ScienceAsia 26 (2000) : **207-211**

Mechanisms of Adaptation to Increasing Salinity of Mulberry: Proline Content and Ascorbate Peroxidase Activity in Leaves of Multiple Shoots

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Received 16 Mar 2000
Accepted 30 Nov 2000

ABSTRACT Multiple shoots of mulberry (*Morus alba* cv Khonpai), a NaCl-tolerant cultivar, were grown on MS media without or with NaCl. On medium with increasing NaCl concentration up to 150 mM, the shoots exhibited slightly reduction in growth. On the other hand, when the concentration of NaCl increased to 200 mM, the growth was completely inhibited and the necrosis of leaves tissue was observed. The relative water content was significantly decreased in leaves treated with NaCl concentration of 150 and 200 mM compared to that in leaves without NaCl treatment. The total chlorophyll content was significantly lower in leaves of salt treated multiple shoots than in leaves of control multiple shoots. Comparing shoots grown on media without to with 150 mM NaCl, the proline content in leaves was increased to approximately 11-fold and the ascorbate peroxidase activity in leaves was increased to approximately 91%.

KEYWORDS: ascorbate peroxidase, mulberry, proline, salinity.

INTRODUCTION

When plants are exposed to high concentration of salt, they can develop various mechanisms for survival. Some tolerate the high concentrations of toxic ion by exclusion[1] or compartmentation of ion in the vacuole[2] and the production of organic solute in the cytoplasm to lower the osmotic potential.[3] It has been reported that the organic solute accumulated when plants were exposed to high concentration of salt and low-water-potential environments is proline.[4,5] Proline has been proposed to act as a compatible solute that adjusts the osmotic potential in the cytoplasm.[6]

Plants have a high capacity for conversion of light energy to chemical energy under favorable conditions by photosynthesis. The excess light energy results in the generation of active oxygen species[7]. In addition, when plant are exposed to environmental stress, such as high concentration of salt, the imbalance between the production of reactive oxygen species and the activity of scavenging system occur. Plants have several mechanisms to suppress the production of these active molecules. One of these mechanisms is ascorbate-glutathione cycle[8] . Enzymes in this cycle had been reported to be response to salt stress by cotton and lupin[9,10] .

Although soil salinisation in northeastern region of Thailand is a severe problem for agriculture, mulberry can grow well in this area. Therefore, mulberry provides us as a good model for studying the mechanisms of adaptation in plant under high salt concentration environment. In the presence work, the tissue culture of mulberry had been developed to examine the effect of high salinity on proline content and ascorbate peroxidase activity in mulberry.

MATERIALS AND METHODS

Tissue culture of mulberry

Mulberry (*Morus alba* L. cv Khonpai), a salt tolerance cultivar, is used. After being sterilized with 0.1% mercuric chloride for 3-5 min and thoroughly washed with sterilized water. Buds were cultured on modified Murashige and Skoog (MS)[11] solid medium supplemented with 1 mg/L 6-Benzylaminopurine (BA). The multiple shoots were subcultured at 40 days intervals and cultured under 150 µmol $m^{-2}s^{-1}$ at 26^0C. For every experiments, multiple shoots were cultured in the same media containing various concentration of NaCl as indicated for 12 days.

After chlorophyll was extracted from leaves with 80% acetone, the contents of chlorophyll a, chlorophyll b and total chlorophyll were culculated according to the method of Hendry and Price.[12] To determine the relative water content (RWC), leaves were measued the fresh weight, dry weight and turgid weight. RWC was calculated according to Rodriguez *et al*.[13]

Protein extraction and ascorbate peroxidase (APX) activity analysis

Approximately 1.00 g of mulberry leaves were extracted with 0.1 M sodium phosphate buffer (pH 7.8) containing 1 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM PMSF (Phenylmethanesulphonyl fluoride), 20 mg PVP (Polyvinylpyrrolidone) and 5 mM ascorbate. The homogenate was centrifuged for 10 min at 15,000 rev/min. Glycerol was added to the supernatant until final concentration of glycerol is 10%. APX activity was determined as reported by Yamaguchi *et al*.[14] Briefly, protein was incubated with 1 ml of 1 mM H_2O_2 and 0.5 mM ascorbate in buffer containing 50 mM potassium phosphate (pH 7.0), 10 mM 3-aminotriazole and 35 mM sucrose. APX activity is monitored spectrophotometrically by measuring the increase in absorbance at 290 nm (UV-VISIBLE Spectrophotometer, UV-1601, SHIMADZU). Protein concentration was determined to Lowry *et al*,[15] using BSA as standard protein. The specific activity of APX is calculated and expressed as µmole of amount of product in min per mg of protein as indicated.

Electrophoresis and detection of APX activity in gels

Samples with equal protein content of 40 µg were subjected to discontinuous PAGE under non-denaturing condition as described by Mittler and Zilinskas.[16] Electrophoretic separation was performed at 4°C for 3 h, using 7% polyacrylamide gels. After the electrophoretic separation, the gel was assayed for enzyme activity. The reaction mixture contained 50 mM sodium phosphate (pH 7.8), 28 mM TEMED (N, N, N^1, N^1 - Tetramethylethylenediamin) and 24.5 mM NBT (Nitroblue tetrazolium). The APX activity was observed as an achromatic band on a purple-blue background.

