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REGULATION OF PROLINE ACCUMULATION IN DETACHED RICE LEAVES

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Regulation of proline accumulation in detached rice leaves was investigated. Under dark conditions, proline content in detached rice leaves remained unchanged 8 h after incubation in distilled water, but increased 2-fold by 24 h. However, proline content did not increase during the entire 24-h incubation period in the light. Cell sap pH did not change at 8 h, but decreased 0.2 units at 24 h after incubation in darkness. No significant decrease in cell sap pH was observed in detached rice leaves incubated in the light. The increase of proline content in ammonium vanadate-treated detached rice leaves was associated with cell sap acidification. However, ammonium chloride also decreased cell sap pH and increased the proline content to some extent. The addition of fusicoccin resulted in an increase in proline content and a decrease in cell sap pH. Although fusicoccin promoted isobutyric acid-induced increase of proline, fusicoccin did not result in further decrease in cell sap pH induced by isobutyric acid. Cycloheximide or cordycepin increased proline content without changing cell sap pH. It is concluded that factors apart from cell sap acidification may also be involved in the regulation of proline accumulation.

Key words: cell sap pH; fusicoccin; isobutyric acid; *Oryza sativa*; proline accumulation; vanadate

Introduction

It is well established that proline accumulates rapidly in excised and intact leaves under water stress [1 – 3]. Proline accumulation can also be induced by abscisic acid in both excised leaves and intact plants [4,5]. Recent reports suggested that decrease of intracellular pH, measured as cell sap pH, was involved in stress-, abscisic acid- and weak acid-induced proline accumulation in wheat coleoptiles and barley leaves [6 – 9]. Venekamp [10] showed that organic acids were the source for drought-induced proline synthesis in field-grown bean plants. Previously, we have reported that proline accumulated in detached rice leaves during dark-induced senescence [11]. We also showed that treatments which retard senescence inhibit proline accumulation, while agents which promote senescence promote proline accumulation [12]. Recently, we demonstrated that the decrease in rate of proton secretion of detached rice leaves played a regulatory role in senescence of detached rice leaves [13]. The consequence of the decrease in rate of proton secretion would be a decrease in intracellular pH. The present investigation was thus conducted to study the possible involvement of lowered intracellular pH in regulating proline accumulation in detached rice leaves.

Materials and Methods

Rice (*Oryza sativa* L. cv. Taichung Native 1) seedlings were grown in a greenhouse with natural light at 30 °C day/25 °C night. The apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. A group of 20 segments were floated in a Petri dish containing 20 ml of distilled water or test solutions. Unless otherwise indicated, all test solutions and distilled water were adjusted to pH 5.5.

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Incubation was carried out at 27 °C in darkness or in the light (16.7 Wm^{-2}) provided by a mixture of cool-white and Grolux lamps.

Proline was extracted and its concentration determined following the method of Bates et al. [15]. Leaf segments were homogenized in 3% (w/v) sulfosalicylic acid and centrifuged. The supernatant fluid was treated with acetic acid and acid-ninhydrin, boiled for 1 h and the absorbance was determined at 520 nm. Proline content was expressed as nmol/20 segments.

For the measurement of the cell sap pH, the method of Pesci and Beffagna [6] was used. Leaf segments were transferred at the end of the treatments directly into syringes and were frozen in liquid nitrogen. After thawing the cell sap was pressed out from the syringe and the pH was measured.

All experiments were repeated one or more times with similar results. The data reported here are from a single experiment.

Results

Table I shows the changes of proline content and the cell sap pH of detached rice leaves incubated in distilled water under dark and light conditions. In darkness, proline content in detached rice leaves did not increase at 8 h after incubation but increased 2-fold at 24 h, which confirmed our previous results [11]. Proline content, however, in detached rice leaves incubated in the light decreased significantly by 8 h and subsequently remained low throughout the incubation period. Cell sap pH in detached rice leaves remained unchanged at 8 h, but had decreased 0.2 units by 24 h of incubation in darkness. No significant decrease in cell sap pH was observed when detached rice leaves were incubated in the light. Clearly, proline accumulation is associated with a decrease in cell sap pH of detached rice leaves.

