

## Characteristics of the Induction of the Accumulation of Proline by Abscisic Acid and Isobutyric Acid in Detached Rice Leaves

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The effects of abscisic acid and isobutyric acid on levels of proline in detached rice leaves were compared. The lowest concentration of abscisic acid that induces accumulation of proline in detached rice leaves was much lower than that of isobutyric acid. The accumulation of proline induced by isobutyric acid was associated with acidification of the cell sap, whereas abscisic acid increased levels of proline without decreasing the pH of the cell sap. Potassium chloride enhanced the accumulation of proline that was induced by abscisic acid, but it did not stimulate that induced by isobutyric acid. Of particular interest is the finding that detached rice leaves treated with fusicoccin showed an increase in levels of proline and a decrease in the pH of the cell sap. A synergistic increase in levels of proline was observed when fusicoccin and abscisic acid applied simultaneously. However, fusicoccin had no effect on the induction of the accumulation of proline by isobutyric acid. Benzyladenine inhibited the induction by abscisic acid of the accumulation of proline, but it did not inhibit the induction by isobutyric acid. It is concluded that the mode of action of abscisic acid in inducing the accumulation of proline differs from that of isobutyric acid. Our results also indicate that factors other than acidification of the cell sap are involved in regulation of the accumulation of proline.

**Key words:** Abscisic acid — Isobutyric acid — Proline accumulation — *Oryza sativa*.

Recent reports have suggested that a decrease in intracellular pH, measured as the pH of the cell sap, is involved in stress-, ABA- and IBA-induced accumulation of proline in wheat coleoptiles and segments of barley leaves (Pesci and Beggagna 1984, 1985, Goring and Plescher 1986). Venekamp (1989) showed that organic acids are the source of drought-induced synthesis of proline in bean plants in the field. Previously, we reported that proline accumulated in detached rice leaves during dark-induced senescence (Wang et al. 1982). More recently, we reported that secretion of protons from detached rice leaves plays a regulatory role in senescence (Chen et al. 1990). Secretion of protons decreases during senescence of detached rice leaves. The present investigation was conducted to compare the effects of ABA and IBA on the levels of proline in detached rice leaves and to test the hypothesis that ABA- and IBA-induced changes in levels of proline might be mediated by changes in intracellular pH, as proposed by Pesci and Beggagna (1985).

Abbreviations: BA, benzyladenine; FC, fusicoccin; IBA, isobutyric acid.

### Materials and Methods

Rice (*Oryza sativa* cv. Taichung Native 1) seedlings were grown as described previously (Kao 1980). Briefly, seedlings were grown on a stainless-steel net floated on half-strength Johnson's modified nutrient solution (Johnson et al. 1975) in a 500-ml beaker. The nutrient solution (pH 4.5) was replaced every three days. Rice seedlings were grown in a greenhouse with natural daylight at 30°C by day and 25°C by night, with 95% humidity.

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 that contained 20 ml of basal medium (1 mM potassium phosphate, pH 5.5) or test solutions. All test solutions were prepared by addition of chemicals of interest to 1 mM potassium phosphate buffer (pH 5.5). Incubations were carried out at 27°C in darkness.

Proline was extracted and its concentration determined by the method of Bates et al. (1973). Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged. The supernatant was treated

with acetic acid and acid ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined. Levels of proline are expressed as  $\mu\text{mol/g}$  fresh weight.

The method of Pesci and Beffagna (1985) was used to measure the pH of the cell sap. Leaf segments were transferred at the end of treatment directly into a syringe and were frozen in liquid nitrogen. After thawing, the cell sap was pressed out from the syringe and its pH was measured.

