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Senescence of rice leaves XXI. Changes of Mg^{2+}-dependent alkaline inorganic pyrophosphatase activity during senescence

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Abstract. The changes of Mg^{2+}-dependent alkaline inorganic pyrophosphatase (Mg^{2+}-IPPase) activity in detached and intact leaves of rice seedlings (*Oryza sativa* L. cv. Taichung Native 1) during senescence were investigated. Mg^{2+}-IPPase activities in freshly excised leaves and excised leaves which had been incubated in darkness for 4 days were monitored with pH optima of 8-9 and 8.6, respectively. Calcium strongly inhibited Mg^{2+}-IPPase activity. Both pyrophosphate and tripolyphosphate were effective substrates for Mg^{2+}-IPPase extracted from freshly excised leaves and senescent excised leaves. Vanadate at 50 and 100 μM was effective, though slightly, in inhibiting Mg^{2+}-IPPase activity. Mg^{2+}-IPPase was present in detached and intact leaves throughout senescence. There seems to be a direct correlation between senescence and decrease of Mg^{2+}-IPPase activity in detached leaves, but not in intact leaves. It seems unlikely that Mg^{2+}-IPPase activity can be used as indicator of leaf senescence or to indicate biosynthetic capacity of leaves.

Key words: Abscisic acid; Benzyladenine; Leaf senescence; Light; Mg^{2+}-dependent alkaline inorganic pyrophosphatase; *Oryza sativa*.

Introduction

Inorganic pyrophosphate is produced as a by-product in several biosynthetic reactions, such as activation of amino acids, polymerization of nucleic acids and synthesis of coenzymes and sugar nucleotides. Both acid and Mg^{2+}-dependent alkaline inorganic pyrophosphatases (acid IPPase and Mg^{2+}-IPPase, EC 3.6.1.1) have been reported in higher plants (Bucke, 1970; Naganna *et al.*, 1955a; Patra and Mishra, 1979). Mg^{2+}-IPPase is probably the enzyme involved in the biosynthetic reactions by degrading inorganic pyrophosphate into orthophosphate (Naganna *et al.*, 1955a; Naganna and Sripathi, 1954; Rauser, 1971; Simmons and Butler, 1969). Theoretically, Mg^{2+}-IPPase activity should decrease during senescence. However, there are conflicting reports on the changes of Mg^{2+}-IPPase activity during leaf senescence. Kisban *et al.* (1964) demonstrated that Mg^{2+}-IPPase activity increased during senescence of detached leaves; but other workers reported that Mg^{2+}-IPPase activity decreaed in attached and detached leaves during senescence (Kar and Mishra, 1975; Patra and Mishra, 1979; Rauser, 1971). The present study was conducted to determine the changes of Mg^{2+}-IPPase activity during senescence of both detached and intact leaves of rice seedlings.

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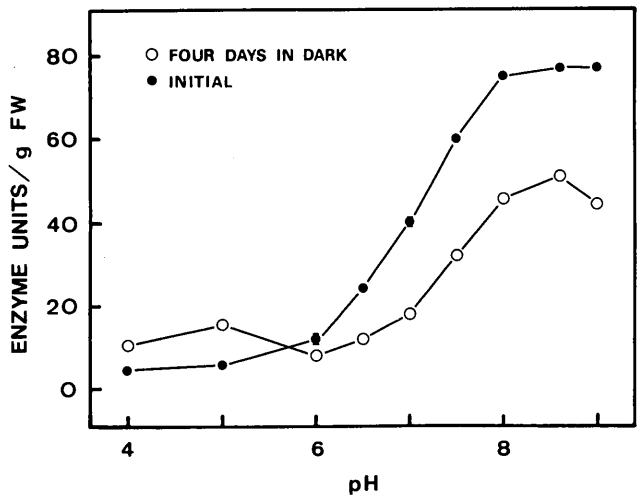

Fig. 1. Effect of pH on Mg^{2+}-IPPase activities in crude extract of freshly excised leaves (initial) and excised leaves which had been incubated in darkness for 4 days (4 days in dark). Aliquots of enzyme extract were incubated with buffers (33 mM final concentration) at various pH levels. The buffers for pH 4.0-5.5, pH 6.0-7.5 and pH 8.0-9.0 are citrate, Tris maleate and Tris, respectively

Materials and Methods

Rice (*Oryza sativa* cv. Taichung Native 1) seedlings were grown as previously described (Kao, 1980). 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 experiment 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 provided with fluorescent lamps (16.7 Wm^{-2}).

