Bot. Bull. Academia Sinica (1990) **31:** 189-195

Senescence of rice leaves XXII. Changes of acid inorganic pyrophosphatase during senescence

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(*Received February 27, 1990; Accepted March 21, 1990*)

Abstract. The changes of acid inorganic pyrophosphatase (acid-IPPase) activity in detached and intact leaves of rice seedlings during senescence were investigated. Acid-IPPase activity increased during dark-induced senescence of detached leaves. Benzyladenine (BA), which retarded senescence of detached leaves in the dark, suppressed the increase of acid-IPPase activity. Senescence of detached leaves in the light was promoted and acid-IPPase activity was increased as the concentration of abscisic acid increased. However, light, which retarded dark-induced senescence of detached leaves, promoted the increase of acid-IPPase activity. Furthermore, acid-IPPase activity remained unchanged during senescence of intact leaves. Hence, it is unlikely that the increase of acid-IPPase activity is of functional significance during senescence of rice leaves. The increase of acid-IPPase activity in dark-, light- and ABA-treated detached leaves was inhibited by cycloheximide, but not by cordycepin, suggesting that the increase activity is owed to de novo synthesis of this enzyme. The Vmax of acid-IPPase extracted from dark-induced senescing detached leaves was much higher than that from freshly excised leaves. The increase of acid-IPPase activity from dark-induced senescing detached leaves does not seem to be due to the occurrence of an activator during senescence.

Key words: Abscisic acid; Acid inorganic pyrophosphatase; Benzyladenine; Light; Leaf senescence; *Oryza sativa.*

Introduction

Acid-IPPase (EC 3.6.1.1) occurs pervasively in higher plants (Naganna *et al.*, 1955a). It has been suggested that acid-IPPase participates in the catabolic processes of leaves (Naganna and Sripathi, 1954; Rauser, 1971). Senescence involves a predominance of the catabolic over the anabolic processes. It has been shown that respiration measured by either O_2 uptake or CO_2 production increased steadily with time in detached leaves (Kao, 1985; Satler and Thimann, 1983; Udvardy and Horvath, 1964). Studies using detached oat and rice leaves also demonstrated that senescence was an active process involving the participation of O_2 (Kao, 1987; Martin and Thimann, 1972). Any actively respiring tissue requires a supply of adequate amounts of NAD and other coenzymes to participate in the oxido-reduction process. The biosynthesis of NAD has been postulated to be mediated via ATP with nicotinic acid as the precursor (Preiss and Handler, 1957; Waygood *et al.*, 1968). Using nicotinamide as the precursor, NAD is also synthesized through other pathways (Claykin, 1967; Kaplan, 1960). Some of the steps in these pathways are equilibrium reaction and involve

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[2] **Abbreviation:** ABA, abscisic acid; acid-IPPase, acid inorganic pyrophosphatase; BA, benzyladenine; Chl, chlorophyll.

the release of pyrophosphate. The enzymatic hydrolysis of the released pyrophosphate would shift the equilibrium towards the synthesis of NAD. Kar and Mishra (1975) suggested that acid-IPPase activity was associated with NAD production in senescing leaves. Furthermore, NAD has been shown to accelerate the senescence of the *Elodea* leaves (Waygood *et al.*, 1985; Yoshida, 1961). It seems that the increase of acid-IPPase activity is to be expected during senescence of leaves. However, there are several conflicting reports on changes in acid-IPPase activity during senescence of attached and detached leaves. Kar and Mishra (1975) reported that acid-IPPase activity increased in both detached and attached rice leaves during senescence, whereas other investigators provided evidence to show that acid-IPPase activity declined with plant age in attached leaves and cotyledons (Naganna and Sripathi, 1954; Rauser, 1971).

The objectives of the present work are (1) to determine the activity of acid-IPPase in detached and intact leaves of rice seedlings during senescence and (2) to elucidate the regulation of the increase of acid-IPPase activity in detached rice leaves.

Materials and Methods

Rice (*Oryza sativa* cv. Taichung Native 1) seedlings were grown as previously described (Kao, 1980). Briefly, seedlings were planted on a stainless net floating on half-strength Johson'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 day light at 30°C day/25°C night and humidity 95%. In experiments with intact leaves, leaf samples (3 cm from tip) were collected from the third leaves of seedlings at 12, 15, 19, 26 and 32 days after planting. For the experiments with detached leaves, the apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. A group of 10 segments was floated on a Petri dish containing 10 ml of distilled water or test solution. Incubation was carried out at 27°C in darkness or the light (16.7 Wm^{-2}) provided by fluorescent lamps.