Since neither increase in proline content nor cell sap acidification was observed at 8 h after incubation of detached rice leaves in darkness or the light and in order to avoid interference by the factors related to senescence in long-term incubations, an 8-h incubation time was used for all experiments discussed below.

Several lines of evidence indicate that a plasmalemma ATPase acts as an electrogenic proton pump in higher plants [16]. Recent work by Beffagna and Romani [17] showed that vanadate inhibited the activity of the proton pump, causing a decrease in the intracellular pH, measured as cell sap pH. The data in Table II show that the addition of vanadate leads to a significant decrease in cell sap pH. If cell sap acidification is of importance in regulating proline accumulation, then the increase in proline content is to be expected in vanadate-treated detached rice leaves. As indicated in Fig. 1, this is indeed the case. Results in Fig. 1 also indicate that ammonium chloride increased the proline content in detached rice leaves although less markedly. This increase is apparently associated with a decrease in cell sap pH (Table II).

Table I. Changes of proline content and cell sap pH in detached rice leaves incubated in distilled water under dark and light conditions. Mean ± S.E., three repetitions.

Time (h)	Proline content		Cell sap pH	
	Light	Dark	Light	Dark
0	47 ± 2		6.24 ± 0.03	
8	22 ± 1	50 ± 3	6.24 ± 0.03	6.24 ± 0.01
24	23 ± 2	92 ± 5	6.18 ± 0.01	6.05 ± 0.04

Table II. Effect of ammonium vanadate on the cell sap pH in detached rice leaves. Cell sap pH was measured at 8 h after incubation. Initial cell sap pH was 6.39 ± 0.04. Mean ± S.E., three repetitions.

Treatment	Cell sap pH	
	Light	Dark
Water	6.35 ± 0.02	6.34 ± 0.02
Ammonium chloride (5 mM)	6.04 ± 0.03	6.09 ± 0.01
Ammonium vanadate (5 mM)	5.94 ± 0.01	5.90 ± 0.02

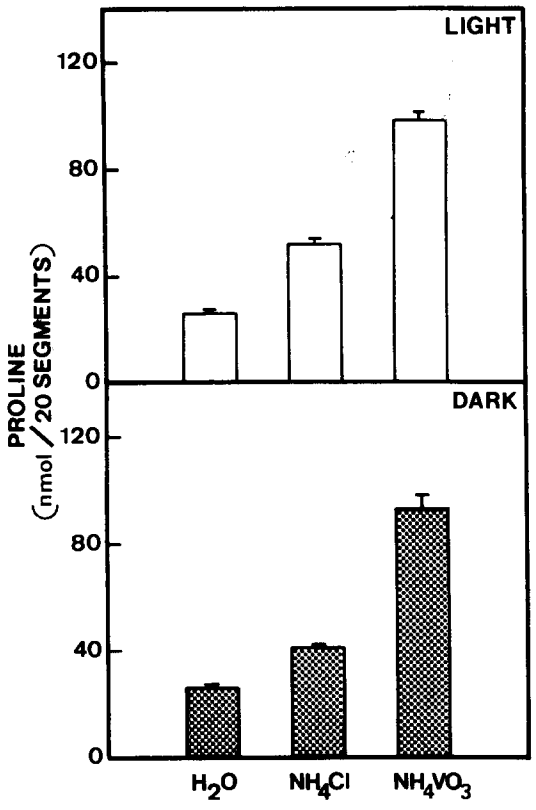

Fig. 1. Effect of ammonium vanadate on proline content in detached rice leaves. Detached rice leaves were incubated in deionized water, ammonium chloride (5 mM) or ammonium vanadate (5 mM) for 8 h. Initial proline content was 29 ± 1 nmol/20 segments. Bars represent S.E., three repetitions.

There is wide consensus that phytotoxin fusicoccin (FC) stimulates active proton export [18]. Surprisingly, treatment with FC caused not only an increase in proline content but also a decrease in cell sap pH (Fig. 2, Table III). Treatment with isobutyric acid (IBA) for 8 h resulted in an increase in proline content (Fig. 3). The treatment with isobutyric acid is accompanied by a decrease in the intracellular pH (Table III). Although FC effectively increased IBA-induced proline accumulation, FC did not result in any additional decrease in cell sap pH greater than that caused by IBA (Fig. 3, Table III).