Estimation of proline content by spectrophotometry

Approximately 50 to 100 mg leaves were homogenized in 10 ml 3% aqueous sulfosalicylic acid and centrifuged for 10 min at 15,000 rev/min. To 2 ml upper aqueous phase, 2 ml acid ninhydrin and 2 ml of glacial acetic acid were added and the reaction mixture was incubated at 100°C for 1 h. The reaction mixture was placed on ice and extracted with 4 ml toluene. Proline content in the extract was measured spectrophotometrically (UV-VISIBLE Spectrophotometer, UV-1601, SHIMADZU) as described by Bate *et al*.[17]

Statistical analysis

The experiments were set up in a completely randomised design. For relative water content experiment and chlorophyll content determination, five treatments were set up (0, 50, 100, 150, 200 mM NaCl). One treatment sample consisted of leaves from 3 plants. Each treatment was replicated three times. Analysis of variance was performed on the data, and significant differences among treatment means calculated by Tukey's test ($\alpha = 0.05$).

Results and Discussion

Mulberry is a good modle for studying the mechanisms of adaptation salt stress in higher plant because of its widespread over salinized northeastern region of Thailand. By using tissue culture techniques, the concentration of salt in the media can be varied and its effect on the plant tissure can be observed in a controlling manner. The leaf tissue was chosen in this study because of its important role in photosynthesis. Therefore, multiple shoots were developed in this investigation to study mechanisms of adaptation to salt stress.

First, the threshold of salt concentration that induced necrosis in leaf tissue was examined as in Fig 1. We found that NaCl at 200 mM concentration completely inhibited growth in tissue culture. In salinity environment, leaf cells loss water to lower osmotic potential. As shown in Table 1, RWC decreased proportionally when the concentration of NaCl increased. The RWC was significantly decreased in leaves treated with NaCl concentration of 150 and 200 mM compared to that in leaves without NaCl treatment (O mM). The decrease in water potential caused by a rapid change in salt concentration, or salt shock, had also been reported in other tissues, *ie*, callus of *Bassica nupus*[18] and roots of *Zea mays*.[13] Although both salt-adapted and salt-unadapted cell cultures of photoautotrophic tobacco had similar chloroplast morphology and similar degree of chlorophyll accumulation under high salinity, but carbondioxide fixation was more resistant to inhibition by NaCl in salt-adapted cells than in salt-unadapted cells.[19] Table 2 shows the

Fig 1. Growth of multiple shoots of mulberry cultured on basal medium supplemented with 0, 50, 100, 150, 200 mM NaCl. for 12 days. The experiment was performed in triplecates.

decrease in the content of chlorophyll a, chlorophyll b and total chlorophyll when the NaCl concentration increased in the media. The total chlorophyll content was significantly lower in leaves of salt treated multiple shoots (50, 100, 150, 200 mM) than in leaves of control multiple shoots. However, the chlorophyll a content in leaves treated with 50 and 100 mM NaCl remained unaltered compared to that of the control, whereas the chlorophyll b content decreased significantly and propotionally when the concentration of NaCl increased. When plants are subjected to environmental salt stress, the balance between the production of reactive oxygen species *ie* superoxide radical, hydroxyl radicals and singlet oxygen and the quenching activity of antioxidants is upset[20]. Singha and Choudhuri[20] have shown that hydrogen peroxide and singlet oxygen may play an important role in the mechanism of NaCl injury in *Vigna catjary* and *Oryza sativa* leaves. The reactive oxygen species are known to be the main mediations of peroxidative damage to various cellular components such as membrane fatty acid, protein, nucleic acids and chlorophyll.[21]

Under high salt environment, higher plants maintain their water content by accumulation of compatible organic solute in their cytoplasms. Proline is one of the compatible organic solute that is used by plant as the osmoprotectant. In several types of plant, the accumulation of proline has been observed in response to salt stress.[6] For example, salt-selected cell lines accumulate proline in cytoplasm to a greater extent than the non-selected cell lines of lucerne[5]. Under salt stress, the novel light-dark change of proline level (high in the light and low in the dark) was reported in leaves of barley and wheat.[4] Proline has been suggested to play a bifunctional role in the adaptation to high salt stress : as osmoprotectant in the light and as substrate that supply energy required for compartmentation of ions in vaccuole in the dark respiration.[4] Table 3 shows that proline content in leaves of mulberry increases 11-folds when compare control to high salinity of

Table 1. Relative water content of leaves derived from multiple shoots of mulberry grown on basal medium supplemented with 0, 50, 100, 150, 200 mM NaCl.

