Proline and Glycinebetaine Ameliorated NaCl Stress via Scavenging of Hydrogen Peroxide and Methylglyoxal but Not Superoxide or Nitric Oxide in Tobacco Cultured Cells

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Efficient detoxification of the reactive oxygen species, nitric oxide (NO) and methylglyoxal (MG), provides protection against NaCl-induced damage in plants. To elucidate the protective mechanisms of proline and glycinebetaine (betaine) against NaCl stress, intracellular levels of hydrogen peroxide (H2O2), superoxide (O2·−), NO, and MG were investigated in tobacco Bright Yellow-2 cells. The Levels of H2O2, O2·−, NO and MG were higher in the short-term and long-term NaCl-stressed cells than in the non-stressed cells, whereas the O2·− level was higher in the long-term stressed cells. Exogenous proline and betaine decreased the H2O2 level in both the short-term and the long-term NaCl-stressed cells and the Mg level in the long-term NaCl-stressed cells, but did not change the O2·− or NO levels. Under salt stress, both proline and betaine increased the transcription levels of glutathione peroxidase, which can contribute to the reduction of H2O2. In conclusion, proline and betaine mitigated salt stress via reduction of H2O2 accumulation during short-term incubation and via reduction of the accumulation of H2O2 and MG during long-term incubation.

Key words: antioxidant defense; glycinebetaine; methylglyoxal; proline; salt stress

Salt stress is one of the major environmental stresses limiting crop productivity. Salinity imposes both ionic toxicity and osmotic stress on plants, leading to oxidative stress.1,2 One of the main adaptive mechanisms to salt stress in plants is accumulation of compatible solutes, such as proline and glycinebetaine (hereafter betaine). Proline and betaine contribute to osmotic adjustment1,3,4 as well as to the protection of membranes, proteins, and enzymes from the damaging effects of various stresses.1–7 Moreover, proline and betaine provide protection against stress by maintaining redox homeostasis.8 Under salt stress, exogenous application of proline or betaine upregulates stress-protective proteins,9 and reduces lipid peroxidation10–12 and protein oxidation.13

Environmental stress, including salinity, induces the production of hydrogen peroxide (H2O2), the superoxide radical (O2·−), and nitric oxide (NO) in plant cells.1,12–18 Excess production of H2O2, O2·−, and NO is toxic to plants, and causes oxidative damage to cellular constituents, leading to cell death.12–15,18–20 In plant cells, salinity also leads to the accumulation of methylglyoxal (MG), a cytotoxic compound that can react with and modify other molecules, including DNA and proteins.21–23 Because of the highly cytotoxic and reactive properties of H2O2, O2·−, NO, and MG, their concentrations must be kept under strict control. Plants possess antioxidant defense and glyoxalase systems to protect their cells against the damaging effects of H2O2, O2·−, and MG.13,19,22,23

Proline, besides functioning as an osmoprotectant, suppresses production of free radicals16,11 and reactive oxygen species (ROS).24,25 Our recent study indicated that both exogenous proline and betaine decrease H2O2 accumulation induced by NaCl12 and cadmium.18 There is increasing evidence that both proline and betaine increase antioxidant defense mechanisms and improve stress tolerance in plants as well as in cultured cells.9,10,18,25–27 We have found that exogenous application of both proline and betaine increases intracellular proline and betaine contents,11 which leads to the suppression of cell death and improvement of salt tolerance in tobacco Bright Yellow-2 (BY-2) cells via increments in the antioxidant defense and MG detoxification systems,8,12,28,29 but protective mechanisms of proline and betaine in plant responses to salt stress remain to be clarified. The present study was therefore conducted to investigate the effects of exogenous proline and betaine on intracellular H2O2, O2·−, NO, and MG levels and the mRNA levels of glutathione peroxidase (GPX) and glyoxalase I genes in tobacco BY-2 cells exposed to salt stress. A very recent study found inhibition of the activity of H2O2-scavenging antioxidant enzymes in Brassica oleracea roots under short-term (1-d) salt stress, whereas long-term (7-d) salt stress resulted in recovery of the activity of certain H2O2-scavenging antioxidant enzymes.
scavenging enzymes, suggesting that induction of antioxidant defenses is one component of the tolerance mechanism to long-term salt stress. In this study, the roles of proline and betaine in early and late responses to salt stress were also investigated.