Leaf segments weighing about 0.4 g were homogenized in a prechilled mortar and pestle with 6 ml cold 50 mM Tris-maleate buffer (pH 7.0) at 4°C. The resulting supernatants were used directly for the assay of acid-IPPase activity. The 5 ml assay mixture contained of 25 μmol pyrophosphate; 350 μmol citrate buffer, pH 5.5; and 0.5 ml enzyme extract. After incubation at 37°C for 10 min, the reaction was stopped by adding 1 ml 20% perchloric acid. After centrifugation, an aliquot of the clear supernatant was taken for inorganic phosphorus determination (Yoshida *et al.*, 1972). To 0.5 ml of the supernatant, 1 ml of 2 N HNO_3, 0.5 ml of molybdate-vanadate solution [5% (w / v) ammonium molybdate plus 0.25% ammonium vanadate in 1 N HNO_3] and 3 ml of distilled water were added. After 20 min, an absorbance at 420 nm was measured. The amount of phosphorus as calculated using a standard curve. Acid-IPPase activity was calculated based on the net amount of phosphorus released during incubation. One unit of enzyme activity is defined as the amount of enzyme which liberates 1 μmol of inorganic phosphorus per min under the assay condition described. Protein content in the supernatant was measured by the method of Lowry *et al.* (1951).

Chlorophyll (Chl) of 10 leaf segments was extracted by boiling in 80% ethanol for 20 min and the final volume of the solution was brought to 10 ml. The Chl content was obtained by reading the absorbance (A) at 665 nm (Kao, 1980).

Results

The senescence of leaves was followed by measuring the decrease of Chl. Figure 1 shows the time courses of Chl level and acid-IPPase activities of

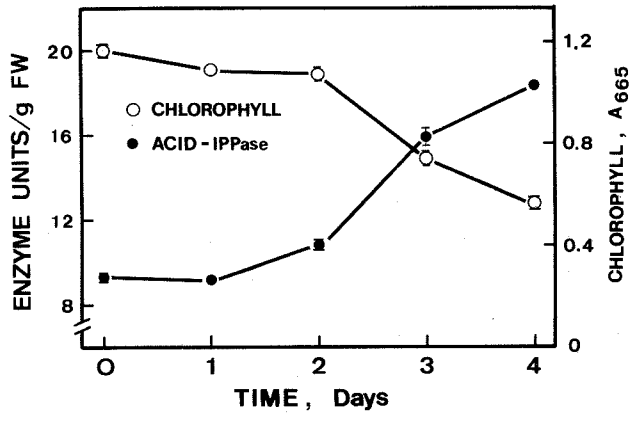

Fig. 1. Chl content and acid-IPPase activity in detached rice leaves during dark-induced senescence. Vertical bars represent ± SE.

detached leaves floating on water in darkness. A decrease of Chl level was evident 3 days after incubation. Acid-IPPase activity remained unchanged at 1 day after incubation and then increased subsequently.

It has long been recognized that cytokinins are effective in retarding the senescence of most, if not all, leaves. The effect of cytokinins on retarding senescence is species- or variety-specific; for the variety used in this investigation, benzyladenine (BA) has been found to be the most active cytokinin in retarding senescence in the dark (Kao, 1978). The effect of BA on senescence and acid-IPPase activity is presented in Fig. 2. BA effectively retarded leaf senescence and suppressed the elevation of acid-IPPase activity in the dark. This result is consistent with that reported by Mishra *et al.* (1973).

Among the known promoters of senescence, abscisic acid (ABA) has been studied most widely. Gepstein and Thimann (1980) claimed that ABA is an endogenous promoter of leaf senescence. Figure 3 shows the effect of ABA on senescence and acid-IPPase activity in the light. Senescence was promoted and acid-IPPase activity increased significantly as the concentration of ABA increased.

Light is known to retard senescence of rice leaves (Hurng *et al.*, 1986). If the increase in acid-IPPase activity is of functional importance during the senescence of detached rice leaves, light is expected to suppress the increase of acid-IPPase activity. Although light indeed retarded senescence as shown in Table 1, it also markedly increased acid-IPPase activity. This result is in disagreement with that of Singh and Mishra (1975), who reported that red light retarded senescence and suppressed the increase of acid-IPPase activity of detached rice leaves.