Table III. Effect of FC and isobutyric acid (IBA) on the cell sap pH in detached rice leaves. Detached rice leaves were incubated in the light for 8 h in 10 mM potassium phosphate buffer in the absence or presence of FC (10^{-5} M), IBA (10 mM) or FC (10^{-5} M) + IBA (10 mM). Initial cell sap pH was 6.14 ± 0.02 and 6.17 ± 0.01, respectively, for Experiments I and II. Mean ± S.E., three repetitions.

Treatment	Cell sap pH
Experiment I	
Control	6.16 ± 0.01
FC	6.05 ± 0.02
Experiment II	
Control	6.19 ± 0.01
IBA	6.02 ± 0.01
FC	6.09 ± 0.01
IBA + FC	6.05 ± 0.02

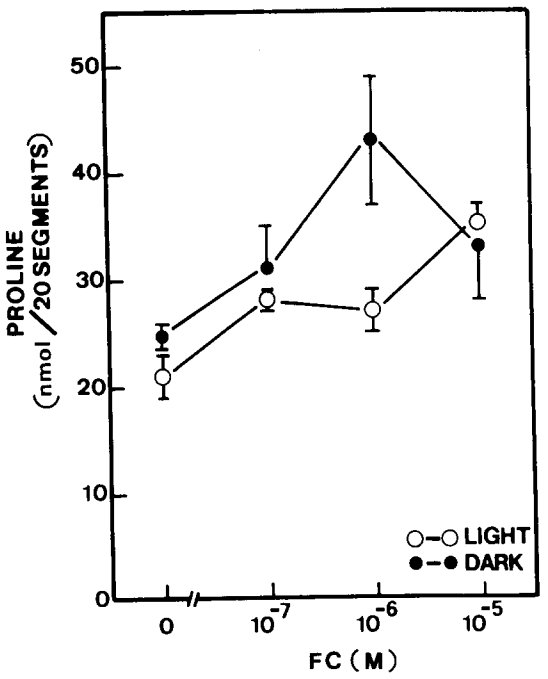

Fig. 2. Effect of different concentrations of FC on proline content in detached rice leaves. Detached rice leaves were incubated for 8 h in 10 mM potassium phosphate buffer in the presence of FC at different concentrations. Initial proline content was 46 ± 6 nmol/20 segments. Bars represent S.E., three repetitions.

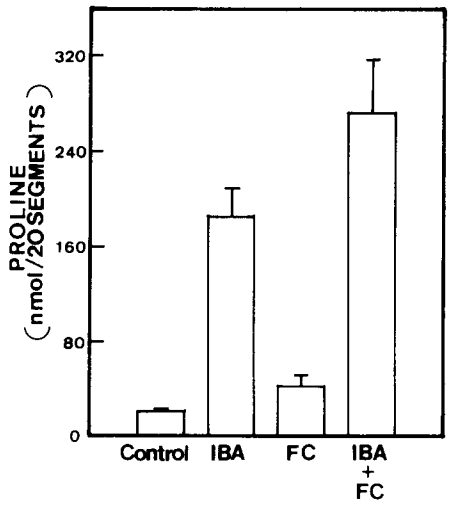

Fig. 3. Effect of fusicoccin (FC) and isobutyric acid (IBA) on proline content in detached rice leaves. Detached rice leaves were incubated in the light for 8 h in 10 mM potassium phosphate buffer in the absence or presence of FC (10^{-5} M), IBA (10 mM) or FC (10^{-5} M) + IBA (10 mM). Initial proline content was 30 ± 3 nmol/20 segments. Bars represent S.E., three repetitions.

Our previous work showed that proton secretion by detached rice leaves is inhibited by cycloheximide (CHI) and suggested that protein synthesis is required for proton secretion [19]. It was expected that treatment of detached rice leaves with CHI would cause a decrease in cell sap pH. Contrary to our expectation, neither CHI nor cordycepin (COR), an inhibitor of RNA synthesis, lowered the cell sap pH (Table IV). Figure 4 shows the effect of CHI and COR on proline content in detached rice leaves. The addition of CHI or COR resulted in a significant increase in proline content. Obviously, CHI- or COR-induced increase of proline content is unrealted to the shift of cell sap pH.

