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Proline and glycinebetaine induce antioxidant defense gene expression and suppress cell death in cultured tobacco cells under salt stress

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Summary

Salt stress causes oxidative damage and cell death in plants. Plants accumulate proline and glycinebetaine (betaine) to mitigate detrimental effects of salt stress. The aim of this study was to investigate the protective effects of proline and betaine on cell death in NaCl-unadapted tobacco (*Nicotiana tabacum*) Bright Yellow-2 suspension-cultured cells subjected to salt stress. Salt stress increased reactive oxygen species (ROS) accumulation, lipid peroxidation, nuclear deformation and degradation, chromatin condensation, apoptosis-like cell death and ATP contents. Neither proline nor betaine affected apoptosis-like cell death and G_1 phase population, and increased ATP contents in the 200 mM NaCl-stressed cells. However, both of them effectively decreased ROS accumulation and lipid peroxidation, and suppressed nuclear deformation and chromatin condensation induced by severe salt stress. Evans Blue staining experiment showed that both proline and betaine significantly suppressed increment of membrane permeability induced by 200 mM NaCl. Furthermore, among the ROS scavenging antioxidant defense genes studied here, mRNA levels of *salicylic acid-binding* (SAbind) *catalase* (CAT) and *lignin-forming peroxidase* (POX) were found to be increased by proline and betaine under salt stress. It is concluded that both proline and betaine provide a protection against NaCl-induced cell death via decreasing level of ROS accumulation and lipid

Abbreviations: APX, ascorbate peroxidase; Betaine, glycinebetaine; CAT, catalase; DAPI, 4′, 6-diamidino-2-phenylindole; H_2DCF-DA, 2′, 7′-dichlorofluorescin diacetate; MDA, malondialdehyde; PI, propidium iodide; POX, peroxidase; ROS, reactive oxygen species; SAbind, salicylic acid-binding; SOD; superoxide dismutase.

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peroxidation as well as improvement of membrane integrity.

Introduction

Under environmental stress conditions, many plants accumulate several kinds of compatible solutes such as proline, glycinebetaine (from now on betaine), sugars and polyols. Proline and betaine are well-known compatible solutes that play a pivotal role in the process of osmotic adjustment in different organisms including higher plants (Greenway and Munns, 1980; Rhodes and Hanson, 1993; Hasegawa et al., 2000; Ashraf and Foolad, 2007). Salt stress up-regulates the enzymes involved in proline and betaine biosyntheses in several plant species (Russell et al., 1998; Hare et al., 1999), and increased accumulation of proline and betaine can reduce stress damage to plant cells (Rhodes and Hanson, 1993; Hasegawa et al., 2000; Ashraf and Foolad, 2007). In addition to their roles as osmoprotectants, proline and betaine protect higher plants against salt/osmotic stress by stabilizing membranes, proteins and enzymes (Mansour, 1998; Okuma et al., 2000, 2002; Ashraf and Foolad, 2007). Furthermore, proline decreases amounts of free radicals (Hasegawa et al., 2000; Hong et al., 2000; Okuma et al., 2000, 2004) and reactive oxygen species (ROS) (Chen and Dickman, 2005; Chen et al., 2006). It has been shown that exogenous proline and betaine improve salt tolerance by up-regulating stress-protective proteins (Khedr et al., 2003) and reducing oxidation of lipid membranes (Demiral and Türkan, 2004; Okuma et al., 2004).

Cell death is essential for growth and development of eukaryotes by maintaining tissue and organ homeostasis in concert with cell proliferation, growth and differentiation (Van Breusegem and Dat, 2006). Plant cell death occurs as either necrosis or apoptosis. Necrosis is passive and characterized by a progressive loss of membrane integrity that results in swelling of cytoplasm and release of cellular constituents (Van Breusegem and Dat, 2006). Apoptosis (programmed cell death), on the other hand, is genetically controlled and characterized by specific alterations of cellular morphological and biochemical features such as cell shrinkage, chromatin condensation, nuclear degradation and DNA fragmentation (Lam et al., 2001; Lam, 2004). Apoptosis and its mechanism are fairly well known in animals but it is little understood in plants. Intracellular ATP levels determine cell death occurred by apoptosis or necrosis in plant cells as well as in animal cells. Higher levels of ATP are required for the execution of cell death by apoptosis, while necrosis can proceed in the presence of low levels of ATP (Leist et al., 1997).

Salt stress induces the accumulation of ROS. ROS are toxic to plants because they oxidize lipids, proteins, DNA and carbohydrates, resulting in the breakdown of normal cellular functions (Rodriguez and Redman, 2005) and ultimately trigger apoptosis (Fath et al., 2001; Apel and Hirt, 2004; Van Breusegem and Dat, 2006). Under salt stress, apoptosis-like cell death has been reported in plant cells (Katsuhara and Kawasaki, 1996; Katsuhara, 1997; Deuschle et al., 2004; Lin et al., 2006). It has been shown that H_2O_2 causes apoptosis including cell shrinkage, chromatin condensation and DNA fragmentation in tobacco BY-2 cells (Houot et al., 2001; de Pinto et al., 2006). However, proper regulation of cell death is crucial for organisms in eliminating cells in a variety of developmental, physiological and pathological contexts.

