids [29] by HPLC. The above PCA fraction was also used for ion analysis by ICP [30]. Reduced GSH was measured using 100 mg of cells in 500 μL of 6.3 mM DTPA (Fluka) containing 0.1% trifluoroacetic acid, derivatization and HPLC as described in Thangavel et al. [54]. Total soluble protein content was determined according to Bradford [6] using Bio-Rad dye (Bio-Rad, Hercules, CA).

4.7. Mitochondrial activity and membrane integrity

About 100 mg (FW) cells each were used to measure the mitochondrial activity using MTT assay and cell membrane integrity [31].

4.8. Statistical analysis

For all experiments, three replicate flasks were used for each cell line on a given day. Data shown here are combined from at least two replicate experiments. Data were subjected to analysis of variance using SYSTAT, version 10.2. Significance at $P \leq 0.05$ was determined using Tukeys test.

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