Materials and Methods

Culture of tobacco BY-2 cells. Suspension-cultured cells of tobacco (Nicotiana tabacum L., cv. BY-2) were used as the source of NaCl-unadapted cell lines. The standard medium was a modified LS medium, in which K2HPO4 and thiamine-HCl were increased to 370 and 1 mg/l respectively, supplemented with 3% sucrose and 1 μM 2,4-dichlorophenoxyacetic acid. The 50 mM NaCl, 100 mM NaCl, and 200 mM NaCl media were standard media supplemented with 50 mM NaCl, 100 mM NaCl, and 200 mM NaCl respectively. The proline medium was the 200 mM NaCl medium supplemented with 20 mM proline, and the betaine medium was the 200 mM NaCl medium supplemented with 20 mM betaine. The cells were cultured and maintained as described previously. Briefly, they were subcultured weekly by transfer of the suspension cells of a 7-d-old culture into 30 ml of various fresh media. The culture was incubated on a rotary shaker at 100 rpm at 25°C in the dark. Four-d-old BY-2 cells cultured in the various media were used.

Isolation and preparation of protoplasts. In order to measure intracellular H2O2, O2·−, and NO levels using a flow cytometer, protoplasts were enzymatically isolated from the cells. The cells were subjected to occasional gentle swirling at 30°C for about 1 h in an enzyme solution, adjusted to pH 5.5, that contained 1% cellulase Onozuka RS (Yakult Honsha, Tokyo), 0.1% pectolyase Y-23 (Seishin Pharmaceutical, Tokyo), and 0.64 mM sorbitol for the cells grown in the standard medium; 0.7 M sorbitol for the cells grown in the 50 mM NaCl medium; 0.8 M sorbitol for the cells grown in the 100 mM NaCl medium; 0.8 M sorbitol for the cells grown in the 100 mM NaCl medium or proline or betaine medium; and 1.0 M sorbitol for the cells grown in the 200 mM NaCl medium. Protoplasts were collected by centrifugation at 100 × g for 1 min.

Assay of intracellular H2O2, O2·−, and NO levels during short-term incubation. Protoplasts isolated from 4-d-old non-stressed cells were incubated for 3 h at 37°C with 50, 100, or 200 mM NaCl in the presence and the absence of 20 mM proline or betaine. An additional incubation was performed for 30 min at 37°C with 25 μM 2′,7′-dichlorofluorescin diacetate (H2DCF-DA), 10 μM dihydroethidium (DHE), and 10 μM 4,5-diaminofluorescein diacetate (DAF-2DA) for measurements of the H2O2, O2·−, and NO levels, respectively. The protoplasts were then washed and resuspended in sorbitol solution. The intracellular H2O2, O2·−, and NO levels were measured by flow cytometry. Similar procedures were followed for the control (non-stress) treatment.

Assay of intracellular H2O2, O2·−, and NO levels during long-term incubation. Protoplasts were isolated from 4-d-old BY-2 cells cultured in the various media. Isolated protoplasts were incubated with 25 μM H2DCF-DA, 10 μM DHE and 10 μM DAF-2DA for measurement of H2O2, O2·−, and NO levels, respectively, for 30 min at 37°C. Thereafter, the protoplasts were washed and resuspended in sorbitol solution. The intracellular H2O2, O2·−, and NO levels were measured by flow cytometry.

Assay of MG contents during short-term incubation. Four-d-old non-stressed cells were incubated with 50, 100, or 200 mM NaCl in the presence and the absence of 20 mM proline or betaine for 3 h on a rotary shaker at 100 rpm at 25°C in the dark. The MG content was measured by the method of Yadav et al., with modifications. An aliquot of cells (0.5 g fresh weight) was homogenized in 2 ml of PBS. The homogenate was centrifuged at 4°C at 11,000 × g after incubation for 15 min on ice. Thereafter, the MG content was assayed and calculated by the method as described above.

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from freeze-dried ground cells using TRIzol® reagent (Invitrogen, Carlsbad, CA) following to the manufacturer’s instructions. The concentration and purity of the RNA were assayed spectrophotometrically at 260 and 280 nm. Five μg of total RNA were reverse-transcribed into first-strand cDNA using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Takara Bio, Ohtsu, Japan). PCR was carried out in a reaction mixture containing 1 μl of cDNA, 200 μM dNTPs, 0.5 μM primers (forward and reverse), 1.25 units of Ex Taq polymerase (Takara Bio) and 1 × Ex Taq buffer. The gene-specific primers used in this study with the PCR conditions are listed in Table 1. An equal volume of PCR products was subjected to electrophoresis on 1.5% agarose gel prior to staining with ethidium bromide. The gels were photographed under UV light.