NaCl (mM)	Relative water content
0	73.60 ± 2.25 a
50	71.98 ± 2.44 ab
100	70.08 ± 2.36 ab
150	68.42 ± 2.85 b
200	65.06 ± 2.54 b

Values shown are means of triplecates ± SE. In the column, values with the same letter are not significantly different according to Tukey's test ($\alpha = 0.05$). Confidence intervals (95%) were used for all pairwise comparisons.

Table 2. Chlorophyll content of leaves derived from multiple shoots of mulberry grown on basal mudium supplemented with 0, 50, 100, 150, 200 mM NaCl.

NaCl (mM)	Chlorophyll a (mg/L)	Chlorophyll b (mg/L)	Total Chlorophyll (mg/L)
0	18.64 ± 1.46 a	23.91 ± 2.72 a	42.53 ± 2.10 a
50	17.60 ± 0.73 a	18.85 ± 2.37 b	35.62 ± 2.53 b
100	15.84 ± 0.48 a	11.03 ± 1.49 c	26.95 ± 1.95 c
150	11.74 ± 1.08 b	7.70 ± 2.41 c	19.44 ± 3.48 cd
200	9.53 ± 0.81 b	5.78 ± 1.24 c	15.47 ± 2.02 d

Values shown are means of triplecates ± SE. In each column, values with the same letter are not significaty different according to Tukey's test (a = 0.05). Confidence intervals (95%) were used for all pairwise comparisons.

Table 3. Proline content and ascorbate peroxidase activity of leaves derived from multiple shoots of mulberry grown on basal medium supplemented with 0 and 150 mM NaCl.

NaCl (mM)	Proline (µmol/g fr wt)	Ascorbate peroxidase activity (µmol/min/mg protein)
0	326.11 ± 7.90	0.11 ± 0.01
150	3647.65 ± 81.73	0.21 ± 0.03

Values shown are means of triplecates ± SE. Three plants per replication

150 mM NaCl condition. This result may reflect the important role of proline as osmoprotectant in mulberry.

Plants have been demonstrated to possess the ascorbate-glutathione cycle that capable of scavenging the reactive oxygen species under favorable condition.[8] Therefore, we examine the response of ascorbate peroxidase (APX), one of the enzyme in this cycle, to high salt environment. Hydrogen peroxide produced by superoxide dismutase (SOD) was scavenged by APX. Yu and Rengel reported that salt stress enhanced the activity of Cu/Zn SOD by 145% without affecting the activity of FeSOD and MnSOD.[10] Benavides *et al*[22] have studies the relationship between the antioxidant defense system and salt tolerance in two clones of potato (*Solanum tuberosum* L) differing in salt sensitivity. The antioxidant enzymes, *ie*, ascorbate peroxidase, superoxide dismutase and dehydroascorbate reductase were significantly elevated in the tolerant clone compared to the sensitive one when both were grown in the absence of NaCl. Under 100 and 150 mM NaCl treatments no changes in the antioxidant stress parameters were detected in the tolerant clone whereas in the sensitive clone, the superoxide dismutase activity was 63% increase under 100 mM NaCl treatment. However, as shown in Table 3, there was 91% induction of APX activity when the control (O mM NaCl) was compared to the high salinity of 150 mM NaCl. This result was confirmed by the zymogram of APX in Fig 2. Two isozymes of APX have been reported in spinach, tea and pumpkin.[14,23,24] One isozyme of APX with molecular weight of about 30 kDa has been shown to be localized in cytosol, stroma and membrane of microbody, whereas the other isozyme with molecular weight of 40 kDa has been shown to be localized in the thylakoid membrane. The zymogram of APX in Fig 2 shown two bands that were induced in response to high salinity. This result may reflect the important role of the ascorbate-glutathione cycle in adaptation of plant under salinity. Other enzymes in the ascorbate-glutathione cycle will be examined later. In summary, plants possess mechanisms of adaptation in response to the change in salt concentration in environments. In this paper, we demonstrated that when the concentration of salt in the environment increased, proline content in mulberry leaves as well as the APX activity in the tissues also increased.

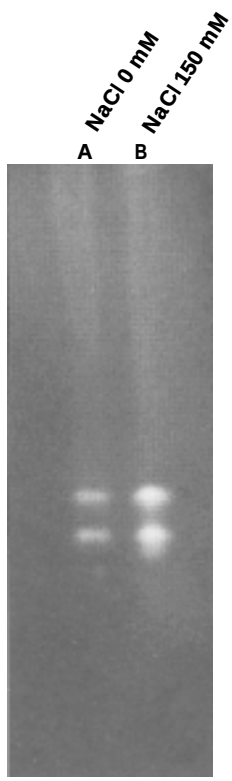

Fig 2. Zymogram of ascorbate peroxidase in extracts from leaves of multiple shoots grown on basal media supplemented with 0 mM NaCl (A) and 150 mM NaCl (B).

Acknowledgements

We are grateful to Dr. Somchai Pornbanlualap, Department of Biochemistry, Faculty Of Science, Kasetsart University, for valuable suggestions and Ms.Apinya Hirunwong, Department Of Statistic, Faculty Of Science, Kasetsart University for statistic analysis.

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