Plants employ antioxidant defense mechanisms against oxidative damage of ROS. Proline and betaine enhance antioxidant defense systems in plant responses to various oxidative stresses (Khedr et al., 2003; Demiral and Türkan, 2004; Okuma et al., 2004; Park et al., 2006; Molinari et al., 2007). Our earlier reports have shown that both proline and betaine improve salt tolerance in tobacco BY-2 cells by increasing the activity of enzymes involved in the antioxidant defense and methylglyoxal detoxification systems (Hoque et al., 2007a, b, c). Recent research has focused the protective roles of proline and betaine in cell death against various oxidative stresses. It has been demonstrated that proline inhibits ROS-mediated apoptosis in fungal pathogenesis (Chen and Dickman, 2005). In animal cells, betaine has a protective effect on oxidative stress-induced apoptosis (Horio et al., 2001; Alfieri et al., 2002). However, to our knowledge, insufficient information is available about the protective effects of proline and betaine on NaCl-induced cell death in plant cells. To clarify whether proline and betaine protect plant cells against NaCl-induced cell death, we investigated the effects of proline and betaine on cell death as well as on intracellular ROS and ATP levels, lipid peroxidation, and expression of antioxidant defense genes in tobacco Bright Yellow-2 (BY-2) suspension-cultured cells under salt stress.

Materials and methods

Culture of tobacco BY-2 cells

Suspension-cultured cells of *Nicotiana tabacum* L. cv. BY-2 were used as the sources of NaCl-unadapted cell lines (Murata et al., 1994a, b). The standard medium was a modified LS medium (Linsmaier and Skoog, 1965) in which the levels of KH_2PO_4 and thiamine-HCl were increased to 370 and 1 mg L^{-1}, respectively, supplemented with 3% sucrose and 1 μM 2,4-dichlorophenoxyacetic acid (Nagata et al., 1981). The 50 mM NaCl, 100 mM NaCl and 200 mM NaCl media were the standard medium 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 by Murata et al. (1994a, b). Briefly, the cells were subcultured weekly by transferring suspension cells of a 7-d-old culture into 30 mL of different fresh media. The culture was incubated on a rotary shaker at 100 rpm and at 25 °C in the dark. Four-d-old BY-2 cells cultured in the different media were used for experiments.

Cell death assay

An aliquot of BY-2 suspension cells was incubated with 0.025% (w/v) Evans Blue at room temperature for 10 min and subsequently washed with 100 μM $CaCl_2$ (pH 5.6) solution. The stained cells were observed and photographed by light microscopy. Evans Blue-positive cells were evaluated using Adobe Photoshop program.

4′,6-diamidino-2-phenylindole (DAPI) staining

Nuclei of BY-2 cells to be observed by epifluorescence microscopy were stained with DAPI. Cells were fixed briefly in 70% (v/v) ethanol, incubated with 1 μg mL^{-1} DAPI in PBS at room temperature for 15 min, and subsequently rinsed twice with PBS. The morphology of stained nuclei was observed and photographed using an epifluorescence microscope with an appropriate filter.

Hoechst 33342 staining

Tobacco BY-2 cells were incubated with 10 μg mL^{-1} Hoechst 33342 stain at 37 °C for 1 h in an incubator in the dark and subsequently washed twice with PBS. The morphology of stained nuclei was visualized and photographed using an epifluorescence microscope with an appropriate filter.

Apoptosis-like cell death and cell cycle analyses

Apoptosis-like cell death and cell cycle were examined by measuring DNA content with propidium iodide (PI) stain using the procedure of Kadota et al. (2004). Cells were collected by centrifugation, chopped with a razor blade and incubated in PBS at room temperature for 10 min. Nuclei were separated from cells by filtering the mixture through a 60-μm nylon filter, fixed in 70% (v/v) ethanol and kept at 4 °C until analysis. Nuclei were washed with PBS and incubated in phosphate citrate buffer at room temperature for 90 min with some vortexes. Nuclei were again incubated with 100 μg mL^{-1} RNase A at 37 °C for 60 min. Fluorescence intensity was measured by flow cytometry after staining with 50 μg mL^{-1} PI. The percentage of apoptosis-like cell death and cell cycle population was calculated using WinMDI 2.9 package program (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

DNA fragmentation assay

Cells were collected by centrifugation and DNA was extracted from freeze-dried ground cells by the cetyl trimethyl ammonium bromide method (Murray and Thompson, 1980). DNA was subjected to electrophoresis on 2% (w/v) agarose gels and stained with ethidium bromide.

Measurement of intracellular ATP

Intracellular ATP level was measured by using an ATP Bioluminescence Detection Kit (Promega Corporation, Madison, USA). Aliquots of BY-2 cells were incubated with lysis buffer containing 100 mM Tris–HCl (pH 8.0) and 4 mM EDTA at 70 °C for 10 min in a water bath. The cell lysate was heated in boiling water for 2 min. After centrifuging at 2000*g*, 500 μL of supernatant was transferred into a cuvette, and then 50 μL of rLuciferase/Luciferin reagent was added to it. Luminescence was measured using a luminometer (model AB-2200-R, Atto Corporation, Japan). ATP content was calculated from an ATP standard curve and expressed as nmol mg^{-1} protein.

