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The effect of polyethylene glycol on proline accumulation in rice leaves

S.Y. HSU, Y.T. HSU and C.H. KAO

Department of Agronomy, National Taiwan University, Taipei 106, Taiwan, Republic of China

Abstract

The regulation of proline accumulation in polyethylene glycol (PEG, -1.5 MPa) treated rice leaves was investigated. PEG treatment resulted in a decrease in relative water content, indicating that PEG treatment caused water stress in rice leaves. Proline accumulation caused by PEG was related to protein hydrolysis, an increase in ornithine-δ-aminotransferase activity, an increase in the content of ammonia, and an increase in the contents of the precursors of proline biosynthesis, glutamic acid, ornithine, and arginine. Results also show that abscisic acid accumulation is not required for proline accumulation in PEG-treated rice leaves.

Additional key words: abscisic acid, arginine, glutamic acid, ornithine, ornithine-δ-aminotransferase, *Oryza sativa*, relative water content, water stress.

Introduction

Drought and salinity are two major stresses that dramatically limit plant growth and productivity (Boyer 1982). Many higher plants accumulate proline in response to osmotic stress (Aspinall and Paleg 1981, Delauney and Verma 1993, Yoshiba *et al.* 1997, Yang *et al.* 2000a,b, Demir and Kocacaliskan 2001, Singh *et al.* 2001), a phenomenon first observed by Kemble and MacPherson (1954) in wilted rye grass. Different roles have been suggested for proline accumulation, although the actual role remains unclear (Rhodes *et al.* 1999). In addition to acting as an osmoprotectant (Pollard and Wyn Jones 1979), proline also serves as a sink for energy to regulate redox potectials (Blum and Ebercon 1976, Saradhi and Saradhi 1991), as a hydroxyl radical scavenger (Smirnoff and Cumbes 1989), as a solute that protects macromolecules against denaturation (Schobert and Tschesche 1978), and as a means of reducing the acidity in the cell (Venekamp *et al.* 1989).

Proline accumulation in plant tissues has been suggested to result from *1*) an increase in proline biosynthesis, *2*) a decrease in proline degradation, *3*) an increase in protein hydrolysis, and *4*) a decrease in proline utilization (Charest and Phan 1990, Yoshiba *et al.* 1997). In plants, proline is synthesized from glutamic acid via Δ^1-pyrroline-5-carboxylate (P5C) by two enzymes, P5C synthetase and P5C reductase (P5CR, EC 1.5.1.2) (Delauney and Verma 1993, Yoshiba *et al.* 1997). Plants also synthesize proline from ornithine, by ornithine-δ-aminotransferase (OAT, EC 2.6.1.13). Arginine can also contribute to proline biosynthesis, and the pathway from arginine proceeds via ornithine as a result of catalytic activity of arginase (Brown and Fowden 1966, Lingnowski and Splittstoesser 1971). On the other hand, the content of proline also depends on its degradation, which is catalysed by the enzyme proline dehydrogenase (PDH, EC 1.5.99.8) (Yoshiba *et al.* 1997).

There have been reports that proline accumulation in salt-stressed plants correlated with increases P5CR activity (Huber 1974, Madan *et al.* 1995, Mattioni *et al.* 1997, Sudhakar *et al.* 1993, Treichel 1986), and content of P5CR mRNA is elevated in salt-stressed plant tissues (Delauney and Verma 1990, Williamson and Slocum 1992). However, in a study of P5CR activity in tobacco cells undergoing salt stress, La Rosa *et al.* (1991) concluded that the P5CR reaction was not rate-limiting and was not involved in NaCl-dependent regulation of proline synthesis. Szoke *et al.* (1992) also demonstrated that a 50-fold increase in soybean P5CR activity in

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Abbreviations: ABA - abscisic acid; f.m. - fresh mass; OAT - ornithine-δ-aminotransferase; P5C - Δ^1-pyrroline-5-carboxylate; P5CR - Δ^1-pyrroline-5-carboxylate reductase; PDH - proline dehydrogenase; PEG - polyethylene glycol; RWC - relative water content.

