Auxin autotrophic tobacco callus tissues resist oxidative stress: the importance of glutathione S-transferase and glutathione peroxidase activities in auxin heterotrophic and autotrophic calli

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Received February 3, 2003 · Accepted July 17, 2003

Summary

Auxin autotrophic and heterotrophic tobacco callus lines were grown on MS medium with or without 100 mmol/L NaCl and growth and some of the stress-related activities, such as GPX, SOD, CAT, GST, GSH-PX, as well as the concentration of ethylene and H2O2, were measured and compared with each other. The auxin autotrophic calli grew slower, however, on the NaCl-containing medium the growth rate was higher than that of the heterotrophic cultures after two weeks of culturing. The stress-related ethylene production was lower in the autotrophic cultures and, contrary to the heterotrophic tissues, its level did not change significantly upon NaCl treatment. The guaiacol peroxidase (GPX) activities were higher in the autotrophic tissues in all cell fractions regardless of the presence of NaCl. Treated with NaCl, the GPX activities elevated in the soluble and covalently-bound fractions in the heterotrophic calli, but were not further increased in the autotrophic line. SOD and CAT activities were higher in the heterotrophic tissues, and were increased further by 100 mmol/L NaCl treatment. The GST and GSH-PX activities were higher in the autotrophic line, which might explain their enhanced stress tolerance. In the autotrophic tissues, the elevated antioxidant activities led to reduced levels of H2O2 and malondialdehyde; under mild NaCl stress, these levels decreased further. The lower growth rate and the effective protection against NaCl stress-induced oxidative damage of the autotrophic line can be explained by the cell wall-bound peroxidase and GSH-PX activities in the auxin autotrophic tissues. Their maintained growth rate indicates that the autotropic cultures were more resistant to exogenous H2O2.

Key words: Auxin-dependent and independent calli – ethylene – glutathione peroxidase – oxidative stress resistance – scavenging enzymes

Abbreviations: ALA = δ-aminolevulinic acid. – AOS = active oxygen species. – APX = ascorbate peroxidase. – BHT = butylhydroxytoluene. – CAT = catalase. – CDNB = 1-chloro-2,4-dinitrobenzene. – DTNB = 5,5′-dithio-bis(2-nitrobenzoic acid). – HRP = horseradish peroxidase. – HVA = 4-hydroxy-3-methoxyphenylacetic acid or homovanillic acid. – IAA = indole-3-acetic acid. – GPX = guaiacol peroxidase. – GR = glutathione reductase. – GSH = reduced glutathione. – GSH-PX = glutathione peroxidase. – GST = glutathione S-transferase. – MDA = malondialdehyde. – MS medium = Murashige-Skoog medium. – NBT = nitro blue tetrazolium. – SOD = superoxide dismutase. – TCA = trichloroacetic acid

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Introduction

Auxin-independent autotrophic and auxin-dependent heterotrophic lines of tobacco calli may differ, not only in their indoleacetic acid (IAA) synthesizing abilities and in their sensitivities to exogenous auxins (Nakajima et al. 1979, Szabó et al. 1981, Michalchuk and Druart 1999), but also in their stress tolerance. Levels of low molecular weight antioxidants (e.g., glutathione or ascorbate) and activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR), generally increase in plants under stress conditions and correlate well with the enhanced tolerance (Foyer et al. 1997). Peroxidases, such as those participating in lignin biosynthesis or the degradation of IAA, have long been identified in plants (García-Florenziano et al. 1991, Quiroga et al. 2000). Peroxidases in this group utilize a wide range of electron donors and can be referred to as GPXs (guaiacol is widely applied for their assay). Plant peroxidases are able to catalyze a variety of different reactions and may participate in the scavenging of active oxygen species (AOS) because they can utilize and eliminate H$_2$O$_2$. Examples include APX and glutathione peroxidase (GSH-PX) (Roxas et al. 2000, Noctor et al. 2002, Shigeoka et al. 2002). Identification of GSH-PX activity in plants, the isolation of plant genes with sequence homology to the animal counterparts, and isolation and characterization of the protein product has proven that the enzymes existing in plants belong to the phospholipid hydroperoxide GSH-PX family. The function of phospholipid hydroperoxide GSH-PXs is the reduction of alkyl hydroperoxides, such as fatty acid hydroperoxides (Eshdat et al. 1997). Some glutathione S-transferases (GSTs) also exhibit GSH-PX activity. GSTs are ubiquitous enzymes that catalyze the addition of reduced glutathione (GSH) to various hydrophobic electrophilic substrates, which tags them for vacuolar sequestration. GSTs protect against environmental stress and disease by detoxifying reactive products generated by oxidative stress. GSTs with a high affinity for auxins and cytokinins have also been suggested to contribute to hormone homeostasis (Marrs 1996, Edwards et al. 2000).