Measurement of lipid peroxidation

Lipid peroxidation was assayed as described by Okuma et al. (2004) by measuring the malondialdehyde (MDA) content. An aliquot of BY-2 cells was homogenized with 5% (w/v) trichloroacetic acid. After centrifuging at 12,000*g* for 15 min, supernatant was diluted twice with 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was heated at 95 °C for 25 min and centrifuged at 7500*g* for 5 min. The absorbance of the supernatant was measured at 532 nm, and the value for non-specific turbidity at 600 nm was subtracted. MDA content was calculated using extinction co-efficient of 155 mM^{-1} cm^{-1}.

Measurement of intracellular ROS level

In order to measure intracellular ROS levels by using a flow cytometer, protoplasts were isolated enzymatically from BY-2 cells as described previously (Murata et al., 1994a, b). Isolated protoplasts were incubated with 25 μM 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) at 37 °C for

Table 1. Gene specific primers, accession number and PCR conditions

Gene and accession no.	Primer sequences	PCR conditions
Superoxide dismutase (*SOD*, X55974)	Forward 5′-AAGATGGAGATGCACCAACC-3′ Reverse 5′-CCAGCATTTCCAGTGGTTTT-3′	94 °C/1 min; 94 °C/30 s, 58 °C/30 s, 72 °C/1 min, 25 cycles; 72 °C/10
Manganese superoxide dismutase (*MnSOD*, X14482)	Forward 5′-GTGAGCAGACGGACCTTAGC-3′ Reverse 5′-CCCAAACGTCTATTCCCAGA-3′	94 °C/1 min; 94 °C/30 s, 59 °C/30 s, 72 °C/1 min, 30 cycles; 72 °C/10
Catalase1 (*CAT1*, U93244)	Forward 5′-AGCCGGTGGGAAGATTAGTT-3′ Reverse 5′-AAGCAAGCTTTTGACCCAGA-3′	94 °C/1 min; 94 °C/30 s, 59 °C/30 s, 72 °C/1 min, 30 cycles; 72 °C/10
Salicylic acid-binding catalase (SAbind CAT, U03473)	Forward 5′-TGACAACAAATGCTGGTGGT-3′ Reverse 5′-TAAAGAAGACGGGGACGTTG-3′	94 °C/1 min; 94 °C/30 s, 58 °C/30 s, 72 °C/1 min, 35 cycles; 72 °C/10
Lignin-forming peroxidase (*POX*, J02979)	Forward 5′-GGTTGCAATTTTTGGAGCAT-3′ Reverse 5′-TCCATCCCCTTATTGGTGAA-3′	94 °C/1 min; 94 °C/30 s, 59 °C/30 s, 72 °C/1 min, 30 cycles; 72 °C/10
Cationic peroxidase (*cationic POX*, D42064)	Forward 5′-TTAGCTGCTCGTGATGCTGT-3′ Reverse 5′-AGTTGAGCTCTGAGGGTCCA-3′	94 °C/1 min; 94 °C/30 s, 58 °C/30 s, 72 °C/1 min, 25 cycles; 72 °C/10
Cytosolic ascorbate peroxidase (cytosolic APX, D85912)	Forward 5′-GTTCAAGGCTGAGCAAGGAC-3′ Reverse 5′-GGGGATTGGTAGTCCAAGGT-3′	94 °C/1 min; 94 °C/30 s, 58 °C/30 s, 72 °C/1 min, 25 cycles; 72 °C/10
Actin (AB158612)	Forward 5′-AAGGTTACGCCCTTCCTCAT-3′ Reverse 5′-CATCTGTTGGAAGGTGCTGA-3′	94 °C/1 min; 94 °C/30 s 59 °C/30 s, 72 °C/1 min, 30 cycles; 72 °C/10

30 min. Protoplasts were washed and re-suspended in sorbitol. Fluorescence intensity was measured by flow cytometry.

Total RNA isolation

Total RNA was isolated from freeze-dried ground cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The concentration and purity of RNA were assayed spectrophotometrically at 260 and 280 nm.

Reverse transcription (RT)-PCR

Five micrograms of total RNA was reverse-transcribed into first-strand cDNA using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Takara Bio Inc., Otsu, 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 Inc.) and 1 × Ex Taq buffer. Gene-specific primers used in this study with PCR conditions are listed in Table 1. Equal volume of PCR products was subjected to electrophoresis on 1.5% (w/v) agarose gels prior to staining with ethidium bromide. Gels were photographed under UV light.

Determination of protein

Protein contents were measured as described by Bradford (1976) using BSA as a standard.

Statistical analysis

The significance of differences between mean values was compared by Student's *T*-test. Differences at $P<0.05$ were considered significant.

Results

Cell death

To investigate the protective role of proline and betaine in cell death against NaCl stress, BY-2 cells were stained with Evans Blue and photographed by light microscopy (Figure 1). Salt stress increased the number of Evans Blue-positive cells. Evans Blue-positive cells increased with the increasing concentration of NaCl (Figure 1A–D). Exogenous application of proline or betaine decreased Evans Blue-positive cells under 200 mM NaCl stress (Figure 1E–F). The uptake of Evans Blue into BY-2 cells was quantitatively analyzed (Figure 1G).