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Fax: (+886) 2 23620879, e-mail: kaoch@ccms.ntu.edu.tw

transgenic tobacco plants expressing soybean P5CR did not result in any significant increase in proline formation. It has been shown that proline accumulation in response to NaCl and water stress could be attributed to an increase in OAT activity (Lutts *et al.* 1999, Madan *et al.* 1995, Sundaresan and Sudhakaran 1995) or a decrease in PDH activity (Kiyosue *et al.* 1996, Mattioni *et al.* 1997, Peng *et al.* 1996, Verbruggen *et al.* 1996).

In recent studies, we found that exogenous NH_4Cl and methionine sulfoximine, which caused an accumulation of ammonia in detached rice leaves, increased proline content (Yang and Kao 1999). It is not known whether ammonia accumulation is linked to proline accumulation in detached rice leaves caused by water stress.

Abscisic acid (ABA) is known to increase rapidly in plant tissues under water stress (Hsiao 1973). Proline accumulation can be induced by ABA in plant tissues (Aspinall and Paleg 1981). Thus, proline accumulation in response to water stress may be a response to accumulated ABA. In fact, Ober and Sharp (1994) reported that increased content of ABA is required for proline accumulation in maize primary roots at lower water potentials. However, proline accumulation in the absence of ABA accumulation has also been reported (Stewart and Voetberg 1987).

In the present investigation, we used polyethylene glycol (PEG) to induce water stress. Four main questions have been addressed in this paper: *1*) Is the accumulation of glutamic acid, ornithine, and arginine related to proline accumulation in rice leaves caused by water stress? *2*) Is the accumulation of ammonia related to proline accumulation caused by water stress? *3*) Is proline accumulation caused by water stress regulated by P5CR, ODC, and PDH activities? *4*) Is ABA accumulation required for proline accumulation caused by water stress?

Materials and methods

Rice (*Oryza sativa* L. cv. Taichung Native 1) was cultured in a stainless net floating on half-strength Johnson's modified nutrient solution (pH 4.2) in a 500-cm^3 beaker (Lin *et al.* 1999). The nutrient solution was replaced every 3 d. Rice plants were grown for 12 d in a greenhouse, under natural light and the day/night temperature of 30/25 °C. The apical 3 cm of the third leaf of 12-d-old seedling was used for the experiment. A group of 10 segments floated in a Petri dish containing 10 cm^3 of distilled water served as controls. For induction of water stress, leaf segments were exposed to PEG-6000 solution of osmotic potential -1.5 MPa. All samples were kept at temperature at 27 °C and irradiance of 40 μmol m^{-2} s^{-1} for 4, 8 and 12 h.

RWC, defined as water content of leaf tissue as a percentage that of the fully turgid tissue, was determined by the method of Weatherley (1950). For determination of proline, glutamic acid, ornithine, arginine, NH_3, and total amino acids, leaf samples were extracted with 2 % sulfosalicylic acid and the homogenates was centrifuged at 15 000 *g* for 20 min. The supernatant was used directly for amino acid analysis (amino acid analyzer, *Beckman 63000*, Palo Alto, USA). For protein determination, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 6.8). The homogenate was centrifuged at 17 600 *g* for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976). ABA and enzymes were extracted and assayed as described previously (Chen *et al.* 2001).

Amino acids, protein, NH_3, protein and ABA were expressed on the basis of initial fresh mass (f.m.). Enzyme activity was expressed on the basis of protein. All experiments were repeated three times; within each experiment, treatments were replicated 4 times. Similar results and identical trends were obtained in all experiments. The data reported here are from a single experiment.

Results and discussion

Basically, PEG does not enter the cell wall space (Foster and Walters 1991). It is generally thought that PEG molecules with a molecular mass greater than 3 000 are not absorbed at all (Mexal *et al.* 1975). Thus, PEG is commonly used as a water stress agent (Hanson and Nelsen 1978). RWC of detached rice leaves exposed to PEG (-1.5 MPa) decreased considerably during 12-h treatment (Fig. 1), indicating that PEG treatment in our study did indeed cause water stress in detached rice leaves. Proline content in detached rice leaves exposed to PEG increased significantly with the increase of

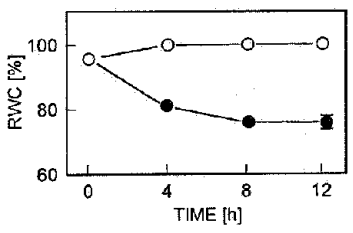

Fig. 1. Relative water content (RWC) in detached rice leaves floating on water (*open circles*) or PEG solution (-1.5 MPa; *closed circles*) for 4, 8, and 12 h. Means ± SE, $n = 4$.

incubation time, increased about 25-fold at 12 h in the light (Fig. 2).

