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BRIEF COMMUNICATIONS

Mechanical Stimulation-Induced Chilling Tolerance in Tobacco Suspension Cultured Cells and Its Relation to Proline[1]

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Abstract—Mechanical stimulation (MS), widely existing but usually ignored in nature, is one of the major environmental stress factors. MS by increasing the rotational speed of shaker incubator could alleviate a decrease in vitality of tobacco (*Nicotiana tabacum* L.) suspension cultured cells and reduce the accumulation of MDA under chilling stress at 1°C, which in turn improved survival percentage under chilling stress and regrowth ability of tobacco suspension cells after chilling stress. In addition, MS could increase the activity of Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and induce the accumulation of endogenous proline in tobacco cells; exogenously applied proline also could enhance its endogenous level under normal culture conditions and survival percentage of the cells under chilling stress. These results suggest that MS could improve chilling tolerance of tobacco suspension cells and the acquisition of this chilling tolerance was related to proline.

Keywords: Nicotiana tabacum, chilling tolerance, mechanical stimulation, proline, tobacco suspension cells

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INTRODUCTION

Plants, due to sessile and poikilothermatic nature, are constantly exposed to various abiotic and biotic stresses. Abiotic stresses, including extreme in temperature, high salinity, drought, and mechanical stimulation, are the major causes of crop failure worldwide, and low temperature is one of the main abiotic factors affecting the growth, development, geographical distribution, and even survival of plants [1]. Mechanical stimulation (MS), such as wind, raining, touching, hailing, trampling, and soil obstruction as well as animal nibble, widely existing but usually ignored in nature, is one of the primary environmental stress factors, which significantly affects plant growth, development, reproduction, and even survival [2–4]. Keller and Steffen [5] showed that MS by brushing with a wooden bar could increase chilling tolerance of tomato seedlings. Walleye et al. [6], using mechanical wounding as the stress stimulus and performing the whole genome microarray analysis of *Arabidopsis thaliana* leaf tissues, identified a number of rapid wound-responsive (RWR) genes involved in the tolerance to abiotic and biotic stresses. In our previous work, we have illustrated that mechanical stimulation by increasing the rotational speed in a shaker incubator could improve heat tolerance of tobacco suspension cultured cells, and mechanical stimulation-induced heat tolerance was related to H_2O_2 and Ca^{2+} as well as calmodulin [3, 4].

A key adaptive mechanism in many plants grown under various kinds of abiotic and biotic stresses like low temperature, drought, oxidative stress, excess light, and ultraviolet radiation, is the intense accumulation of proline [7–11]. Many researchers found that low temperature tolerance was correlated with increased proline concentrations in many crops, including bermudagrass, centipedegrass, alfalfa, barley, potato, maize, and winter wheat [1, 7, 12]. The primary mechanisms for increased freeze tolerance in the presence of proline have been discovered, such as osmotic adjustment, ROS scavenging, or redox buffering; proline can also function as a low-molecular chaperone as well as plant development signal [1, 7, 12]. In higher plants, there are at least two alternative routes of proline biosynthesis, e.g., the glutamate and the ornithine pathways. Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is a key enzyme in the glutamate pathway, while ornithine-δ-aminotransferase (OAT) is the rate-limiting enzyme in the ornithine pathway [13–17]. Thus, the biosynthesis pathways of proline may vary in dependence of the plant organ, species, developmental phase, and the type of environmental stress. However, in tobacco suspension cells, the effect of mechanical stimulation on chilling tolerance and

[1] This text was submitted by the authors in English.

Abbreviations: MS—mechanical stimulation; OAT—ornithine-δ-aminotransferase; P5CS—Δ^1-pyrroline-5-carboxylate synthetase; RWR—rapid wound responsive.