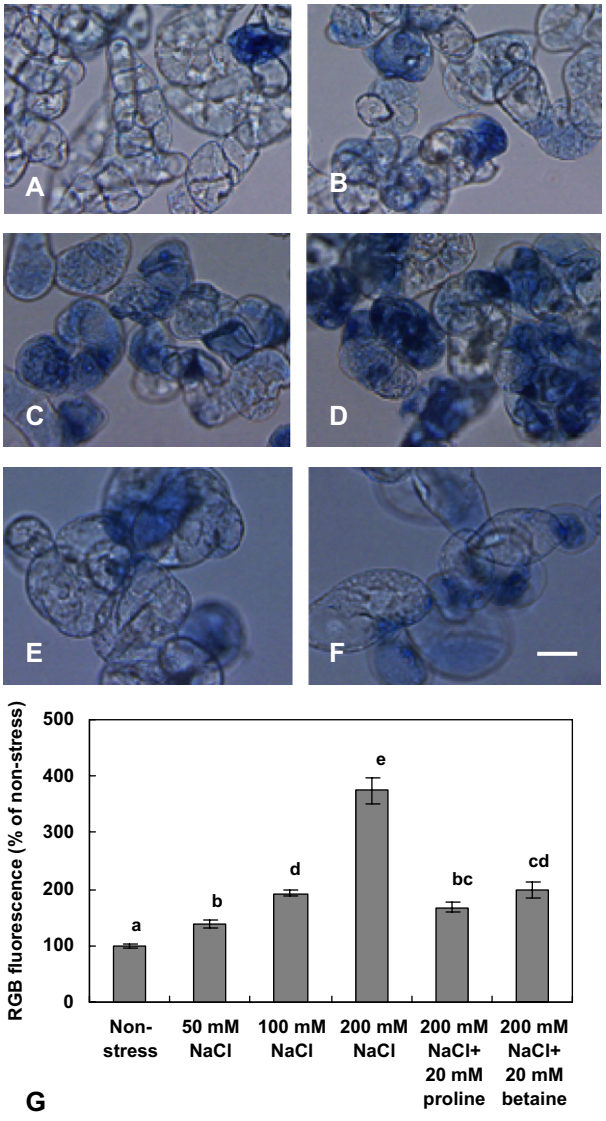

Figure 1. Inhibition of NaCl-induced cell death by proline and betaine in tobacco BY-2 suspension cells. Cell death was evaluated by Evans Blue staining. Photographs shown are representative of five independent experiments. Scale bar represents 20 μm for all of the photographs. (A) Non-stress, (B) 50 mM NaCl, (C) 100 mM NaCl, (D) 200 mM NaCl, (E) 200 mM NaCl+20 mM proline and (F) 200 mM NaCl+20 mM betaine. (G) Uptake of Evans Blue into the cells was calculated using Adobe Photoshop program. Values represent the mean ± SE from five independent experiments. Bars with the same letters are not significantly different at $P<0.05$. Non-stress, 50 mM NaCl, 100 mM NaCl, 200 mM NaCl, 200 mM NaCl+20 mM proline and 200 mM NaCl+20 mM betaine indicate the standard medium, 50 mM NaCl medium, 100 mM NaCl medium, 200 mM NaCl medium, proline medium and betaine medium, respectively.

A significant increase in uptake of Evans Blue into BY-2 cells under salt stress was observed, whereas both proline and betaine significantly suppressed the uptake of Evans Blue into cells under 200 mM NaCl stress. These results suggest that both proline and betaine improved membrane integrity to increase the viability of cells under salt stress.

Morphological changes of nuclei

To investigate whether proline or betaine suppresses nuclear deformation and chromatin condensation induced by salt stress, we used DAPI that can specially bind double-stranded DNA (Figure 2A–F). Nuclear deformation and chromatin condensation were observed in BY-2 cells under salt stress. Exogenous proline or betaine suppressed nuclear deformation as well as chromatin condensation under severe salt stress.

To confirm morphological changes of nuclei observed using DAPI staining, BY-2 cells were also stained with Hoechst 33342 (Figure 2G–L). Like DAPI staining, Hoechst 33342 staining showed deformed nuclei and condensed chromatin in BY-2 cells under salt stress. Severe salt stress caused an increase in nuclear deformation and degradation, and chromatin condensation, whereas both proline and betaine reduced deformation of nuclei and chromatin condensation under salt stress.

Apoptosis-like cell death and cell cycle population

To test the effect of proline and betaine on apoptosis-like cell death and cell cycle population induced by NaCl stress, DNA contents were measured with PI staining by flow cytometry (Table 2). Salt stress caused a significant increase in population of apoptosis-like cell death. Severe salt stress caused a remarkable increase (approximately 7-fold) in population of apoptosis-like cell death and a decrease in G_1 phase population. Neither proline nor betaine suppressed apoptosis-like cell death and affected G_1 phase population induced by severe salt stress (Table 2).

DNA fragmentation

To investigate whether DNA fragmentation occurred in BY-2 cells under salt stress either in the presence or absence of proline and betaine, DNA was extracted from BY-2 cells and subjected to electrophoresis on agarose gel prior to staining with ethidium bromide. No DNA fragmentation was detected in the stressed cells irrespective of the presence or absence of proline and betaine (data not shown).