Hormone autotrophic or habituated callus tissues have been observed to exhibit substantial changes, not only in hormone metabolism, but also in primary metabolic pathways. Bisbis et al. (1999) found that sugarbeet calli gradually lost their peroxidase activities and their organogenic capacity during neoplastic progression of cultures. They found that the reduction of the synthesis of aminolevulinic acid (ALA), an obligatory intermediate of tetrapyrrole biosynthesis, through reduction of the plastidial Beale pathway, might be responsible for the substantial decrease of the heme-containing compounds. Hemoprotein synthesis of autotrophic cultures could additionally be decreased by inhibiting ALA-dehydratase via a disturbance of the phenol metabolism. Besides peroxidases, the heme-containing CAT, which removes H$_2$O$_2$ in peroxi-

Materials and Methods

Plant material

The callus cultures originated from protoplasts of Nicotiana tabacum SR1 plants (Csiszár et al. 2001). The auxin-requiring cultures were grown on solid MS medium (Murashige and Skoog 1962) containing 2 µmol/L kinetin, 17.5 µmol/L IAA and 0.45 µmol/L 2,4-D, and the auxin autotrophic cultures were transferred onto the same medium without auxin. Each Petri dish contained 25 inocula. The weight of one inoculum was approximately 20 mg. The salt treatment was carried out by adding 100 mmol/L NaCl to the culture media. The H$_2$O$_2$ was added aseptically just before the medium became solid, and the inocula were plated immediately. The cultures were kept in a growth chamber at 25 °C, under 8.4 W m$^{-2}$ warm white fluorescent light (Tungsram F29 lamps, Hungary) and were analyzed over a 3-week period.

Ethylene measurements

The ethylene production of calli of different ages was measured as described earlier (Tari and Mihalik 1998). Callus tissues (0.5 g) were kept in closed tubes for 1 h. The ethylene-containing samples were withdrawn from the gas phase above the plant material and were analyzed in a gas chromatograph equipped with a flame ionization detector.

Na$^+$ contents

Two-week-old callus tissues were dried at 70 °C for 72 h. Na$^+$ concentrations were analyzed after wet digestion of the dry material in a mixture of HNO$_3$ and 30 % H$_2$O$_2$ (5 : 4; v/v) at 200 °C for 3 h; the samples were measured with a Hitachi Z-8200 Zeeman polarized atomic absorption spectrophotometer.
Activity measurements of antioxidant enzymes

The enzyme activities were determined two weeks after the transfer onto the NaCl-containing medium. Two grams of callus tissue was homogenized on ice in 4 mL extraction buffer (50 mmol/L phosphate buffer pH 7.0, containing 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride and 1% polyvinyl-poly-pyrrolidone). The homogenate was filtered through two layers of cheese-cloth and centrifuged for 25 min at 15,000 g at 4°C. The supernatant was used for enzyme activity assays. In the case of SOD, CAT, GPX, and GR, refinding experiments were made for the control samples with standard enzymes. The percent of recovery was 92.3 (± 3.72) for SOD (from horseradish, Sigma), 78.38 (± 7.58) for catalase (from bovine liver, Sigma), 56.62 (± 5.51) for GPX (from horseradish, Reanal), and 75.43 (± 6.18) for GR (Type II from wheat, Sigma).