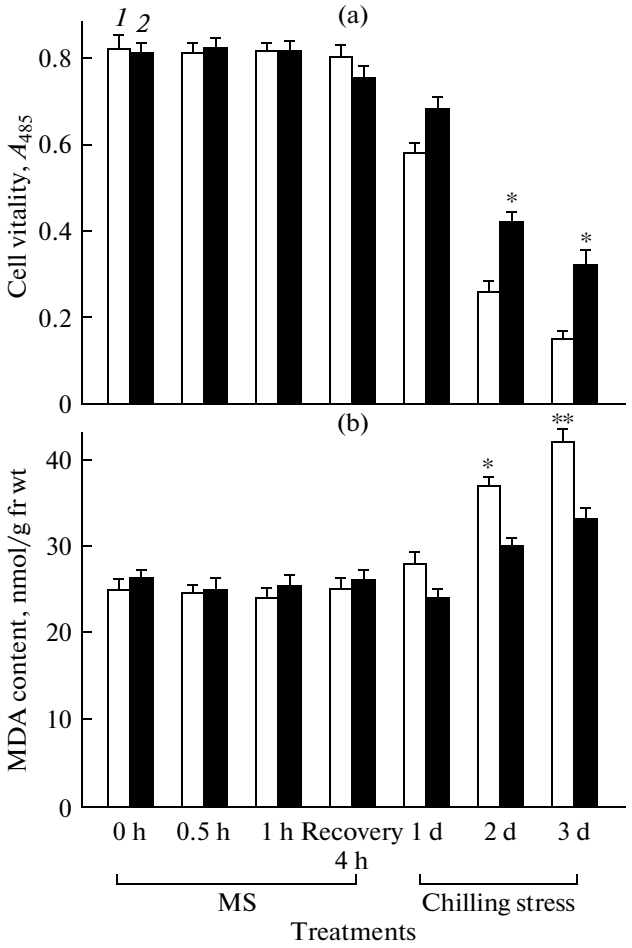

Fig. 1. Effects of mechanical stimulation on vitality (a) and MDA content (b) of tobacco suspension cells under chilling stress at 1°C.

(*1*) Non-MS; (*2*) MS. Asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$ from the control.

proline accumulation are poorly known. In this paper, the effect of mechanical stimulation on chilling tolerance and the involvement of proline were investigated, and the results indicated that mechanical stimulation could induce chilling tolerance and the acquisition of this chilling tolerance was related to proline.

MATERIALS AND METHODS

Calli were originated from young stem pith of Bright Yellow variety of tobacco (*Nicotiana tabacum* L.), and detailed protocols of suspension cell culturing were described by us previously [18].

Four-day-old tobacco cultured cells in logarithmic phase were transferred to the other shaker incubator with the raised rotational speed of 150 rpm for 1 h as a mechanical stimulation treatment and then recovered at 120 rpm for 4 h; the control cells without mechanical stimulation treatment (non-MS) always grew under normal culture conditions at 120 rpm. Treated and nontreated (control) suspension cells were then subjected to chilling stress at 1°C for 3 days. After MS treatment and chilling stress, cell vitality (triphenyl tetrazolium chloride reduction), MDA content, and survival percentage were measured as previously described [19, 20] and expressed as A_{485}, nmol/(g fr wt) and %, respectively. Meanwhile, chilled cells were subcultured to fresh culture medium and recovered for 7 days under normal culture conditions; regrowth ability of the cells was determined according to our previous methods and expressed as g fr wt/60 mL [4].

To explore the effect of MS on P5CS activity and proline accumulation, before mechanical stimulation for 30 min or 1 h, as well as after recovery at 120 rpm for 2 or 4 h, the suspension was filtered through a single layer of filter paper and the cells were washed three times with culture medium and collected to detect the activity of P5CS and proline content. The determination of P5CS activity and proline accumulation were performed using the methods of Song et al. [21] and Lin and Kao [22], respectively. P5CS activity and proline content were expressed as μmol/(g fr wt min) and μmol/g fr wt, respectively.

To investigate the effect of exogenous application of proline on P5CS activity, contents of endogenous proline, and chilling tolerance, P5CS activity and proline content were determined as mentioned above after 4-day-old cells were treated with 10, 20, 30, or 40 μM proline for 4 h. Meanwhile, survival percentages of the cells treated with proline was measured according to above methods after chilling stress at 1°C for 3 days.

All data were taken from at least three independent experiments, and each experiment was performed in two parallel repeats. The results were processed statistically using the analysis of variance (ANOVA). The figures were drawn using SigmaPlot 11.0, and each data bar in figure represents the mean ± SE of at least three experiments.