### Results

Figure 1 shows the changes in levels of proline in detached rice leaves incubated in various concentrations of ABA or IBA in darkness for 8 h. Levels of proline increased with the increase in the concentrations of ABA or IBA. The level of proline was first found to increase in  $10 \mu\text{M}$  ABA, but only in 1 mM IBA. The lowest concentration of ABA to cause increased accumulation of proline was 100 times lower than that of IBA. Figure 1 shows the changes in the pH of the cell sap of detached leaves incubated in various concentrations of ABA and IBA in the dark for 8 h. The pH of the cell sap decreased with increases in the concentration of IBA. The pH of the cell sap also decreased during incubation in 1 mM IBA. How-

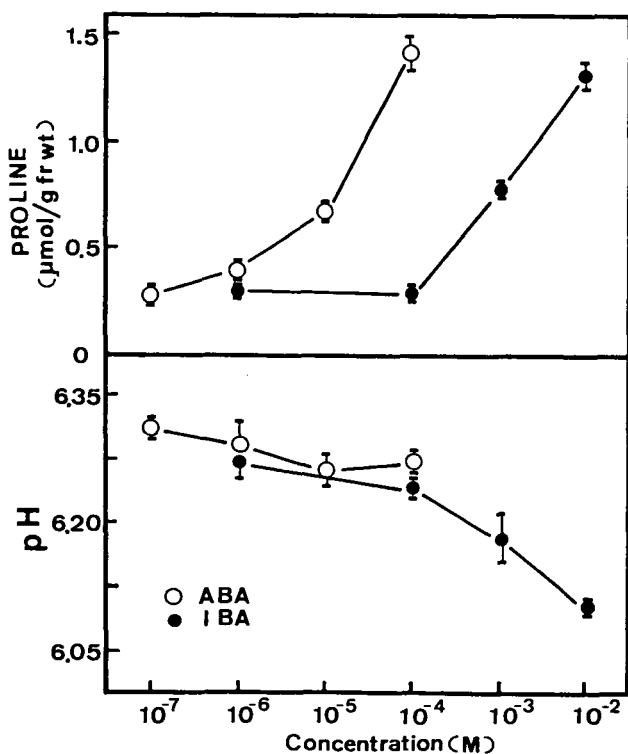


Fig. 1 Effects of various concentrations of ABA and IBA on levels of proline and pH of the cell sap. Detached rice leaves were incubated in darkness for 8 h. Bars represent SE; 3 replicates.

Table 1 Effects of KCl on ABA- and IBA-induced accumulation of proline

Treatment	Proline level ( $\mu\text{mol/g fr wt}$ )
None	$0.25 \pm 0.01$
ABA, $10 \mu\text{M}$	$0.48 \pm 0.04$
ABA, $10 \mu\text{M}$ + KCl, 50 mM	$0.60 \pm 0.04$
IBA, 10 mM	$1.01 \pm 0.09$
IBA, 10 mM + KCl, 50 mM	$1.06 \pm 0.04$

Detached rice leaves were incubated in the dark for 8 h in either ABA or IBA with or without KCl (mean  $\pm$  SE; 4 replicates).

ever, the pH was not significantly affected by addition of ABA at the concentration from  $0.1 \mu\text{M}$  to  $100 \mu\text{M}$ . Clearly, the accumulation of proline induced by IBA, and not that induced by ABA, was associated with acidification of the cell sap. Since leaf segments treated with  $100 \mu\text{M}$  ABA showed slight signs of wilting,  $10 \mu\text{M}$  ABA was adopted for subsequent experiments.

The increase in levels of proline induced by ABA in detached barley leaves was stimulated by potassium chloride (Pesci 1987, 1988, Pesci and Beffagna 1986). The effects of potassium chloride on ABA- and IBA-induced accumulation of proline in detached rice leaves were, therefore, compared. Table 1 shows that potassium chloride at 50 mM stimulated the ABA-induced accumulation of proline, but it did not stimulate the IBA-induced accumulation of proline. These results are in agreement with those observed in