SOD (EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin in light (Dhindsa et al. 1981). One unit (U) of SOD was calculated as the amount causing a 50% inhibition of NBT reduction in light. The enzyme activity was expressed in terms of specific activity (U mg⁻¹ protein). CAT (EC 1.11.1.6) activity was determined by the decomposition of H₂O₂ and was measured spectrophotometrically by following the decrease in absorbance at 240 nm (Upadhyaya et al. 1985). One U = the amount of H₂O₂ (in μmol) decomposed in 1 min. GPX (EC 1.11.1.7) activity was determined by monitoring the increase in absorbance at 470 nm during the oxidation of guaiacol (Upadhyaya et al. 1985). The amount of enzyme producing 1 μmol min⁻¹ of oxidized guaiacol was defined as 1 U. The soluble, cell wall-bound and covalently bound fractions were prepared according to Hendricks et al. (1985), with a slight modification for preparation of the ionically bound fraction with 1 mol/L KCl. GR (EC 1.6.4.2) activity was determined by measuring the absorbance increment at 412 nm when 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) was reduced by GSH, generated from glutathione disulfide (GSSG) (Smith et al. 1988). The specific activity was calculated as the amount of reduced DTNB, in μmol min⁻¹ protein mg⁻¹ protein. GST (EC 2.5.1.18) activity was determined spectrophotometrically by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB), according to Habig et al. (1974). Reactions were initiated by the addition of CDNB, and the increase in A 340 was determined. One U is the amount of enzyme producing 1 μmol conjugated product in 1 min, ε 340 = 9.6 mmol/L⁻¹ cm⁻¹. GSH-PX (EC 1.11.1.9) activity was measured by the method of Awasthi et al. (1975), with cumene hydroperoxide as a substrate. The reaction mixture contained 4 mmol/L GSH, 0.2 mmol/L NADPH, 0.05 U of GR (Type II from wheat, Sigma), 100 μL enzyme extract, and 0.5 mmol/L substrate in phosphate buffer (0.1 mol/L, pH 7.0) in a total volume of 1 mL. The decrease of NADPH was followed by measuring the absorbance at 340 nm. The nonspecific NADPH decrease was corrected for by using additional measurements without substrate, ε 340 = 6.22 mmol/L⁻¹ cm⁻¹. One U = μmol converted NADPH min⁻¹. The protein contents of the extracts were determined by the method of Bradford (1976).

H₂O₂ and malondialdehyde (MDA) assays

H₂O₂ concentration was measured by the fluorometric method of Asada et al. (1974) with some modifications published by Hidieg et al. (2003). A half gram (0.5 g) callus was frozen in liquid N₂ and homogenized with 0.5 mL of 0.1 mol/L phosphate buffer, pH 7.0. After centrifugation, 25 μL of the extract was added to 3 mL of reaction mixture containing 0.5 mmol/L homovanillic acid (HVA) and 0.25 U horseradish peroxidase (HRP). The fluorescence yield due to H₂O₂ was determined in each sample by the increase in fluorescence intensity, using excitation and emission wavelengths of 295 and 415 nm, respectively. During the calculation of the H₂O₂ content, the results of the recovery experiments were taken into consideration. MDA formation was assayed by using the thiobarbituric acid method (Esterl et al. 1997). A half gram (0.5 g) callus was homogenized with 5 mL of 0.1% trichloroacetic acid (TCA). To avoid further lipid peroxidation, 500 μL of 4% butylhydroxytoluene (BHT) was added to the extract. After centrifugation at 15,000 g for 25 min, 1 mL of supernatant was mixed with 4 mL 0.5% thiobarbituric acid in 20% TCA and the mixture was incubated in boiling water for 30 min. The absorbance was read at 532 nm and adjusted for nonspecific absorbance at 600 nm. MDA concentration was estimated by using an extinction coefficient of 155 mmol/L⁻¹ cm⁻¹.

Statistical analysis

The means ± SD were calculated from the data of at least three separate experiments. Differences between treatment means were deter-
mined by Duncan’s multiple range test. Columns denoted by the same letters did not differ significantly at a probability level of $P < 0.05$.

**Results**

The growth rate, Na$^+$ content, and ethylene release of heterotrophic and autotrophic calli under NaCl stress

The growth of auxin heterotrophic calli was compared with that of the autotrophic line of the same tissues two weeks after the transfer onto MS medium containing 100 mmol/L NaCl. At this age the calli were in their exponential growth phase and the difference between the growth rates of the two lines was maximal (Köves and Szabó 1987, and unpublished results). The habituated cultures grew slowly even on NaCl free media, but in the presence of NaCl the basic growth rate did not drop as much as for the heterotrophic line (Fig. 1A). Determination of the Na$^+$ concentrations of the 2-week-old calli revealed that the Na$^+$ uptake of the autotrophic tissues was not inhibited, because their Na$^+$ concentration in the presence of 100 mmol/L NaCl was slightly higher than that of the heterotrophic line (Fig. 1B).

![Figure 2](image)

**Figure 2.** Ethylene evolution of auxin heterotrophic (A) and autotrophic calli (B) growing on MS medium containing 100 mM NaCl. The data are means of one representative experiment in at least 4 replicates. Note the different vertical scales in A and B.