Chl level and acid-IPPase activity in the third leaf from 12- to 32-day-old seedlings are shown in Fig. 4. Chl level in intact leaves decreased with the increasing of age. However, acid-IPPase activity remained unchanged during senescence of intact leaves. This is unexpected, since acid-IPPase activity in intact leaves is generally thought to be either increased or decreased during senescence (Kar and Mishra, 1975; Naganna and Sripathi, 1954; Rauser, 1971).

The effect of cycloheximide and cordycepin on dark-, light- and ABA-induced increase in acid-IPPase activity was presented in Table 2. The increase in acid-IPPase activity that occurred in dark-, light- and ABA

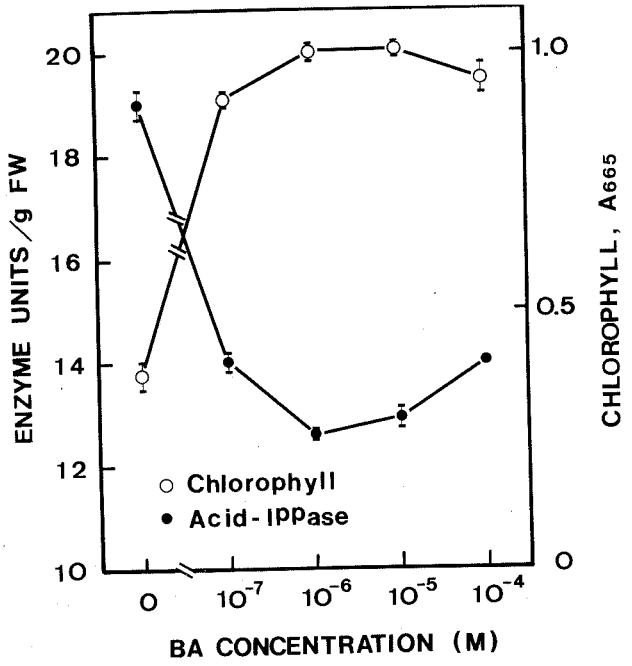

Fig. 2. Effect of BA concentrations on Chl content and acid-IPPase activity of detached rice leaves. Detached rice leaves were incubated in darkness for 4 days. Vertical bars represent ± SE.

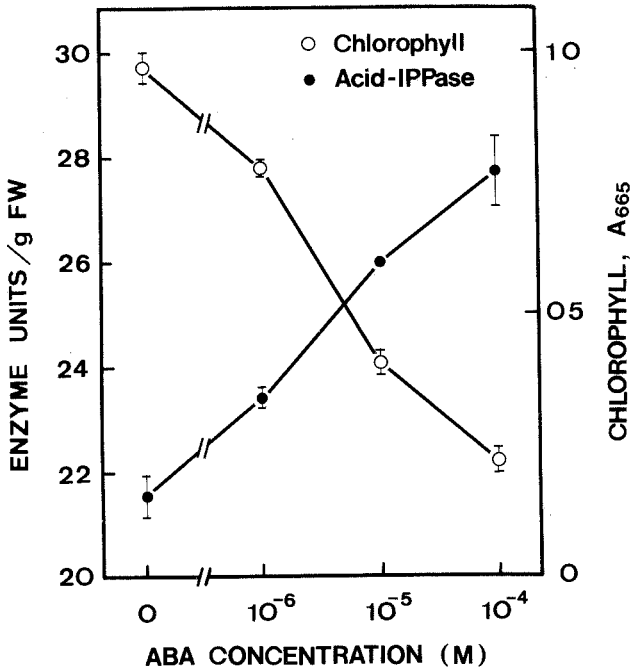

Fig. 3. Effects of ABA concentrations on Chl content and acid-IPPase activity of detached rice leaves. Detached rice leaves were incubated in the light for 3.5 days. Vertical bars represent ± SE.

