Changes in Foliar Proline Concentration of Osmotically Stressed Barley

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The amino acid proline is accumulated in plant tissues in response to a variety of stresses. The existence of two routes for its biosynthesis is well documented. However, little is known about the contribution of each pathway to the accumulation of free proline under stress conditions. In the present study young barley plants were subjected to osmotic stress by treating their roots with 25% polyethylene glycol. Prior to stress imposition roots were incubated for 24 h in nutrient solution containing proline or one of its metabolic precursors: glutamate and ornithine. Free proline quantity in the leaves was measured before and after stress. Relative water content (RWC) was used as a measure of the plant water status. Foliar proline levels showed a significant increase in ornithine- and proline-pretreated plants compared to the control. Nevertheless, no considerable changes in leaf RWC were observed. It was shown that before stress application only ornithine but not glutamate was immediately metabolized to proline. Under stress conditions, however, both precursors were converted into proline. The possible role of this amino acid in the processes of post stress recovery is discussed.

Key words: Barley, Osmotic Stress, Proline Precursors

Introduction

It is well known that one of the first symptoms of abiotic stress in plants is the accumulation of free proline in the tissues. Drought and salinity as well as suboptimal temperatures may all lead to the development of osmotic stress (Hsu *et al.*, 2003; Aziz *et al.*, 1998; Simon-Sarkadi *et al.*, 2006). It is assumed that increased proline content may be due to the following: enhanced biosynthesis, slow rate of degradation, and/or increased proteolysis (protein hydrolysis). In plant cells proline is synthesized from glutamate via Δ^1-pyrroline-5-carboxylate (P5C) in two consecutive reactions catalyzed by P5C synthetase and P5C reductase (Delauney and Verma, 1993). Apart from this metabolic route an alternative biosynthetic pathway is present in plants, which leads to proline from ornithine, involving the catalytic activity of the enzyme ornithine aminotransferase (OAT) (Mestichelli *et al.*, 1979). The amino acid arginine may also contribute to proline synthesis via ornithine and involving the enzyme arginase (Boggess and Stewart, 1976). The two main metabolic pathways (from glutamate and from ornithine) are spatially separated. Synthesis of proline from glutamate is located in the cytosol while the ornithine pathway is compartmentalized in mitochondria. Due to the existence of at least two biosynthetic pathways, the control of proline synthesis is rather complicated. Regulation is accomplished at two levels: enzyme activity and gene expression. Evidence exists for feedback regulation of the proline biosynthesis with the end product acting as an inhibitor of the reaction. Under stress conditions, however, this regulation is disturbed and despite the high level of proline its synthesis continues (Zhang *et al.*, 1995; Yoshiba *et al.*, 1997). In the present study we examined the contribution of two proline precursors (ornithine and glutamate) to stress-induced proline accumulation in leaves of young barley plants.

Materials and Methods

Barley seeds (cv. Pamina) were superficially sterilized with sodium hypochlorite (NaOCl) and germinated on wet filter paper in a thermostat (25 °C) in the dark for 24–48 h. Seedlings were grown in a phytostat chamber (12 h photoperiod, 25 °C, 60% relative humidity) on full strength Knop nutrient solution until the stage of the first fully developed leaf was reached. Macronutrient concentration was: 3.0 mM $Ca(NO_3)_2$, 1.5 mM KH_2PO_4, 2.0 mM KNO_3, 0.8 mM $MgSO_4$, 1.3 mM KCl. In the different variants 10-day-old barley

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plants were pretreated with either proline or one of its metabolic precursors (glutamate or ornithine), which were introduced in 10 mM concentration in the nutrient solution. Plants were incubated in the presence of the amino acids for 24 h and the foliar proline content was subsequently determined. After this pretreatment, plants were transferred to 25% polyethylene glycol (PEG) 8000 dissolved in nutrient solution. Proline levels were measured again after 24 h of stress. Control plants were left on nutrient solution.

Relative water content (RWC) of the leaves was estimated as described by Turner (1981) and calculated according to

$$RWC = [(FW - DW) / (TW - DW)] \cdot 100,$$

where FW is the fresh weight of the leaves, TW is the weight at full turgor, measured after floating the leaves for 24 h on water in the light at room temperature, and DW is the weight estimated after drying the leaves for at least 4 h at 80 °C or until a constant weight is achieved. Free proline content was assessed in leaf tissue by the ninhydrin method of Bates *et al.* (1973). Fresh leaf material (0.5 g) was stirred with 5 ml distilled water and boiled in a water bath for 30 min. After centrifugation samples were filtered through paper and 1 ml of the supernatant was mixed with 2 ml freshly prepared ninhydrin reagent (0.5 g ninhydrin in 50 ml of 60% acetic acid). The colour reaction developed after incubation of the samples for 1 h in a boiling water bath. Registration was performed spectrophotometrically after toluene extraction, and estimation of proline concentration was based on a previously prepared standard curve. The equivalent dry weight was obtained by drying an identical leaf quantity at 80 °C to a constant weight. Foliar concentration of free amino acids (without proline) was determined according to Yemm and Cocking (1955). Ethanol extracts of leaf tissue were evaporated to dryness and subsequently dissolved in water (1:1) with respect to the fresh weight of the sample. The method is based on the reaction of ninhydrin with the amino group of amino acids. Proline reacts with ninhydrin in an entirely different way and is not included in the presented data for total amino acids. All values are means of at least 3 replications ± SD.

Results

Pretreatment with proline, ornithine and glutamate *per se* did not lead to an increase in the content of total amino acids (Fig. 1A). Nevertheless, 24 h of osmotic stress caused a drastic increase in foliar levels of these solutes in all variants. Highest values were observed in stressed plants pretreated with proline. After PEG stress total amino acids content in glutamate-pretreated plants was somewhat lower than in stressed controls.