![Figure 3](image)

**Figure 3.** Guaiacol peroxidase activities in the auxin heterotrophic and autotrophic tobacco calli after 2 weeks of NaCl treatment. A: GPX activity of the soluble fraction; B: ionically bound peroxidase activity; C: activity of the covalently bound peroxidases. Means denoted by the same letters were not significantly different ($P < 0.05$, Duncan test).
Auxin autotrophic calli and oxidative stress

The ethylene production of the two lines followed the pattern observed earlier for auxin-dependent and auxin autotrophic cultures. The heterotrophic tissues evolved much more ethylene with an early maximum on an auxin-containing culture medium and the cells increased the ethylene production in the presence of the salt. The ethylene formation was 20 times less in the autotrophic cultures, and its level did not change significantly upon the NaCl treatment (Fig. 2).

The effect of salt stress on the activities of the antioxidative enzymes and H2O2 concentrations of heterotrophic and autotrophic tissues

Comparison of the GPX activities in the heterotrophic and autotrophic calli revealed approximately two-fold higher activities in the autotrophic cultures in the soluble and ionically bound fractions, and the fraction bound covalently to the cell walls also exhibited a slight increase in peroxidative capacities (Figs. 3 A, B, C). The NaCl stress did not elevate the peroxidative capacity in the autotrophic line, and the level did not change significantly upon the NaCl treatment (Fig. 2).

The activity of GR, which reduces GSSG to GSH, was higher in the auxin-requiring cultures, but in response to a NaCl stress the activity increased only in the heterotrophic line (Fig. 6A). Interestingly, the GST and especially the GSH-PX activities were higher in the autotrophic calli, which essentially contribute to the elevated stress tolerance of habituated tissues (Figs. 6 B, C). While the auxin autotrophic line enhanced the GSH-PX activity during salt stress, the enzyme activity remained exceptionally low in the heterotrophic cultures. The level of MDA, a lipid peroxidation product in the tissues, was less in the autotrophic line and, similar to the level of H2O2, the amount of this product decreased further on NaCl treatment (Fig. 7), suggesting a membrane-protecting role of glutathione peroxidases during salt stress.

Growth of calli in the presence of exogenous H2O2

In order to reveal the sensitivities of the two lines to exogenous H2O2, the calli were transferred onto fresh MS medium...
Figure 6. GR (A), GST (B) and GSH-PX (C) activities of auxin heterotrophic and autotrophic calli after 2 weeks of salt treatment. Means denoted by the same letters were not significantly different (P < 0.05, Duncan test).

Figure 7. MDA level in the 2-week-old auxin heterotrophic and autotrophic tobacco cultures after 100 mmol/L NaCl treatment. Means denoted by different letters indicate a significant difference between the treatments (P < 0.05, Duncan test).

Figure 8. Growth of 2-week-old auxin heterotrophic and autotrophic tobacco callus cultures in the presence of different H$_2$O$_2$ concentrations. The data are means ± SD of four measurements.

The growth of the auxin heterotrophic calli decreased significantly at 1 mmol/L H$_2$O$_2$, while the autotrophic line was inhibited only by 10 mmol/L H$_2$O$_2$ (Fig. 8). This proves that the autotrophic calli really do possess a more effective H$_2$O$_2$-scavenging mechanism.

Discussion

The data presented here suggest that tobacco calli that have passed through several steps of neoplastic progression and
have become habituated are very resistant to NaCl stress treatment. During the habituation process, the cells regain their auxin-synthesizing capacity and they are able to grow on an auxin-free culture medium. The phenotype of our cultures is slightly different from other habituated cultures, such as those from fully auxin- and cytokinin habituated sugar beet cell lines, which involve very friable and achlorophyllous tissues and, according to Bisbis et al. (1999), can be considered true cancer cells. Our auxin autotrophic calli are compact and may contain even more chlorophyll than the heterotrophic cells. It seems that in this line tetrpyrrole synthesis is not inhibited. To decide whether this is due to a maintained ALA accumulation or to the synthesis of cinnamic acid derivatives, instead of ALA-dehydratase-inhibiting benzoic acid derivatives in the autotrophic calli, requires further investigations.

The heterotrophic line exhibited a high ethylene production with an early maximum after subculturing, which may be due to the exogenous growth regulators (auxins and cytokinins) in the growth medium. This ethylene release was further enhanced by the NaCl treatment.

Ethylene, as a stress hormone, induces an acclimatization process involving the activation of defence mechanisms at a transcriptional level or may activate those processes, such as the generation of AOS including H$_2$O$_2$, which result in increased peroxidase activities (Ishige et al. 1993, levins et al. 1995). The steady-state activity of GPX (based on the steady-state hormonal and environmental factors) were even higher in the 2-week-old auxin autotrophic line, but the tissues did not enhance the peroxidative capacity significantly in response to 100 mmol/L NaCl stress, which may be due to the lower ethylene production of these cells in the absence or presence of NaCl than in the heterotrophic calli.