Mg^{2+}-IPPase was extracted and assayed following the method of Rauser (1971) with some modification. Leaf segments weighing about 0.4 g were homogenized in prechilled mortar and pestle with 6 ml cold 50 mM Tris-maleate buffer (pH 7.0) at 4°C. The homogenate was centrifuged at 5,000 g for 30 min at 4°C. The resulting clear supernatant fractions were used directly for enzyme assay. The 5 ml of the assay mixture consisted of 25 μmol tetrasodium pyrophosphate; 100 μmol $MgCl_2$; 165 μmol Tris buffer, pH 8.6; and 0.2 ml enzyme extract. After incubation at 37°C for 10 min, the reaction was stopped by adding 1 ml 20% perchloric acid. After centrifugation at 3,500 g for 20 min, an aliquot of the clear supernatant was taken for inorganic phosphorus determination (Yoshida *et al.*, 1972). Mg^{2+}-IPPase activity was calculated on the basis of the net 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.

Chlorophyll was extracted and determined as described previously (Kao, 1980) and expressed as A_{665} per 10 segments.

Results

General Properties of Mg^{2+}-IPPase

The pH profiles of Mg^{2+}-IPPase activity of the crude extracts from freshly excised leaves and excised leaves which had been incubated in darkness for 4 days (senescent detached leaves) are shown in Fig. 1. The pH optimum for freshly excised leaves ranged from 8.0 and 9.0, whereas that for senescent detached leaves was 8.6. In the pH range from 6.5 to 9.0, Mg^{2+}-IPPase activities in senescent detached leaves were lower than those in freshly excised leaves. However, at pH 4.0 and pH 5.0, Mg^{2+}-IPPase activities in senescent detached leaves were higher than those in freshly excised leaves. Calcium inhibition of Mg^{2+}-IPPase activity from various sources has been conclusively shown and studied in detail by several workers (Gavalas and Manetas, 1980; Moe and Butler, 1972; Naganna *et al.*, 1955b; and literature therein). When calcium was included in the assay mixture, it was found that calcium inhibition of Mg^{2+}-IPPase activity only observed at pH higher than 6.0 (Fig. 2). Since calcium does not inhibit acid IPPase (unpublished data), it seems that Mg^{2+}-IPPase activities observed at low pH in the present study turn out to be acid IPPase activities.

In the presence of 20 mM $MgCl_2$, the activity of Mg^{2+}-IPPase was found to be a function of pyrophosphate concentration (Fig. 3). The optimum pyrophosphate concentration was 5 mM. The hydrolytic activities of a series of potential substrates by the crude extract from freshly excised and senescent detached leaves are presented in Table 1. Though pyrophosphate was the best substrate among the substrates tested, tripolyphosphate was also an effective substrate, indicating that Mg^{2+}-IPPase in rice-leaf extract did not

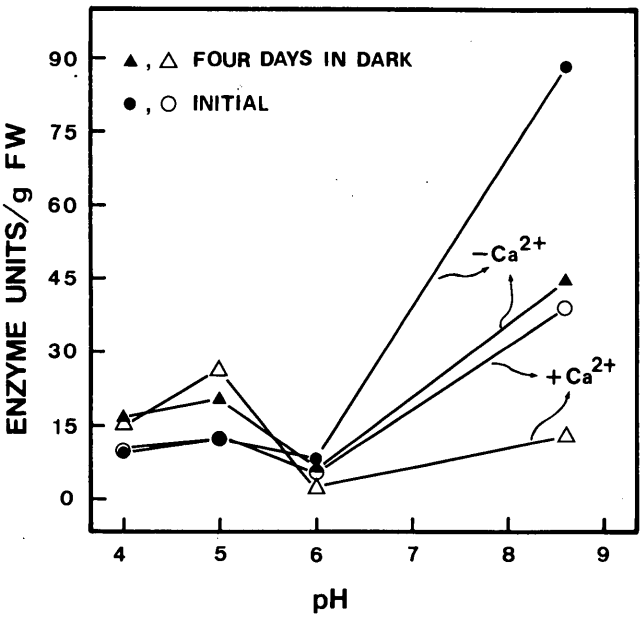

Fig. 2. Effect of calcium on the Mg^{2+}-IPPase activity at different pH in rice-leaf extracts. Enzyme extracts were prepared from freshly excised leaves (initial) and excised leaves which had been incubated in darkness for 4 days (4 days in dark).