Statistical analysis. Statistical analysis was carried out by Analysis of Variance (ANOVA). The means of the various treatments were tested for level of significance at 5% probability by Duncan’s Multiple Range test (DMRT).

Results

Intracellular H2O2 levels during short-term and long-term incubation

To clarify the mechanisms of protection of proline and betaine in plant responses to NaCl stress, the intracellular H2O2 levels in tobacco BY-2 cells were measured after 3 h (short-term) and 4 d (long-term) of incubation with 0 to 200 mM NaCl in the presence and the absence of 20 mM proline or betaine (Fig. 1A and B). Compared with the intracellular H2O2 level in non-stressed protoplasts, the intracellular H2O2 levels were significantly increased under each stress condition (50 to 200 mM NaCl) during short-term incubation (Fig. 1A). The level of H2O2 increasing with increasing concentrations of NaCl, but no significant differences (p > 0.05) were observed between the 50 mM and 100 mM NaCl treatment (Fig. 1A). Compared with the intracellular H2O2 level in the non-stressed protoplasts, intracellular H2O2 levels were significantly increased under every stress condition (50 to 200 mM NaCl) during long-term incubation (Fig. 1B). The level of H2O2 increased with increasing concentrations of NaCl, but no significant differences (p > 0.05) were observed as between 50 mM and 100 mM NaCl treatments. During both the short and the long period of incubation, exogenous application of proline and betaine significantly decreased (p < 0.05) the H2O2 accumulation in the BY-2 cells under 200 mM NaCl stress (Fig. 1A and B).

Prior to incubation with 0 to 200 mM NaCl in the presence and the absence of 20 mM proline or betaine for 3 h, we added 25 μM diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, to the protoplast suspension to determine the effects of DPI on NaCl-induced H2O2 accumulation in the presence and the absence of proline and betaine, but DPI did not affect H2O2 production in the tobacco BY-2 cells (data not shown).
Intracellular $O_2^{-}$ levels during short-term and long-term incubation

As shown in Fig. 2A, no significant changes ($p > 0.05$) in intracellular $O_2^{-}$ levels were observed in response to the short-term NaCl treatment, even with addition of proline or betaine to the protoplast suspension. On the other hand, a significant increase ($p < 0.01$) in the intracellular $O_2^{-}$ level was observed in response to 200 mM NaCl stress after long-term incubation (Fig. 2B). We investigated to determine whether proline or betaine would reduce the $O_2^{-}$ level in long-term NaCl-stressed cells, but neither contributed to the reduction in intracellular $O_2^{-}$ level in 200 mM NaCl-stressed cells after long-term incubation (Fig. 2B).

Intracellular NO levels during short-term and long-term incubation

Short-term NaCl treatments caused a significant increase ($p < 0.01$) in NO production in the BY-2 cells (Fig. 3A). The level of NO was significantly higher in the 200 mM NaCl-stressed cells than in the 50 mM and 100 mM NaCl-stressed cells during short-term incubation. No significant changes ($p > 0.05$) in intracellular NO levels were observed in the short-term 200 mM NaCl-stressed cells, irrespective of the presence of proline or betaine (Fig. 3A). We also investigated to determine whether proline or betaine would affect NO production in NaCl-stressed cells after long-term incubation. Significant increases ($p < 0.05$) in intracellular NO levels were observed in response to long-term NaCl treatment (Fig. 3B), but an elevated level of NO was recorded in the long-term 200 mM NaCl-stressed cells. Similarly to short-term incubation, exogenously applied proline and betaine did not affect NO production in the 200 mM NaCl-stressed cells during long-term incubation (Fig. 3B).

Methylglyoxal contents during short-term and long-term incubation

A significant increase ($p < 0.01$) in MG content was observed in the BY-2 cells exposed to short-term 200 mM NaCl stress (Fig. 4A). After long-term NaCl treatment, the MG contents increased with increasing NaCl concentrations (Fig. 4B). Long-term exposure to 100 mM ($p < 0.05$) or 200 mM NaCl ($p < 0.01$) produced significantly higher MG contents in the BY-2 cells. Exogenous application of proline and of betaine resulted in a significant reduction of MG contents in the 200 mM NaCl-stressed cells during long-term incubation (Fig. 4B), but not during short-term incubation (Fig. 4A).
Expression of the genes involved in the detoxification of H$_2$O$_2$ and MG

Since both proline and betaine suppressed the accumulation of H$_2$O$_2$ and MG during long-term 200 mM NaCl stress, we investigated to determine whether proline or betaine would induce transcription levels of the GPX and glyoxalase I genes under long-term 200 mM NaCl stress (Fig. 5). NaCl stress produced a slight increase in the mRNA level of GPX. Importantly, both proline ($p < 0.05$) and betaine ($p < 0.05$) significantly increased mRNA levels of GPX under NaCl stress (Fig. 5). The NaCl-stressed cells showed an approximately 2.2-fold higher ($p < 0.01$) glyoxalase I mRNA level than the non-stressed cells, but exogenous proline and betaine did not result in an increase in the mRNA level of glyoxalase I in the NaCl-stressed cells (Fig. 5).