RESULTS

Effect of Mechanical Stimulation on Chilling Tolerance of Tobacco Suspension Cells

Four-day-old cultured cells were exposed to chilling stress at 1°C in a shaker incubator after subjection to mechanical stimulation at 150 rpm for 1 h. The results showed that, before chilling stress, mechanical stimulation had no obvious effect on cell vitality, MDA content, survival percentage, and regrowth ability of tobacco cells, namely, difference was insignificant, which is shown in Figs. 1 and 2. Under chilling stress at 1°C, however, mechanical stimulation could alleviate a decrease in vitality of tobacco suspension cells (Fig. 1a) and reduce the accumulation of MDA, the marker of membrane lipid peroxidation (Fig. 1b), which in turn improved survival percentage of tobacco suspension cells (Fig. 2a) and regrowth ability during

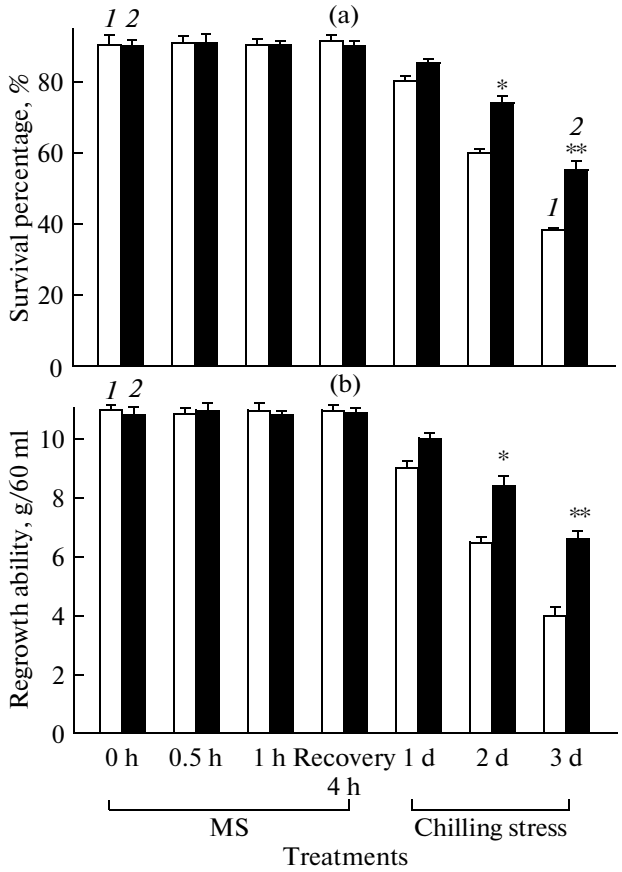

Fig. 2. Effects of mechanical stimulation on survival percentage under chilling stress at 1°C (a) and regrowth ability of tobacco suspension cells during recovery at 26°C (b). (*1*) Non-MS; (*2*) MS. Asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$ from the control.