Table IV. Effect of cycloheximide (CHI) and cordycepin (COR) on the cell sap pH in detached rice leaves. Detached rice leaves were incubated in the light for 8 h. Initial cell pH was 6.07 ± 0.02. Mean ± S.E., three repetitions.

Treatment	Cell sap pH
Water	6.08 ± 0.01
COR (0.1 mM)	6.08 ± 0.02
CHI (0.1 mM)	6.12 ± 0.03

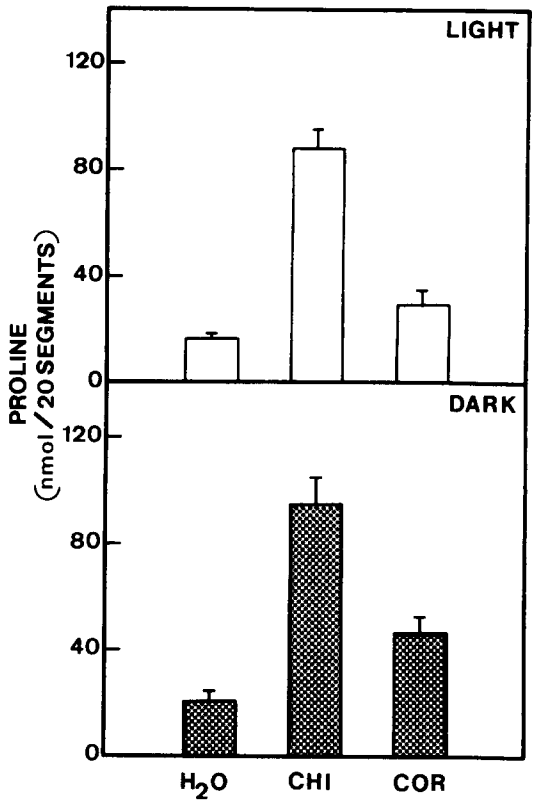

Fig. 4. Effect of cycloheximide (CHI) and cordycepin (COR) on proline content in detached rice leaves. Detached rice leaves were incubated in CHI (0.1 mM) or COR (0.1 mM) for 8 h. Initial proline content was 21 ± 1 nmol/20 segments. Bars represent S.E., three repetitions.

Discussion

The present investigation was initiated to elucidate the possible involvement of intracellular pH, measured as cell sap pH, in regulating proline accumulation in detached rice leaves. Several lines of evidence suggested that cell sap acidification was involved in regulating proline accumulation [6–10]. The results presented in this paper are generally consistent with this suggestion.

The result that ammonium chloride

increased the proline content was unexpected. This further suggests that proline accumulation depends on cell sap pH, since acidification of the cell sap has been established on treating detached rice leaves with ammonium chloride.

Of particular interest is the finding that FC increased proline content and decreased cell sap pH. However, Pesci and Beffagna [6] reported that FC decreased proline content and increased cell sap pH in detached barley leaves. The use of a different species may have led to this discrepancy. The decrease of intracellular pH following FC application has also been reported by other investigators [20–23]. Although FC promoted IBA-induced proline accumulation in detached rice leaves, FC did not cause a further decrease in cell sap pH induced by IBA. It appears that factors apart from cell sap acidification may also be involved in regulating proline accumulation. This conclusion was further supported by the observation that CHI or COR increased proline content without lowering cell sap pH.

Using detached tobacco leaves. Bogges and Stewart [24] demonstrated that CHI treatment resulted in a striking increase in proline content. They attributed the elevated proline content observed to water stress rather than to reduced protein synthesis. Since, in the present work, no curling and loss of turgor were evident in CHI- or COR-treated detached rice leaves, it seemed unlikely that proline accumulation induced by CHI or COR was due to water stress. It seems most likely that CHI- or COR-induced proline accumulation is a consequence of the inhibition of protein synthesis and/or synthesis of specific enzymes responsible for proline oxidation. At the present time, it remains unclear whether cell sap acidification regulates proline accumulation through stimulation of proline synthesis, reduction of proline utilization, or both. Further work along this line would help in elucidating the mechanism of proline accumulation.

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