Discussion

NaCl-unadapted tobacco BY-2 cell lines were grown in non-stress and NaCl media with and without proline or betaine. The non-stressed cell cultures were light yellow in colour, while cell cultures under NaCl stress were brighter yellow in colour. Our previous studies indicated that exogenous proline and betaine induce accumulation of more than 200 mM proline and betaine respectively in tobacco BY-2 cells under 200 mM NaCl stress$^{11}$ and mitigate NaCl-induced growth inhibition after 4 d of incubation,$^{11,28}$ indicating that uptake of compatible solutes plays an important role in the adaptation to osmotic stress due to salinity. Exogenously applied solutes play an important role in the adaptation to osmotic stress due to salinity.
lipid membranes and protein under 200 mM NaCl stress,12 suggesting that both compounds are efficient at mitigating most of the deleterious consequences of salt stress after 4 d of incubation,12 but proline mitigates the inhibitory effects of salt stress more than betaine due to differences in the antioxidant defense and MG detoxification systems.8,28,29 In the present study, we attempted to clarify the protective mechanisms of proline and betaine in cultured tobacco cell responses to salt stress.

ROS are known to be primarily responsible for the impairment of cellular functions under abiotic and biotic stress conditions. Salt stress-induced accumulation of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) and a reduction in cell viability have been reported in plants.14,16 In BY-2 cells, both the short-term (3 h) and the long-term (4 d) NaCl treatment induced \( \text{H}_2\text{O}_2 \) accumulation (Fig. 1A and B), while only the long-term NaCl treatment increased \( \text{O}_2^- \) levels (Fig. 2B). Similarly, Hernandez et al.17 observed a higher accumulation of \( \text{H}_2\text{O}_2 \) in \( \text{B. oleracea} \) roots under both short-term and long-term NaCl treatment. The higher accumulation of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) in NaCl-stressed cells might be associated with decreased activities of \( \text{H}_2\text{O}_2 \)- and \( \text{O}_2^- \)-scavenging antioxidant enzymes, inhibiting cell growth and increasing cell death.3,12,28,29 Tight regulation of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) homeostasis is necessary to protect cells against the damaging effects of salt stress. Proline is a potent ROS scavenger associated with inhibition of apoptosis-like cell death and increasing activity of catalase.24 Similarly to the protective effects

**Fig. 4.** Effects of Proline and Betaine on MG Contents in Cultured Tobacco BY-2 Cells Exposed to Short-Term (A) and Long-Term (B) NaCl Stress.

Values represent the mean \pm SE for five independent experiments. (A) Four-d-old non-stressed cells were incubated with 0 to 200 mM NaCl in the presence and the absence of 20 mM proline or betaine for 3 h on a rotary shaker at 100 rpm at 25°C in the dark. (B) After 4 d of incubation, MG contents were measured from cells cultured in the various media.

**Fig. 5.** Effects of Proline and Betaine on the mRNA Levels of GPX and Glyoxalase I in Cultured Tobacco BY-2 Cells Exposed to Long-Term NaCl Stress.

Values represent the mean \pm SE for at least five independent experiments. Actin was used as internal control to normalize the mRNA levels of each gene. NS, non-stress; NaCl, 200 mM NaCl; Proline, 200 mM NaCl + 20 mM proline; Betaine, 200 mM NaCl + 20 mM betaine.
of proline and betaine against long-term NaCl stress,12) both proline and betaine suppressed H$_2$O$_2$ accumulation in both short-term and long-term NaCl-stressed cells (Fig. 1A and B). The protective effects of proline and betaine were not observed in the production of O$_2^\cdot$- induced by long-term NaCl stress (Fig. 2B). Our previous studies have indicated that both proline and betaine improve salt tolerance without increasing the activity or expression of superoxide dismutase (SOD).12,28) The main function of SOD is to scavenge the O$_2^\cdot$- radicals generated by various stresses. This suggests that both proline and betaine provide protections against salt stress by preventing the O$_2^\cdot$- radicals by increasing the activity and/or expression of SOD.