One of the most important roles of peroxidases in plant cells is to catalyze the cross-linking of cell wall components (Hatfield et al. 1999) and, in this manner, to restrict the expansion of the cells (Andrews et al. 2002). The differences in cell wall-bound peroxidase activity may be responsible for the slower growths of the autotrophic line. The covalently bound peroxidase activity increased considerably in the presence of 100 mmol/L NaCl in the heterotrophic tissues, but not in the autotrophic cells. The elevated enzyme activity may explain why the auxin autotrophic line grew slowly, but the growth inhibition caused by NaCl was smaller in the autotrophic than in the heterotrophic tissues.

A crucial difference was found between the heterotrophic and autotrophic cultures in the CAT activity. The cells are supplied with reduced sugar exogenously and their photosynthetic activity is highly suppressed (Cséplő and Medgyesy 1986). Hence, there is no need for the detoxification of H$_2$O$_2$, which is derived as a by-product of the photosynthetic electron transport and photorespiration. The auxin-requiring tissues increased the CAT activity during the NaCl stress and this activation contributed considerably to the effective elimination of H$_2$O$_2$. However, the auxin autotrophic cultures must activate other pathways to scavenge H$_2$O$_2$.

The GSTs play an important role in the detoxification mechanism by catalyzing the GSH conjugation of toxic substances under stress. GST-encoding genes are selectively expressed during abiotic or biotic stress, but some isoenzymes are involved in conjugating natural plant products and can mediate isomerase reactions or function as carrier proteins. GST isoenzymes are also capable of acting as GSH-PX and reduce organic hydroperoxides to their monohydroxy derivatives by using GSH (Edwards et al. 2000). Some isoenzymes of GSTs have been shown to exhibit considerable GSH-PX activity with linolenic acid hydroperoxide as substrate (Gronwald and Plaisance 1998). Plant GSH-PXs are induced by different stresses, and transgenic tobacco plants overproducing a GST gene with GSH-PX activity exhibit significant stress tolerance (Roxas et al. 2000).

Genes encoding GSTs are induced by several factors, including ethylene, IAA, and H$_2$O$_2$ (Marrs 1996). Callus cultures grow on a medium containing exogenous IAA and 2,4-D; these hormones may induce the transcription of genes encoding GSTs, which, in turn, can influence the level of active auxin hormones, by conjugation for example (Takahashi et al. 1989, Ulmasov et al. 1994, Bianchi et al. 2002). GSTs, and especially the isoenzymes with GSH-PX activity, can scavenge lipid hydroperoxides and maintain membrane integrity under different stress conditions. Higher GST and GSH-PX activities may provide some advantages for the growth of cells in the presence of exogenous hormones, and especially in the presence of the synthetic auxin 2,4-D and/or auxin-induced oxidative stress. Our previous results indicated that the GST activity of the auxin heterotrophic tissues is inhibited by ethylene. When the binding of ethylene to the receptor was blocked with norbornadiene, the total GST activity increased, whereas in the autotrophic calli, norbornadiene did not influence the extractable GST activity (Csiszar et al. 2001). The lower ethylene production of autotrophic cells on an auxin-free culture medium could result in the enhanced GST and GSH-PX activities of these tissues.

We found a high GSH-PX activity in our autotrophic line, but not in the auxin-requiring cells. This seems to prove a very effective acclimatisation mechanism. Despite the lower SOD, CAT, and GR activities, the growth of the autotrophic calli was not inhibited significantly in the presence of 100 mmol/L NaCl as compared with the autotrophic control, and their H$_2$O$_2$ and MDA contents were lower than those in the heterotrophic tissues. The growth of calli measured via the fresh mass demonstrated that the autotrophic line was the more resistant to exogenous H$_2$O$_2$. These are the first data to date indicating that an auxin autotrophic plant culture that has passed through a certain degree of neoplastic transformation exhibits a lower H$_2$O$_2$ sensitivity than an auxin heterotrophic line.

In contrast with the considerable activities of the AOS-scavenging SOD and CAT enzymes in the auxin heterotrophic calli, it is the peroxidases and mainly the GSH-PX activity in the autotrophic tissues that can ensure protection against
NaCl stress and presumably other stress-induced oxidative damage.

Acknowledgement. This work was supported by National Scientific Foundation OTKA grants T030259 and T038392.

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