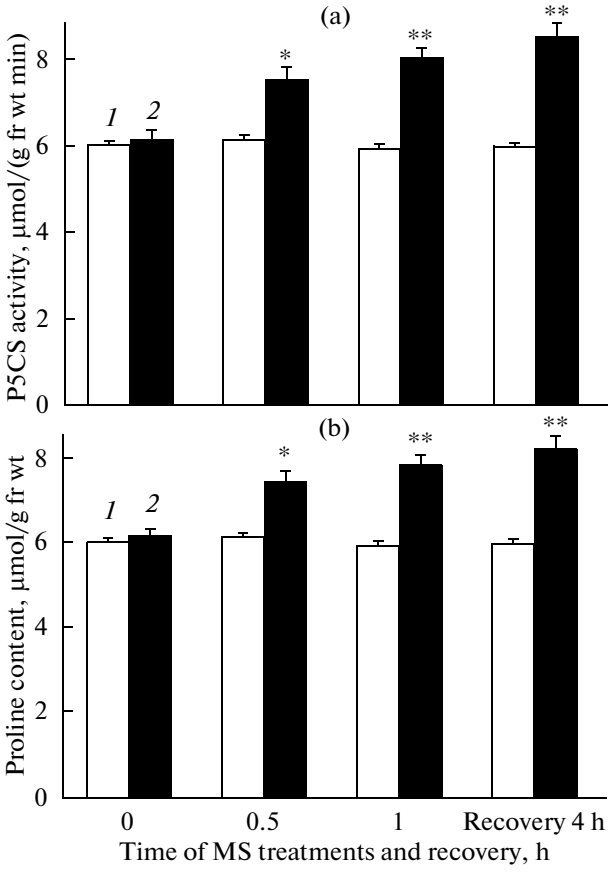

Fig. 3. Effects of mechanical stimulation on P5CS activity (a) and content of proline (b) in tobacco suspension cells. (*1*) Non-MS; (*2*) MS. Asterisks indicate significant difference at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$ from the control.

recovery at 120 rpm after chilling stress (Fig. 2b) as compared with the control without mechanical stimulation. These results illustrate that mechanical stimulation could increase tolerance of tobacco suspension cultured cells to chilling stress.

Effect of Mechanical Stimulation on P5CS Activity and Proline Content of Tobacco Suspension Cells

P5CS is the rate-limiting enzyme of proline biosynthesis in the glutamate pathway. In the process of mechanical stimulation treatment, P5CS activity and the content of proline in tobacco suspension cells were determined. Figure 3a shows that P5CS activity increased with the prolongation of mechanical stimulation time and reached to maximum value after mechanical stimulation for 1 h ($P < 0.01$). During 4-h recovery at 120 rpm after mechanical stimulation, P5CS activity still enhanced and maximized at 8.15 ± 0.31 μmol/(g fr wt min), which was by 23% higher than in control (5.65 ± 0.31 μmol/(g fr wt min)] ($P < 0.01$, Fig. 3a). Additionally, change in proline accumulation was similar to that in P5CS activity during mechanical stimulation and recovery at 120 rpm, especially in recovery for 1 and 4 h, respectively, which was much more significant compared with the control ($P < 0.01$, Fig. 3b).

Effect of Exogenous Application of Proline on P5CS Activity, Content of Endogenous Proline, and Chilling Tolerance of Tobacco Suspension Cultured Cells

The activity of P5CS and the content of endogenous proline were determined after the cells were treated with 10, 20, 30, or 40 μM proline for 4 h. The results showed that exogenous application of proline could increase its endogenous level, in particular, after treatments with ≥20 μM proline ($P < 0.01$), but change in P5CS activity was not obvious (Fig. 4a). In addition, after the cells treated with different concentration of proline were subjected to chilling stress at 1°C, the survival percentage was measured. As shown in Fig. 4b, exogenously applied proline could improve survival percentage of tobacco suspension cells under

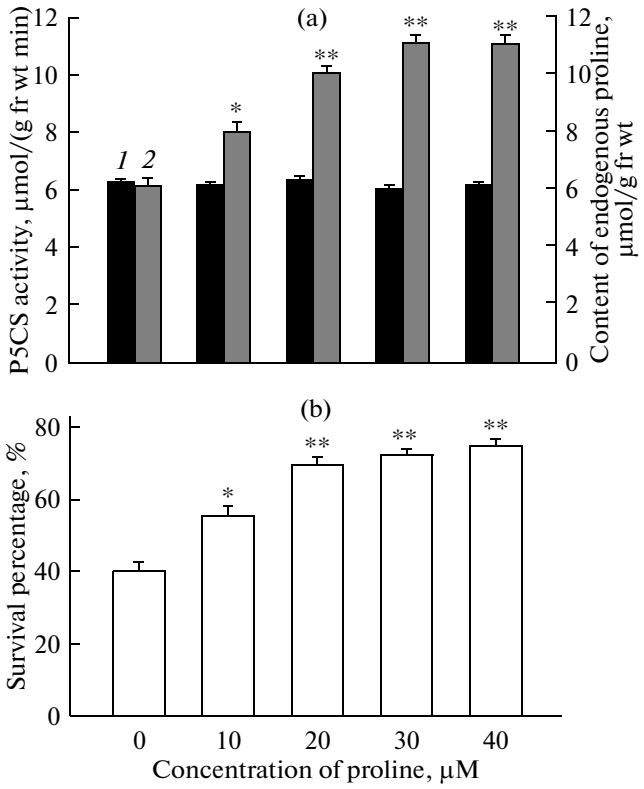

Fig. 4. Effects of pretreatment with exogenous proline on P5CS activity and the content of endogenous proline (a) and survival percentage (b) of tobacco suspension cells.