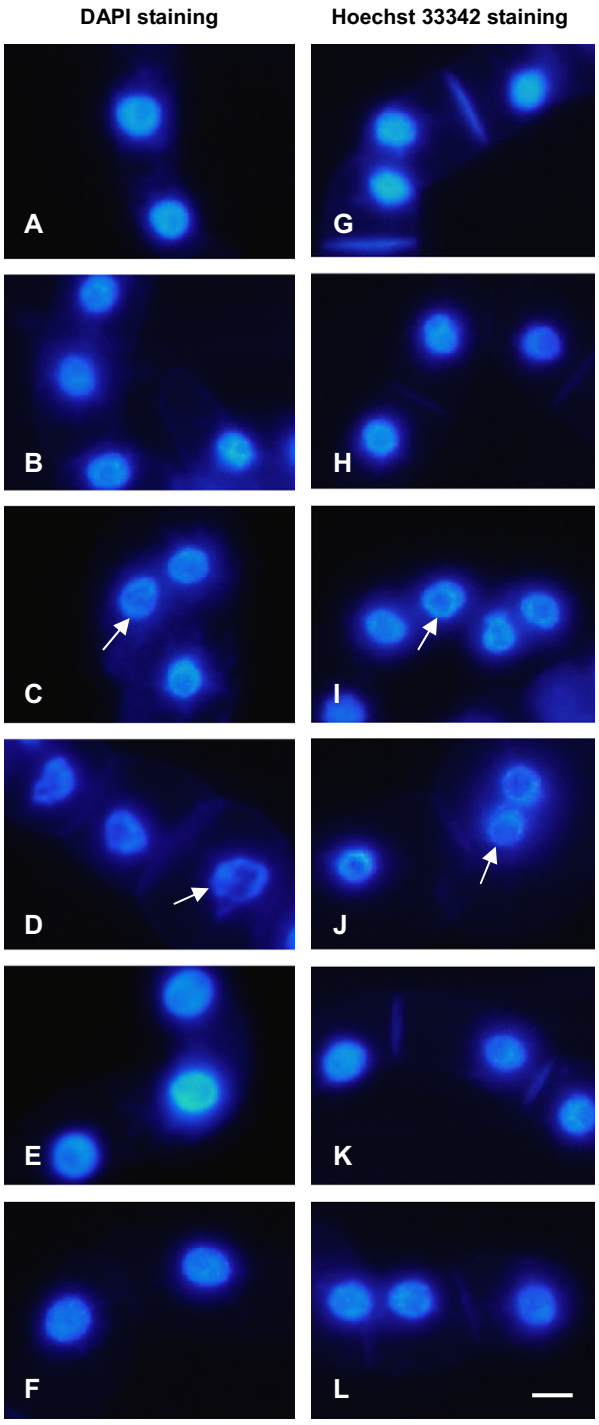

Figure 2. Effect of proline and betaine on morphological changes of nuclei in BY-2 suspension cells under NaCl stress. The nuclei of cells were stained with DAPI (A–F) and Hoechst 33342 (G–L). Photographs shown are representative of five independent experiments. Arrows indicate the deformed nuclei. Scale bar represents 10 μm for all of the photographs. (A, G) Non-stress, (B, H) 50 mM NaCl, (C, I) 100 mM NaCl, (D, J) 200 mM NaCl, (E, K) 200 mM NaCl+20 mM proline and (F, L) 200 mM NaCl+20 mM betaine. Details of culture media are shown in the legend of Figure 1.

Intracellular ATP contents

Severe salt stress caused a significant increase in intracellular ATP levels (Figure 3A), which was accompanied by an increase in apoptosis-like cell death (Table 2). No significant difference was observed in ATP levels when cells were subjected to 0–100 mM NaCl stress. Neither proline nor betaine had significant effect on 200 mM NaCl-induced intracellular ATP levels (Figure 3A).

Lipid peroxidation

As shown in Figure 3B, severe salt stress caused a significant increase in lipid peroxidation in BY-2 cells. There was no significant difference in MDA levels among cells subjected to 0–100 mM NaCl stress. Both proline and betaine resulted in a significant reduction of lipid peroxidation induced by severe salt stress (Figure 3B).

Intracellular ROS levels

We investigated whether proline or betaine could inhibit NaCl-induced ROS accumulation (Figure 4). Salt stress resulted in a significant increase in accumulation of intracellular ROS in BY-2 cells. Intracellular ROS levels increased with the increasing concentration of NaCl. Proline as well as betaine, on the other hand, significantly inhibited 200 mM NaCl-induced ROS accumulation (Figure 4).

Expression of antioxidant defense genes

To verify the relationship between cell death and antioxidant protection offered by proline and betaine under salt stress, mRNA levels of antioxidant defense genes were assayed (Figure 5). Antioxidant defense genes showed differential patterns in mRNA expression under salt stress either in the presence or absence of proline and betaine. Levels of superoxide dismutase (*SOD*) and *cationic peroxidase* (*POX*) mRNAs were higher in salt-stressed cells than in non-stressed cells, but neither proline nor betaine had effect on *SOD* and *cationic POX* mRNAs under salt stress. Levels of *MnSOD*, catalase (*CAT*)*1* and *cytosolic APX* mRNAs were not increased by salt stress irrespective of the presence or absence of proline and betaine. However, salt stress either in the presence or absence of proline and betaine induced mRNA level of salicylic acid-binding (*SAbind*) *CAT*. Under salt stress, betaine remarkably increased mRNA level of