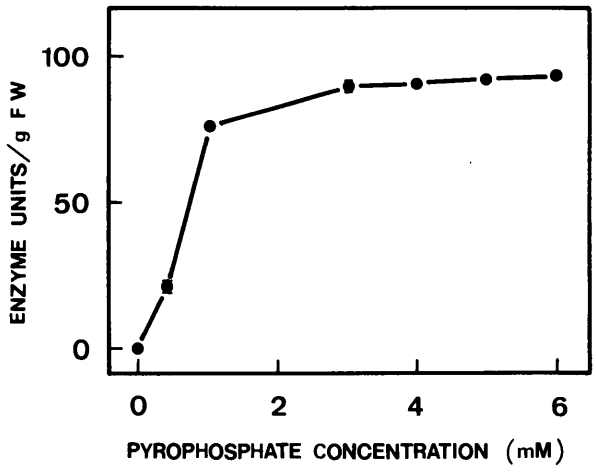

Fig. 3. Effect of pyrophosphate concentration on Mg^{2+}-IPPase activity in rice-leaf extract. Enzyme extract was prepared from freshly excised leaves.

seem to be substrate specific.

Table 2 summarizes the effect of inhibitors of ATPases and phosphatase on Mg^{2+}-IPPase activity. Vanadate has been shown to be a noncompetitive inhibitor of the plasma membrane ATPase (Gallagher and Leonard, 1982). Addition of vanadate (50-100 μM) to the assay mixture, Mg^{2+}-IPPase activity showed 9-18% inhibition. However, Mg^{2+}-IPPase was completely insensitive to molybdate, an inhibitor of phosphatase (Shaw, 1966), and azide, which is a potent inhibitor of mitochondrial type, F_1-ATPase (Bowman *et al.*, 1978).

Table 1. *Effect of different substrates on Mg^{2+}-IPPase activity of rice-leaf extract. Enzyme extract was prepared from freshly excised leaves (initial) and excised leaves which had bee incubated in darkness for 4 days (4 days in dark). The assay mixture contained 5 mM of respective substrates*

Substrate	Enzyme units/g FW	
	Initial	4 days in dark
Sodium pyrophosphate	87.4±2.1	45.1±0.2
Sodium tripolyphosphate	72.7±0.3	36.1±0.3
Adenosine 5'-triphosphate	2.2±0.1	2.5±0.1
Adenosine 5'-diphosphate	2.1±0.1	2.3

Table 2. *Effect of azide, molybdate and vanadate on Mg^{2+}-IPPase activity of rice-leaf extract. Exnyme extract was prepared from freshly excised leaves. The assay mixture contained sodium salts of azide, molybdate and vanadate, respectively*

Concentration (μM)	Enzyme units/g FW		
	Azide	Molybdate	Vanadate
0	105.4±1.6	104.1±0.4	85.8±0.3
10	105.1±0.1	ND*	83.9±1.8
50	106.1±0.9	ND	78.0±1.3
100	103.6±2.1	103.4±1.0	70.5±0.9

*ND, not determined

Changes of Mg^{2+}-IPPase During Senescence of Detached Rice Leaves

The senescence of rice leaves was followed by measuring the decrease of chlorophyll. Figure 4 shows the time courses of chlorophyll level and Mg^{2+}-IPPase activities of detached leaves floating on water in the dark. A decrease of chlorophyll level was evident 2 days after leaf detachment. Mg^{2+}-IPPase activity decreased immediately after excision. The decrease of Mg^{2+}-IPPase activity in dark-induced senescent detached leaves is possibly resulted from the occurrence of inactivator. This possibility was tested by

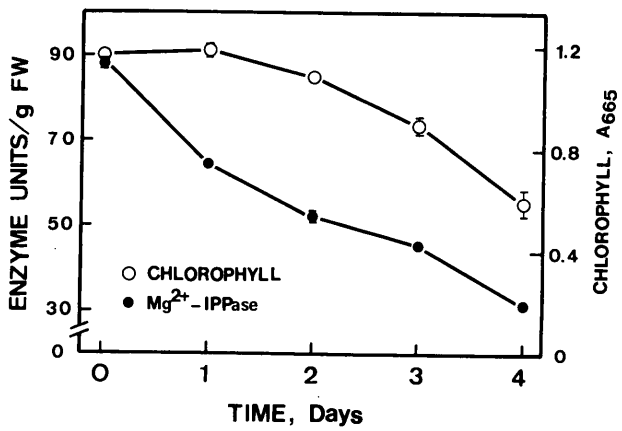

Fig. 4. Chlorophyll content and Mg^{2+}-IPPase activity in detached rice leaves during dark-induced senescence.