(*1*) P5CS activity; (*2*) content of endogenous proline. Asterisks indicate significant differences at $P < 0.05$ and double asterisks indicate significant differences at $P < 0.01$ from the control.

chilling stress, and along with the increase in the concentration of proline, the survival percentage of tobacco suspension cells was enhanced ($P < 0.01$), similarly to the accumulation of endogenous proline (Fig. 4a), demonstrating that pretreatment with exogenous proline could improve tobacco cell resistance to chilling stress.

DISCUSSION

As was mentioned earlier, mechanical stimulation affecting plant growth, development, reproduction, and even survival throughout their life cycle, is a primary abiotic stress factor occurring widely in nature. Keller and Steffen [5] found that mechanical stimulation by brushing with wooden bar could improve chilling tolerance of tomato seedlings; this is the earliest report concerning the enhancement of higher plant chilling tolerance by mechanical stimulation, but its mechanism was not completely clear. Walley et al. [6] also demonstrated that mechanical wounding could induce a series of gene expression associated with abiotic and biotic stress responses, followed by an increase in the resistance of arabidopsis to adverse environment. Capiati et al. [23] discovered that mechanical wounding could increase salt tolerance of tomato plants. Previous results from our laboratory also have shown that mechanical stimulation could enhance heat tolerance of tobacco suspension cells and H_2O_2 could act as a signaling molecule to induce the heat tolerance by MS [3, 4]. In this work, it was shown that, in four-day-old cultured tobacco cells, mechanical stimulation by increasing the rotation speed in a shaker incubator could alleviate the loss in cell vitality and the accumulation of MDA in tobacco suspension cells under chilling stress (Figs. 1a, 1b), which in turn increased the survival percentage under chilling stress and regrowth ability of the cells during recovery under normal culture conditions (Figs. 2a, 2b). These results imply that mechanical stimulation could improve tolerance of plant cells to adverse environments, including chilling stress.

A large number of researchers found that the strong proline accumulation can be induced by various environmental stresses [8–11]. In many crop species, such as bermudagrass, centipedegrass, alfalfa, barley, potato, maize, and winter wheat, the acquisition of low temperature tolerance was closely correlated with proline accumulation [1, 7, 12]. Our previous results also designated that short-term heat shock at 42°C could induce proline accumulation in maize seedlings, and exogenous application of proline could improve the level of endogenous proline and activities of antioxidant enzymes, followed by the increase in

heat tolerance of maize seedlings [24]. Present results also showed that mechanical stimulation could enhance P5CS activity of tobacco suspension cells, which in turn triggered endogenous proline accumulation (Figs. 3a, 3b). In addition, exogenously applied proline also could enhance its level in the cells, especially in pretreatments with ≥20 μM proline, but this did not affect P5CS activity (Figs. 4a, 4b). Chen and Li [25] indicated that pretreatment with proline could alleviate chilling injury of maize cultured cells; Hoque et al [26] also showed that exogenous application of proline could enhance salt tolerance in tobacco suspension cells. In contrast, proline exogenously applied to arabidopsis seedlings exhibited obvious toxic effect: inhibition of seedling growth, change in the ultrastructure of chloroplast and mitochondria, and triggering programmed cell death [16, 17]. However, our present results imply that pretreatment with proline not only had no toxic effect on the cells, namely, no obvious effect on survival percentage of the cells (data not shown) in normal culture conditions, but also could improve survival percentage of tobacco cells under chilling stress (Fig. 4b). Many studies illustrated that, under environmental stress such as chilling stress, multiple-function proline could maintain integration of biomembranes and compartmentalization of organs, as well as keep metabolic balance of cells by osmotic adjustment, redox buffering, and ROS scavenging. Proline could also function as a low-molecular chaperone, which in turn enhanced tolerance of plant cells to various environmental stresses [16, 17].

In summary, mechanical stimulation could improve chilling tolerance of tobacco suspension cells, and the acquisition of this chilling tolerance was related to proline.

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