Table 2. Apoptosis-like cell death and cell cycle populations in tobacco BY-2 suspension cells induced by proline and betaine under NaCl stress

Culture media	Apoptosis-like cell death (%)	G_1 phase (%)	S phase (%)	G_2 phase (%)
Non-stress	4.1 ± 0.71^{a}	77.1 ± 3.5^{c}	2.2 ± 0.40^{a}	16.4 ± 2.1^{b}
50 mM NaCl	10.3 ± 1.2^{b}	74.7 ± 2.9^{c}	1.7 ± 0.19^{a}	12.8 ± 1.3^{ab}
100 mM NaCl	11.9 ± 0.84^{b}	72.2 ± 3.4^{bc}	1.4 ± 0.47^{a}	12.6 ± 1.4^{ab}
200 mM NaCl	28.4 ± 2.4^{c}	58.4 ± 3.9^{a}	2.8 ± 0.43^{a}	10.1 ± 1.2^{a}
200 mM NaCl+20 mM proline	28.6 ± 2.1^{c}	59.4 ± 2.4^{a}	1.8 ± 0.27^{a}	9.9 ± 0.93^{a}
200 mM NaCl+20 mM betaine	26.2 ± 2.0^{c}	63.2 ± 2.3^{ab}	1.7 ± 0.30^{a}	8.8 ± 1.3^{a}

Isolated nuclei were stained with PI. Apoptosis-like cell death and cell cycle population were analyzed on the basis of flow cytometry analysis to measure DNA contents. Values represent the mean ± SE from five independent experiments. Same letters in a column represent insignificant difference at $P < 0.05$. Details of culture media are shown in the legend of Fig. 1.

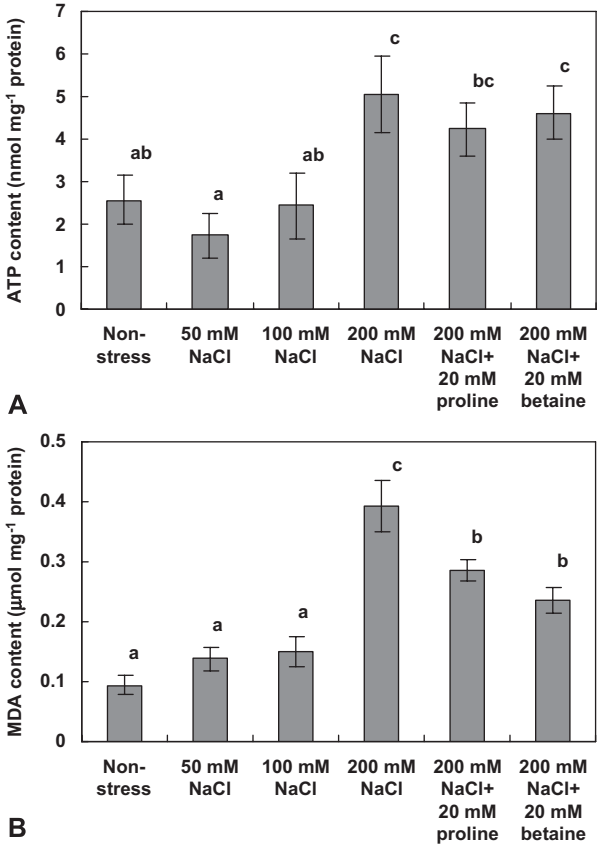

Figure 3. (A) Intracellular ATP levels and (B) MDA content (lipid peroxidation) in BY-2 suspension cells under NaCl stress in the presence or absence of proline or betaine. Values represent the mean ± SE from at least five independent experiments. Bars with the same letters are not significantly different at $P < 0.05$. Details of culture media are shown in the legend of Figure 1.

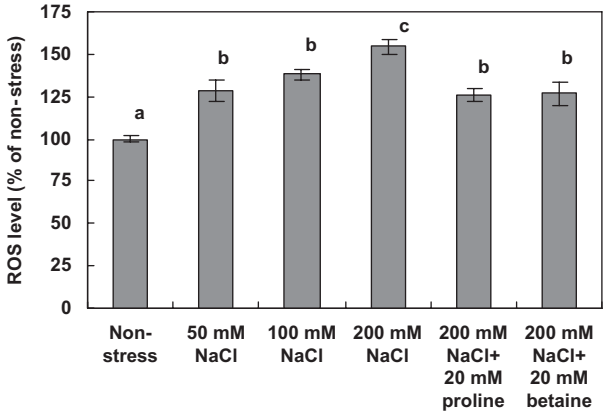

Figure 4. Intracellular ROS levels in BY-2 suspension cells induced by NaCl stress in the presence or absence of proline or betaine. Isolated protoplasts were stained with H_2DCF-DA and the fluorescent intensities were measured by flow cytometry. The fluorescent intensity is shown as a ROS level. Values represent the mean ± SE from five independent experiments. Bars with the same letters are not significantly different at $P < 0.05$. Details of culture media are shown in the legend of Figure 1.

SAbind CAT but proline did not. Salt stress also induced *POX* mRNA level. Importantly, both proline and betaine increased *POX* mRNA level under salt stress.