Salt stress may trigger H$_2$O$_2$ production mediated by NADPH oxidase and/or other redox enzymes. NADPH oxidase catalyzes the production of O$_2^\cdot$- by transferring electrons from NADPH to molecular oxygen, followed by dismutation of O$_2^\cdot$- to H$_2$O$_2$. An inhibitor of NADPH oxidase, DPI, did not affect intracellular H$_2$O$_2$ levels in the salt-stressed cells (data not shown). This suggests that NADPH oxidase is not involved in the generation of H$_2$O$_2$ through dismutation of O$_2^\cdot$- by SOD.

NO is protective or cytotoxic, depending on the concentration. In reaction with ROS, NO can produce peroxynitrite (ONOO$^-$), a powerful oxidant, which can cause cellular damage.35,36) Increases in the NO level have been observed under NaCl stress (Fig. 3A and B). On the other hand, NaCl has been found to decrease the NO level by inhibiting NO synthase activity in Arabidopsis mutant (Atnos1) plants with impaired in vivo NO synthase activity, and in wild-type plants as well,35 but simultaneous increases in NO and H$_2$O$_2$ in tobacco cells induce apoptosis-like cell death.20) In BY-2 cells, proline and betaine did not reduce NO production under either short-term or long-term NaCl treatment (Fig. 3A and B), although both compounds suppress H$_2$O$_2$ accumulation (Fig. 1A and B) and cell death.12) Decreased levels of H$_2$O$_2$ in the NaCl-stressed cells due to proline and betaine might be more important than those of O$_2^\cdot$- and NO in avoiding cellular damage and improving cell growth. The reduction in H$_2$O$_2$ accumulation due to proline and betaine may be a reason for the increased activity and/or expression of H$_2$O$_2$-scavenging antioxidant enzymes and/or genes,8,12,28,29) that can inhibit the accumulation of extra H$_2$O$_2$ in cells.

Besides acting as a signaling molecule,22) MG in cells can cause oxidative damage to cellular constituents.21,23) In the present study, both short-term and long-term NaCl treatment induced MG accumulation in BY-2 cells (Fig. 4A and B). After long-term incubation, both proline and betaine reduced the NaCl-induced MG content (Fig. 4B). Increased activity of glyoxalase I, an MG detoxifying enzyme, might result in a reduction in MG content in NaCl-stressed-cells in the presence of proline or betaine.81)

Our earlier study indicated that both proline and betaine increase the transcript levels of salicylic acid-binding catalase and lignin-forming peroxidase, which are correlated with inhibition of H$_2$O$_2$ accumulation and cell death under salt stress.12) GPX is considered to be an important ROS scavenger because of its broader substrate specifications and stronger affinity for H$_2$O$_2$ than catalase.37) Arabidopsis thaliana GPX3 plays a dual role in H$_2$O$_2$ homeostasis, acting as a general scavenger and in H$_2$O$_2$ signal transduction.38) In eukaryotes, the glyoxalase system is the main MG catabolic pathway. Since both proline and betaine caused lower accumulation of H$_2$O$_2$ and MG in long-term NaCl-stressed cells, we studied the transcription levels of GPX and glyoxalase I after long-term 200 mM NaCl stress. Both exogenous proline and betaine increased the transcription levels of GPX under NaCl stress (Fig. 5). Similarly to the transcription levels, proline and betaine have been to increase the activity of GPX under NaCl stress.8) Neither proline nor betaine affected transcription levels of glyoxalase I under NaCl stress (Fig. 5), although both increased activity of glyoxalase I under such stress,8) which can contribute to the detoxification of MG. Overexpression or higher activity of GPX in plants increases antioxidant activity and improves tolerance of oxidative stress.39,40) Taken together, the increased activity and/or expression of H$_2$O$_2$- and MG-scavenging enzymes and/or genes in tobacco BY-2 cells grown with exogenous proline or betaine under NaCl stress suggests greater protection against the damaging effects of H$_2$O$_2$ and MG.8,12,28,29)

In conclusion, proline and betaine do not modulate the early response to salt stress and production of O$_2^\cdot$- and NO, but do mitigate late response to salt stress and the production of H$_2$O$_2$ and MG. Proline and betaine can alleviate a variety of environmental stresses, since the late response to salt stress appears to be similar to the response elicited by various environmental stresses, but the reduction in accumulation of H$_2$O$_2$ and MG due to proline and betaine might be imperative in avoiding cellular damage and increasing salt tolerance.

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