Ammonia has been shown to accumulate in detached rice leaves in response to desiccation (Lin and Kao 1998). Rabe (1990) postulated that any stress causing reduced growth or impaired plant health will result in ammonia accumulation early and suggested that the ammonia detoxification results in the accumulation of nitrogen-containing compounds, such as putrescine and proline. In recent studies, we observed that exogenous NH_4Cl and methionine sulfoximine, which caused an accumulation of ammonia in detached rice leaves, increased proline content (Yang and Kao 1999). In the present investigation, we also observed that PEG treatment resulted in an ammonia accumulation (Fig. 3).

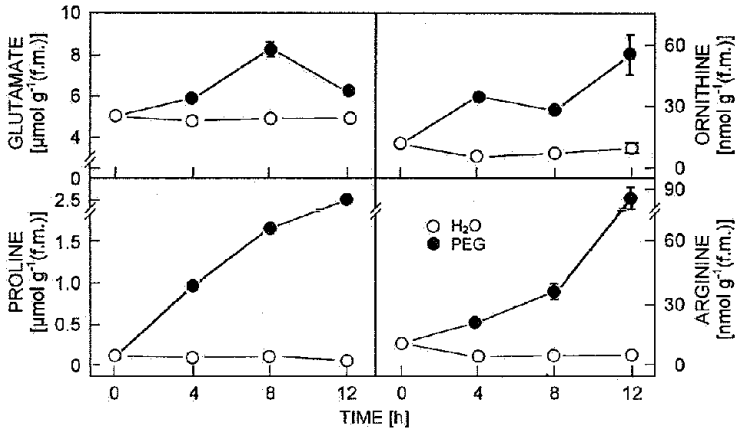

Fig. 2. The contents of proline, glutamic acid, arginine, and ornithine in detached rice leaves floating on water (H_2O) or PEG (-1.5 MPa) solution for 4, 8, and 12 h. Means ± SE, $n = 4$.

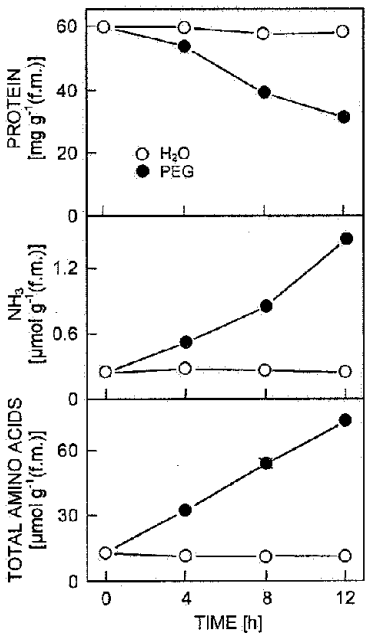

Fig. 3. The contents of protein, ammonia, and total amino acids in detached rice leaves floating on water (H_2O) or PEG (-1.5 MPa) solution for 4, 8, and 12 h. Means ± SE, $n = 4$.

The decrease in protein content was faster in PEG-treated detached rice leaves than in control leaves (Fig. 3). Therefore, protein degradation might contribute to PEG-induced proline accumulation in detached rice. This suggestion is supported further by the observation that content of total amino acids was higher in detached rice leaves exposed to PEG than in control leaves (Fig. 3). It is generally considered that glutamic acid, ornithine, and arginine can contribute to the accumulation of proline (Delauney and Verma 1993). PEG treatment resulted in an increase in glutamic acid, ornithine, and arginine contents in detached rice leaves (Fig. 2), which is most likely a result of protein degradation. Glutamine synthetase catalyses the conversion of glutamic acid and ammonia to glutamine (Ireland and Lea 1999). Our unpublished data indicate that PEG treatment markedly decreased glutamine synthatase activity in detached rice leaves. Thus, the possibility that the increase in ammonia and glutamic acid contents (Fig. 2 and 3) in detached rice leaves treated with PEG is due to PEG-inhibited glutamine synthatase activity cannot be excluded.