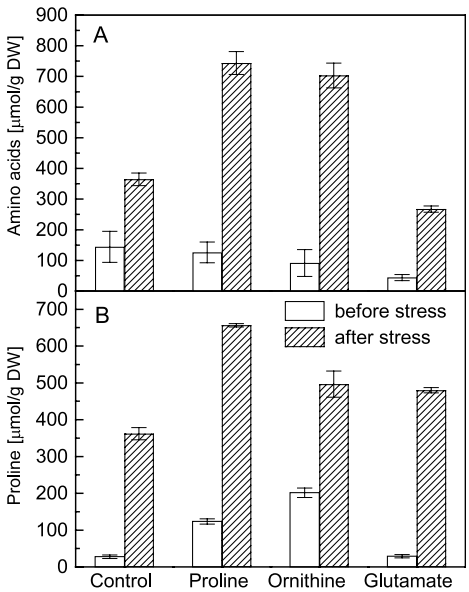

Fig. 1. Changes of total amino acids (A) and free proline content (B) in the leaves of young barley plants pretreated with proline, ornithine, or glutamate and subsequently stressed with PEG. Control plants were not pretreated with amino acids.

After incubation of young barley plants in nutrient solution to which proline was added the content of free proline in the leaves increased 4.5-times in comparison with control values (Fig. 1B). Ornithine-pretreated plants had a 7-times higher proline content than the untreated control. Pretreatment with glutamate, however, did not lead to an increase in the endogenous proline level. After 24 h of PEG treatment foliar concentrations of free proline in plants grown on pure nutrient solution showed a 13-fold increase as compared to untreated controls. The osmotically induced rise in endogenous proline for variants pretreated with

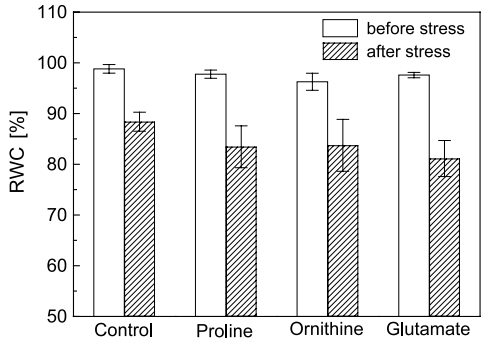

Fig. 2. Changes of relative water content (RWC) in the leaves of barley plants after 24 h of osmotic stress with PEG. Different variants have previously been treated with proline, ornithine, or glutamate. Control plants were subjected to PEG stress without pretreatment with amino acids.

proline, ornithine and glutamate was, respectively, 24-, 18-, and 17-times of the control value.

PEG treatment had an impact on the water status of all stressed plants, causing a decrease in the relative water content of their leaves (Fig. 2). The parameter RWC is widely employed as a measure of stress in plants (Turner, 1981). Since in our experiments all variants exhibited only a slight decrease of foliar RWC, the imposed stress could be regarded as mild or moderate.

Discussion

It is generally accepted that stress-related proline accumulation is primarily due to intensified synthesis *de novo* (Claussen, 2005). The biosynthetic route from glutamate is considered to be predominant in the proline metabolism (Zhang *et al*., 1995). In our experiments, however, glutamate pretreatment did not result in higher proline accumulation in the leaves of unstressed plants (Fig. 1B). One possible explanation is that glutamic acid is a key metabolite, involved in many biochemical reactions in the cell and under normal conditions it is not directly assimilated to proline. Exogenously added ornithine, on the other hand, enters through the roots and is most likely metabolized to proline. After PEG treatment we observed higher proline levels in plants pretreated with proline and its precursors than in stressed controls. This is in accordance with the idea that endogenous proline levels can be altered by the addition of related amino acids (Aziz *et al*., 1998). The presented data suggests that both biosynthetic routes contribute to proline accumulation. While ornithine is the main source for proline synthesis under normal conditions, glutamate is involved predominantly after stress. Amino acids are viewed as compatible solutes, which contribute to the process of osmotic adjustment under stress conditions (Raggi, 1994; Rai, 2002). As it was shown by other authors, the regulation of amino acid levels is rather complex and manipulation of a single amino acid may affect not only its own metabolism and the metabolism of others directly related to it, but also the amino acid composition in general (Simon-Sarkadi *et al*., 2006). In our experiments, although accumulated to high concentrations, amino acids and free proline did not seem to contribute to the maintenance of foliar RWC (Fig. 2). This is in accordance with the suggestion that increased proline levels are associated not only with osmoregulation. Furthermore, its degradation after stress relief provides carbon, nitrogen, and energy for the recovery processes (Hare and Cress, 1997). We have already shown (Kocheva and Georgiev, 2005) that after rehydration proline levels fall to control values, which is probably due to fast metabolization. However, a number of experimental evidences demonstrate that osmotically induced proline accumulation is not a prerequisite for immediate water stress tolerance, but a symptomatic disorder induced by dehydration (Larher *et al*., 2003). Indications that stress encountered by roots may be necessary in order to observe changes in the proline content of the leaves suggest a tissue-specific division of labour in mediating osmolyte accumulation (Raymond and Smirnoff, 2002). As a whole, contradictory opinions exist in the literature regarding the exact role of free proline accumulated in plant tissues in stress response. Nevertheless, this particular amino acid can be considered as a certain stress-marker being among the first signals of stress development. Results presented here elucidate the contribution of the glutamate pathway for proline synthesis and accumulation especially under stress rather than normal conditions.

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