Discussion

Our previous reports have shown that exogenous proline and betaine induce the accumulation of more than 200 mM proline and betaine in BY-2 cells under salt stress, respectively, (Okuma et al., 2004) and mitigate the inhibition of cell growth under salt stress (Okuma et al., 2004; Hoque et al., 2007a). Okuma et al. (2004) also observed that exogenous proline alleviated the inhibition of NaCl-induced cell growth without improving a ratio of K^+ to Na^+, and that proline mitigated the detrimental effects of salinity more than betaine in part because of difference in the antioxidant protection activity. It is interesting to note that exogenous proline or betaine causes an inhibition of cell growth in the

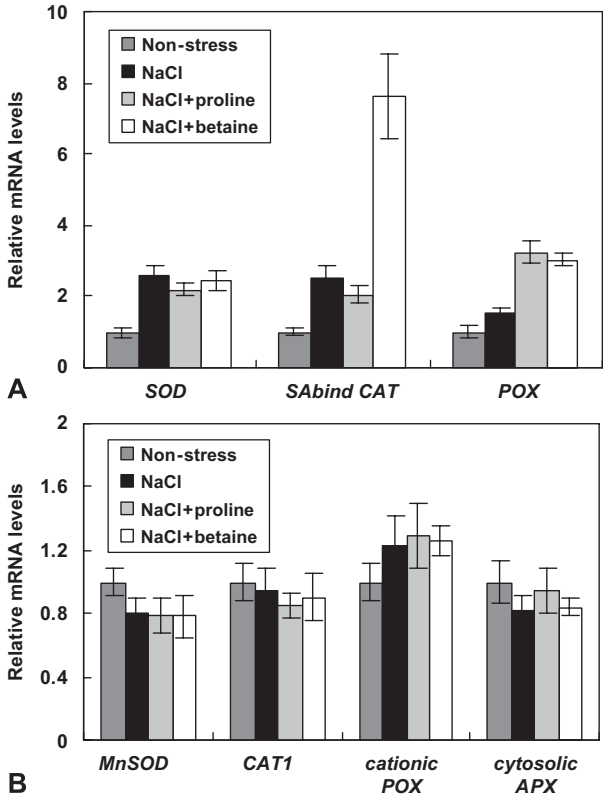

Figure 5. The mRNA levels of antioxidant defense genes in BY-2 suspension cells under NaCl stress in the presence or absence of proline or betaine. Values represent the mean ± SE from at least five independent experiments. *Actin* was used as an internal control to normalize the mRNA levels of each gene. NaCl (200 mM NaCl), NaCl+proline (200 mM NaCl+20 mM proline) and NaCl+betaine (200 mM NaCl+20 mM betaine). Details of culture media are shown in the legend of Figure 1.

absence of NaCl stress (data not shown). Similarly, Deuschle et al. (2004) have reported that in the absence of NaCl stress, exogenous proline inhibits growth and induces cell death in BY-2 cells, and that proline improves cell growth under NaCl stress.

Plants exposed to abiotic and biotic stresses accumulate extra ROS that can damage proteins, lipids, DNA and carbohydrates, and can lead to apoptosis-like cell death and/or necrosis (Fath et al., 2001; Apel and Hirt, 2004; Van Breusegem and Dat, 2006). A tight regulation of ROS homeostasis is necessary to avoid cellular damage and to suppress cell death. Salt stress leads to an enhanced ROS accumulation (Lin et al., 2006) in BY-2 cells (Figure 4), whereas both proline and betaine significantly decrease 200 mM NaCl-induced ROS accumulation (Figure 4) probably due to increased activity of antioxidant enzymes to scavenge ROS (Hoque et al., 2007a, b, c). These results are supported by previous results that proline effectively reduced intracellular ROS levels (Chen and Dickman, 2005; Chen et al., 2006).

Cell death is induced by salt stress (Qiao et al., 2002). Staining with Evans Blue, which can penetrate into dead cells but not living cells, demonstrates that salt stress induced cell death in BY-2 cells (Figure 1). Under 200 mM NaCl stress, both proline and betaine resulted in a reduction of cell death in BY-2 cells (Figure 1) accompanied by decreased oxidative damage to lipid membranes (Figure 3B; Demiral and Türkan, 2004; Okuma et al., 2004) and protein (Hoque et al., 2007c). These results are also supported by the result of Mansour (1998), who reported that NaCl enhanced membrane permeability and cell death, whereas both proline and betaine prevented NaCl-induced cellular alterations. Taken together, results suggest that both proline and betaine mitigate cellular damage and clearly improve survival rate of cells under NaCl stress.

Apoptosis-like cell death is accompanied with alteration of nuclear structure. Nuclear deformation and chromatin condensation occurred in BY-2 cells under salt stress (Figure 2). Salt stress induced deformation and degradation of nuclei in meristematic cells of barley roots (Katsuhara and Kawasaki, 1996; Katsuhara, 1997). H_2O_2-induceed apoptotic-like nuclei and chromatin condensation were observed in BY-2 cells (Houot et al., 2001; de Pinto et al., 2006). Osmotic stress due to salinity is assumed to be responsible for cellular dehydration, resulting in deformation of nuclei and chromatin condensation, whereas compatible solutes facilitate the osmotic adjustment and prevent cellular components against salt stress. Betaine plays an osmoprotective role in preventing cell from dehydration under salt stress (Demiral and Türkan, 2004). In this study, both proline and betaine effectively contributed to suppression of nuclear deformation and chromatin condensation induced by 200 mM NaCl stress (Figure 2).