Table 1. *Effects of light on Chl content and acid-IPPase activity of detached rice leaves*

Treatment	Chl (A665)	Enzyme units/g FW
Initial	1.15±0.02	10.1±0.1
4 days in dark	0.66±0.01	18.3±0.2
4 days in light	0.91±0.01	21.6±0.3

Table 2. *Effects of cycloheximide and cordycepin on acid-IPPase activity. Cycloheximide, cordycepin and ABA concentrations are 20 mg/l, 100 μM and 10 μM, respectively. In Experiment II, under light condition, leaf segments were first incubated for 12 h in distilled water, cycloheximide and cordycepin, respectively, and then transferred to ABA for another 24 h. Light (16.7 Wm^{-2}) was provided with fluorescent lamps*

Treatment	Enzyme units/g FW
Experiment I	
Zero time	9.5±0.1
48 h Water, Dark	14.0±0.1
48 h Cycloheximide, Dark	11.1±0.3
48 h Cordycepin, Dark	14.2±0.1
48 h Water, Light	15.6±0.5
48 h Cycloheximide, Light	10.3±0.1
48 h Cordycepin, Light	16.1±0.2
Experiment II	
Zero time	9.7
12 h water+24 h ABA	14.0±0.2
12 h Cycloheximide+24 h ABA	8.5±0.1
12 h Cordycepin+24 h ABA	13.6±0.2

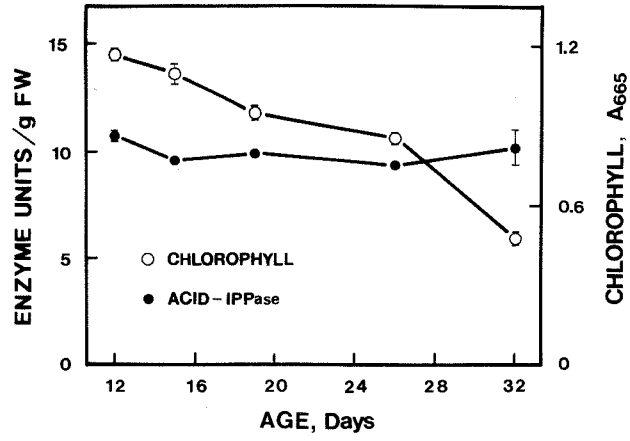

Fig. 4. Chl content and acid-IPPase activity in intact leaves of rice seedling during senescence. Vertical bars represent ± SE.

Table 3. *Acid-IPPase activity in crude extract from rice leaves. Enzyme extracts were prepared from freshly excised leaves (0 day extract) and excised leaves incubated in darkness for 4 days (4 days extract), respectively. The acid-IPPase activity in mixed extract (0 day + 4 days, 1:1) was compared with the theoretical activity calculated from the unmixed extracts*

	Enzyme units/g FW
0 day extract	10.6±0.1
4 days extract	20.3±0.1
0 day extract+4 days extract	15.7±0.5
Theoretical activity	15.5±0.4

-treated leaf tissue was inhibited by cycloheximide. In fact, cycloheximide reduced the level of acid-IPPase activity to about the same level as in zero time controls. In contrast to the effect of cycloheximide, cordycepin did not inhibit the increase of acid-IPPase activity.

The increase of acid-IPPase activity in dark-induced senescing excised leaves is possibly due to the occurrence of factors needed for activation of this enzyme. This possibility was tested by mixing enzyme extract of freshly excised leaves with that of leaves incubated in the dark for 4 days (1:1) and the enzyme activity in the mixed extract was then assayed. It was found that acid-IPPase activity in mixed extract was similar to that calculated from unmixed extract, which was the theoretical activity presented in Table 3. Thus, it seems unlikely that the increase of acid-IPPase activity in dark-induced senescing excised leaves is due to the occurrence of an activator.

Figure 5 compares the Km and Vmax of acid-IPPase extracted from freshly excised and dark-induced senescing leaves. The Vmax of acid-IPPase