To determine the role of the biosynthetic pathways for proline accumulation caused by PEG treatment, the effect of PEG on OAT and P5CR activities was examined (Fig. 4). OAT activity was higher in PEG-treated leaves than in control leaves. However, P5CR activity in PEG-treated leaves was lower than in control leaves (Fig. 4). Thus, the increase in OAT activity may have, to some extent, contributed to the elevated content of proline by PEG. Our results are in agreement with the idea that OAT but not P5CR reaction is rate limiting and involved in water stress-dependent regulation of proline synthesis (La Rosa *et al.* 1991, Lutts *et al.* 1999, Madan *et al.* 1995, Sundaresan and Sudhakaran 1995, Szoke *et al.* 1992). P5C synthetase is also involved in proline biosynthesis (Yoshiba *et al.* 1997). Thus, PEG could also have an

effect on this enzyme. However, for unknown reasons, we failed to detect any P5C synthetase activity in crude extracts. Thus, this enzyme was not considered in the present investigation. The enzyme PDH is reported to catalyse proline oxidation (Yoshiba *et al.* 1997). In the present investigation PEG treatment resulted in a decrease in PDH activity in detached rice leaves at longer treatment (8 and 12 h) (Fig. 4). This result suggests that proline oxidation (or degradation) contributes to proline accumulation in detached rice leaves only at 8- and 12-h of PEG treatment.

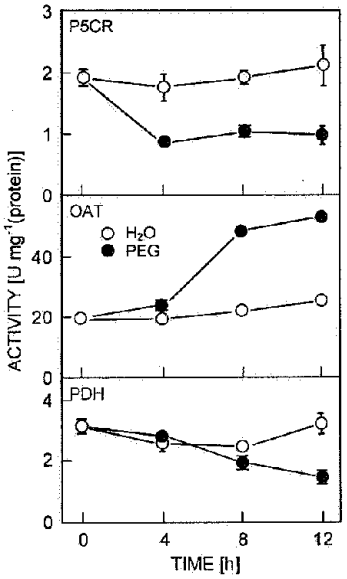

Fig. 4. The activities of P5CR, OAT, and PDH in detached rice leaves floating on water (H_2O) or PEG (-1.5 MPa) solution for 4, 8, and 12 h. Means ± SE, $n = 4$.

PEG treatment resulted in about 5-fold increase in ABA content in detached rice leaves when compared with H_2O-treated leaves (Table 1). To obtain information about the role of ABA in regulating proline accumulation, experiments using inhibitor of ABA biosynthesis were carried out (Table 1). In higher plants, ABA is produced by the oxidative cleavage of the polyene chain of a C_{40} epoxy-xanthophyll precursor, such as 9'-cis-neoxanthin, which is formed from violaxanthin in carotenoid biosynthesis (Kende and Zeevaart 1997). Fluridone is known to inhibit ABA biosynthesis via affecting phytoene desaturase in the carotenogenic pathway (Kowalczyk-Sehröder and Sandmann 1992). Fluridone was observed to inhibit the increase in ABA content in detached rice leaves caused by PEG (Table 1). However, fluridone had no effect on proline accumulation in detached rice leaves induced by PEG (Table 1). It appears that ABA accumulation is not required for proline accumulation in detached rice leaves exposed to PEG. Proline accumulation in the absence of ABA accumulation in wilted leaves has also been reported by Stewart and Voetberg (1987).

Table 1. Effect of fluridone on ABA and proline contents in detached rice leaves exposed to PEG in the light. Detached rice leaves were pretreated with or without 20 μM fluridone for 3 h in the light and then treated with water or PEG for 12 h in the light. Means ± SE, $n = 4.0$

Pretreatment	Treatment	ABA [pmol g^{-1}(f. m.)]	Proline [μmol g^{-1}(f. m.)]
H_2O	H_2O	135.7 ± 3.8	0.16 ± 0.02
Fluridone	H_2O	174.5 ± 35.2	0.10 ± 0.01
H_2O	PEG	780.6 ± 12.7	2.62 ± 0.11
Fluridone	PEG	123.7 ± 4.8	2.70 ± 0.07

In conclusion, out current results suggest that proline accumulation in detached rice leaves in response to water stress induced by PEG is related to protein hydrolysis an increase in OAT activity, and an increase in the contents of glutamic acid, ornithine, and arginine. Evidence is also provided to show that ammonia accumulation is involved in proline accumulation and ABA accumulation is not required for proline accumulation in PEG-treated rice leaves.

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