In plants, apoptosis-like cell death often involves changes of cellular DNA content and DNA degradation. Lin et al. (2006) have shown that NaCl effectively induced apoptosis-like cell death in tobacco protoplasts, which was detectable by flow cytometry analysis to measure cellular DNA content and genomic DNA degradation. We investigated whether proline or betaine could affect DNA content and DNA degradation. Salt stress resulted in an increase in DNA degradation, suggesting that salt stress induced apoptosis-like cell death in BY-2 cells (Table 2). However, DNA fragmentation was not detected using the DNA ladder experiment (data not shown). Salt stress-induced apoptosis-like

cell death was also observed in barley roots (Katsuhara and Kawasaki, 1996; Katsuhara, 1997) and in BY-2 suspension cells (Deuschle et al., 2004). Proline or betaine reduces lipid peroxidation (Figure 3B) and protein oxidation (Hoque et al., 2007c), suppresses nuclear deformation and chromatin condensation (Figure 2), and improves membrane integrity (Figure 1) under salt stress. These results indicate that both proline and betaine are efficient to mitigate most of the deleterious consequences of salt stress after 4 d of inoculation. However, neither proline nor betaine suppresses 200 mM NaCl-induced apoptosis-like cell death in BY-2 cells (Table 2). Here, it is important to note that both proline and betaine slightly improved cell growth at 4 d after inoculation to salinity medium and remarkably improved cell growth at 7 d after inoculation (Okuma et al., 2004; Hoque et al., 2007a). Interestingly, proline inhibits ROS-mediated apoptosis in the fungal pathogen *Colletotrichum trifolii* (Chen and Dickman, 2005). The drastic inhibition of ROS-mediated apoptosis in fungal pathogenesis by proline occurred probably due to more rapid multiplication of *C. trifolii* than BY-2 cells. In addition, the protective effect of betaine on oxidative stress-induced apoptosis has been reported in animal cells (Horio et al., 2001; Alfieri et al., 2002). Adverse environmental conditions suppress plant growth and cell division, and cell cycle regulation might be involved in this response. It has been reported that betaine alleviates the detrimental effects of salt stress by reducing cell size and accelerating cell division (Ferjani et al., 2003). Our results show that neither proline nor betaine modulated cell cycle population of BY-2 cells under salt stress (Table 2).

It has been shown that the level of ATP remains constant in apoptotic cells, while it decreases in necrotic cells of soybean suspension cultures (Casolo et al., 2005), and apoptosis is accompanied with an increase in ATP levels in animal cells (Atlante et al., 2005). In this study, lower salt stress did not affect ATP levels, whereas higher salt stress led to an increase in ATP levels (Figure 3A). Taken together, cell death in BY-2 cells under severe salt stress is similar to a typical apoptosis of animal cells, which is accompanied with an increase in chromatin condensation and intracellular ATP levels. However, neither proline nor betaine affected apoptosis-like cell death as well as intracellular ATP levels in BY-2 cells treated with 200 mM NaCl (Table 2 and Figure 3A).

Compatible solutes are expected to protect plants from various stresses via scavenging ROS and maintaining redox homeostasis. The viability or death of cells exposed to ROS correlates with the activity of antioxidant enzymes. Fath et al. (2001) showed a direct correlation between enzyme activities and hormonal regulation of cell death in barley aleurone cells. Our previous reports have shown that salt stress inhibited cell growth accompanied with reduction of antioxidant enzyme activities, whereas both proline and betaine improved salt tolerance accompanied with increment of antioxidant enzyme activities (Hoque et al., 2007a, b, c). Proline inhibits apoptosis-like cell death, which is accompanied with an increased CAT (EC: 1.11.1.6) activity in *C. trifolii* under various stresses (Chen and Dickman, 2005). A large body of evidence suggests that proline and betaine enhance antioxidant defense systems to improve stress tolerance (Khedr et al., 2003; Demiral and Türkan, 2004; Park et al., 2006; Molinari et al., 2007). Park et al. (2006) also suggest a positive relationship between betaine-enhanced chilling tolerance and *CAT* expression. To better understand the relationship between cell death and antioxidant protection offered by proline and betaine under salt stress, transcription levels of antioxidant defense genes to detoxify ROS were investigated. Among antioxidant defense genes studied here, transcription levels of *SAbind CAT* and *POX* were found to be increased by proline and betaine under salt stress (Figure 5), suggesting that increased expression of *SAbind CAT* and *POX* genes in salt-stressed cells in the presence of proline or betaine could suppress cell death via reduction of ROS level. However, up-regulation of the antioxidant defense systems provided by proline or betaine could be important in avoiding cellular damage and in inhibiting cell death. Alternatively, proline and betaine might confer stress tolerance through their roles in signal transduction pathways. As expected, Khedr et al. (2003) suggest that proline acts as a component of signal transduction pathways that regulate stress-responsive genes and thereby improve salt tolerance. But the roles of proline and betaine in modulating signal transduction or gene expression are yet to be fully elucidated. Further investigations, including expression of stress-responsive genes, are needed to elucidate the role of proline and betaine in molecular aspects of salt tolerance in higher plants.

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