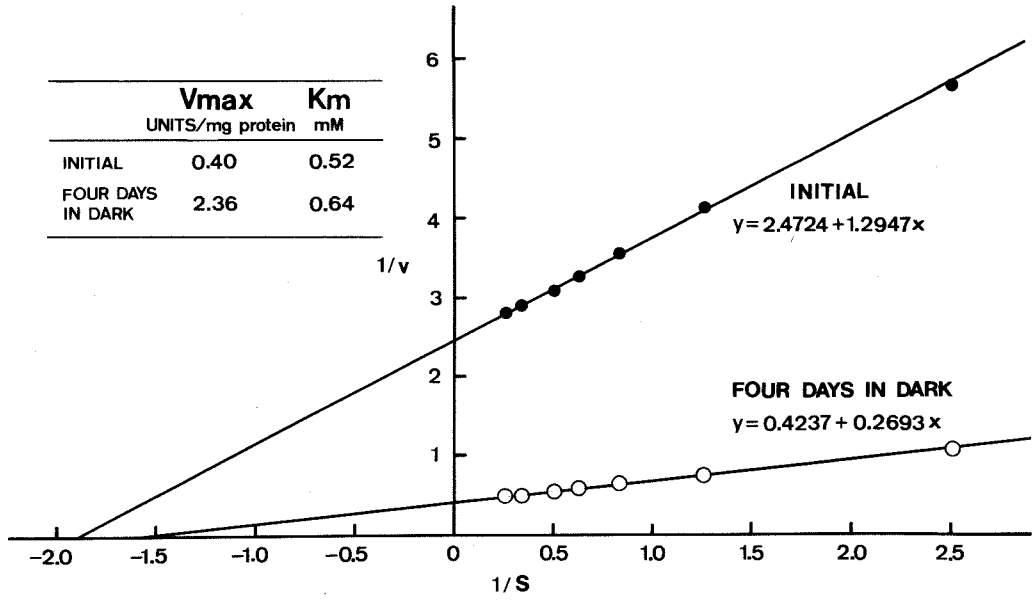

Fig. 5. Lineweaver-Burke plots for acid-IPPase extracted from freshly excised leaves and excised leaves incubated in darkness for 4 days.

extracted from senescing leaves was much higher than that from freshly excised leaves. However, only slight increase of the Km of acid-IPPase extracted from senescing leaves was observed, suggesting that the increase of enzyme activity in dark-induced senescing leaves was resulted from the increase of Vmax.

Discussion

Acid-IPPase was present in detached and intact rice leaves throughout senescence. It has been suggested that acid-IPPase is associated with catabolic processe of leaves (Naganna and Sripathi, 1954). Results of the present investigation do not support this view. This conclusion was based on the observations that (1) light, which retarded senescence, promoted the increase of acid-IPPase activity in detahced rice leaves, and (2) acid-IPPase activity remained unchanged in intact leaves during senescence. It seems unlikely that the increase in acid-IPPase activity is of functional significance during rice leaf senescence. The fact that the changes of acid-IPPase activity in detached leaves differ from those in intact leave during senescence implies that the senescence of leaves attached to plant is not essentially the same as that of excised leaves. Thus, the increase in acid-IPPase activity could not be used as a reliable indicator of rice leaf senescence.

The activity of acid-IPPase in dark-induced senescing detached leaves was inhibited by cycloheximide, but not by cordycepin. Although the specificity of cycloheximide and cordycepin is questionable, results give indication that the increased activity is possibly owed to de novo synthesis of this enzyme, perhaps at the level of translation. Another possibility of the increase in acid-IPPase activity is due to an accumulation of this enzyme as a result of a specific decrease in the rate degradation. Some alteration to the newly synthesized protein might occur, since the Vmax of acid-IPPase extracted from dark-induced senescing detached leaves was significantly increased (about 6 -fold increase). Although we do not know the mechanism of high Vmax of acid-IPPase in dark-induced senescing excised leaves, our data seem to support that the high Vmax is unlikely due to the occurrence of an activator during senescence.

Acknowledgements. This work was supported by the National Science Council, Republic of China under Grant NSC78-0409-002-43.

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水稻葉片老化之研究
(二十二)老化過程酸性焦磷酸鹽水解酵素活性之變化

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本文主要探討台中在來一號水稻幼苗切離與完整葉片，在老化過程酸性焦磷酸鹽水解酵素(IPPase)活性之變化。IPPase 活性變化之可能機制亦一併加以探討。切離葉片在黑暗中老化時，IPPase活性增加。BA 可延緩切離葉片在黑暗中之老化，同時亦可降低 IPPase 活性之增加；ABA 可加速老化，亦可促進 IPPase 活性之增加。然而，光線雖可延緩切離葉片之老化，確促進 IPPase 活性之增加。再者，完整葉片老化過程中，IPPase 活性無明顯之變化。此似乎說明 IPPase 活性之增加，不能作爲水稻葉片老化之指標。Cycloheximide 抑制黑暗、光線與 ABA 處理所誘導之 IPPase 活性之增加，顯示 IPPase 活性之增加，很可能係由於酵素之重新合成。與剛切離之葉片比較，黑暗處理 4 天之老化葉片，其 IPPase 之 Vmax 確顯著的增加，但此 Vmax 之增加，似乎不可能是由於某種活化物質之形成所造成。