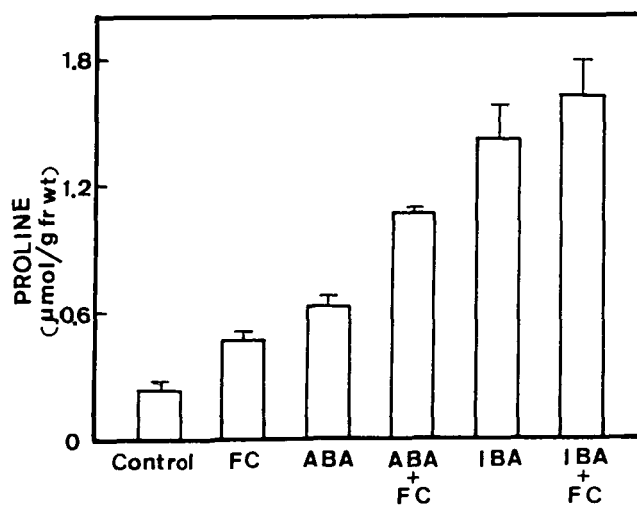


Fig. 2 Effects of fusicoccin (FC) on levels of proline and of FC on ABA- and IBA-induced accumulation of proline. Detached rice leaves were incubated in the dark for 8 h in ABA ( $10 \mu\text{M}$ ), IBA (10 mM), FC ( $10 \mu\text{M}$ ), FC ( $10 \mu\text{M}$ ) + ABA ( $10 \mu\text{M}$ ), FC ( $10 \mu\text{M}$ ) + IBA (10 mM). Bars represent SE; 3 replicates.

**Table 2** Effects of ABA or IBA with or without fusicoccin (FC) and of FC alone on the pH of the cell sap

Treatment	pH of the cell sap
Control	6.20±0.01
FC	6.07±0.01
ABA	6.20±0.02
FC + ABA	6.08±0.02
IBA	6.02±0.01
FC + IBA	6.05±0.02

Detached rice leaves were incubated in the dark for 8 h in ABA (10  $\mu$ M), IBA (10 mM), FC (10  $\mu$ M), FC (10  $\mu$ M) + ABA (10 mM), or FC (10  $\mu$ M) + IBA (10 mM) (mean  $\pm$  SE; 3 replicates).

the barley leaf system (Pesci 1987, 1988, Pesci and Beffagna 1986). However, the enhancement by potassium chloride on the ABA-induced accumulation of proline in detached rice leaves was much lower than that in the barley system.

The phytotoxin fusicoccin (FC) has been shown to stimulate active export of protons (Marre 1979). To our surprise, FC at 10  $\mu$ M caused not only an increase in levels of proline level but also a decrease in the pH of the cell sap in our system (Fig. 2 and Table 2). Similar results were observed when the concentration of FC was only 1  $\mu$ M (data not shown). Figure 2 and Table 2 show the effects of IBA and ABA in the absence and presence of FC on levels of proline and on the pH of the cell sap, respectively. IBA-induced accumulation of proline was clearly associated with a decrease in the intracellular pH. However, such was not

**Table 3** Effects of ABA or IBA with or without benzyladenine (BA) and of BA alone on the pH of the cell sap

Treatment	pH of the cell sap
Control	6.32±0.02
BA	6.31±0.02
ABA	6.36±0.04
BA + ABA	6.28±0.02
IBA	6.14±0.03
BA + IBA	6.17±0.04

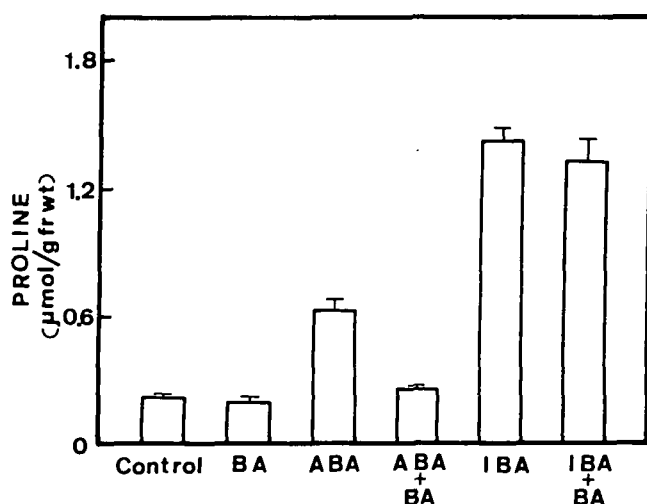
Detached rice leaves were incubated in the dark for 8 h in ABA (10  $\mu$ M), IBA (10 mM), BA (10  $\mu$ M), BA (10  $\mu$ M) + ABA (10  $\mu$ M), or BA (10  $\mu$ M) + IBA (10 mM) (mean  $\pm$  SE; 3 replicates).

the case in ABA-treated segments of rice leaves. FC stimulated the ABA-induced accumulation of proline. However, FC had no effect on the IBA-induced accumulation of proline. A synergistic effect on the increase in levels of proline was observed when FC and ABA were applied together. No difference was observed between the pH of cell sap of detached rice leaves treated with FC alone and the pH of detached leaves treated with FC and ABA together.

Kinetin has been shown to reduce the extent of the accumulation of proline induced by ABA (Eder and Huber 1977). More recently, it was reported that benzyladenine (BA) inhibited the accumulation of proline in ABA-treated segments of barley leaves (Stewart et al. 1986). We found that BA did inhibit the ABA-induced accumulation of proline in detached rice leaves, but it did not inhibit the IBA-induced accumulation of proline (Fig. 3). No difference was observed between the pH of the cell sap of detached rice leaves treated with BA alone and the pH of those treated with BA and ABA together (Table 3).

### Discussion

The mechanism of the ABA-induced accumulation of proline in detached rice leaves differs from that of the IBA-induced accumulation of proline. This conclusion is based on the observations that (a) the lowest concentration of ABA that induces the accumulation of proline was 100 times lower than that of IBA; (b) potassium chloride stimulated the accumulation of proline that was induced by ABA, but it did not stimulate the IBA-induced accumulation of proline; (c) a synergistic increase in levels of proline was found when FC and ABA were applied together, but FC had no effect on the IBA-induced accumulation of proline; (d) BA did inhibit the accumulation of proline by ABA, but it did not inhibit that induced by IBA; (e) the accumulation of proline induced by IBA was associated with acidification of the cell sap, whereas ABA increased levels of proline without any decrease in the pH of the cell sap.



**Fig. 3** Effects of benzyladenine (BA) on levels of proline and of BA on ABA- and IBA-induced accumulation of proline. Detached rice leaves were incubated in the dark for 8 h in ABA (10  $\mu$ M), IBA (10 mM), BA (10  $\mu$ M), BA (10  $\mu$ M) + ABA (10  $\mu$ M), or BA (10  $\mu$ M) + IBA (10 mM). Bars represent SE; 3 replicates.

It is apparent that the effect of ABA is a "hormonal" effect (ABA is effective at very low concentrations; it acts synergistically with FC and BA; the increase in levels of proline is relatively independent of the pH of the cell sap), whereas the effect of IBA can be explained by the decrease of the pH of the cell sap.

Of particular interest is the finding that FC increased levels of proline in detached rice leaves. Although FC is known to promote secretion of protons from detached rice leaves (Chen and Kao 1988), FC was observed to decrease the pH of the cell sap. The decrease in the pH of the cell sap after application of FC has been reported by other investigators (Bertel and Felle 1985, Brummer et al. 1985, Hager and Moser 1985, Roberts et al. 1981). FC is a diacetate ester and, according to Hager and Moser (1985), it can be hydrolyzed like naphthylacetate and other carboxyester compounds. Such hydrolysis might explain why FC decreases the pH of the cell sap. Apparently, the accumulation of proline induced by FC is associated with a decrease in the pH of the cell sap.

Pesci and Beffagna (1985) reported that FC completely suppressed the ABA-induced increase in levels of proline during a 7-h treatment period. In contrast to their results, our data indicate that FC promotes the ABA-induced accumulation of proline in detached rice leaves. ABA- and IBA-induced accumulation of proline has been proposed to be mediated by acidification of the cell sap (Pesci and Beffagna 1985). However, our results demonstrate that the accumulation of proline that is induced by IBA, but not by ABA, is associated with acidification of the cell sap. It appears that, in detached rice leaves, factors apart from acidification of the cell sap may also be involved in regulating the accumulation of proline.

At present, it remains unclear whether acidification of the cell sap regulates the accumulation of proline through stimulation of the synthesis of proline, reduction in the utilization of proline, or both. Further studies should provide valuable information on the mechanism of accumulation of proline.

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