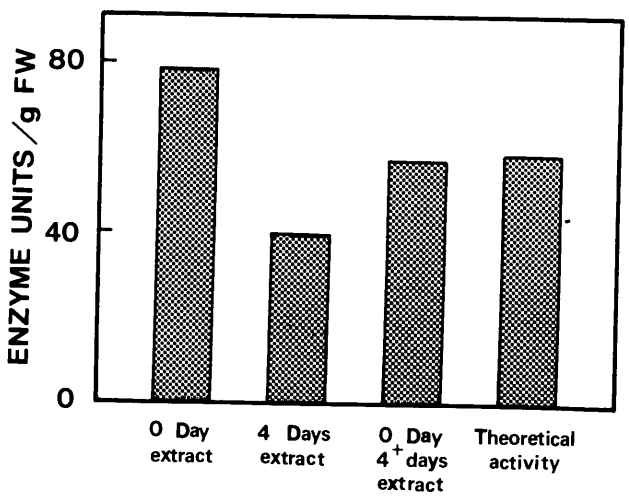

Fig. 5. Mg^{2+}-IPPase activity in the crude extract from rice leaves. Enzyme extracts were prepared from the freshly excised leaves (0 day extract) and excised leaves which had been incubated in darkness for 4 days (4 days extract), respectively. The Mg^{2+}-IPPase activity in mixed extract (1:1) was assayed at 30 min after mixing and was compared with the theoretical activity calculated from unmixed extracts.

mixing enzyme extract of freshly excised leaves with that of detached leaves which had been incubated in darkness for 4 days (1:1). Enzyme activity was assayed at 30 min after mixing. It was found that Mg^{2+}-IPPase activity in mixted extract was similar to that calculated from unmixed extract, which was the theoretical activity in Fig. 5. Thus, it seems unlikely that the decrease of Mg^{2+}-IPPase activity in dark-induced

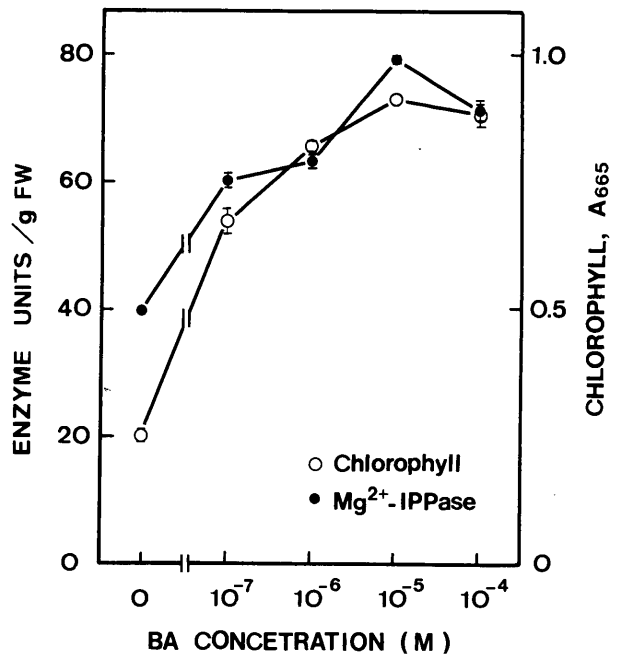

Fig. 6. Effect of BA concentrations on the chlorophyll content and Mg^{2+}-IPPase activity of detached rice leaves. Detached rice leaves were incubated in darkness for 4 days.

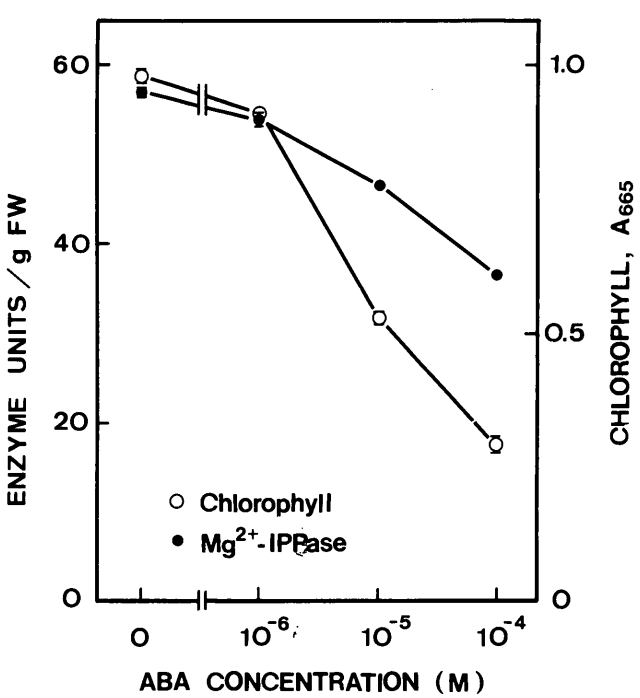

Fig. 7. Effect of ABA concentrations on the chlorophyll content and Mg^{2+}-IPPase activity of detached rice leaves. Datached rice leaves were incubated in the light for 3.5 days.

Table 3. *Effect of light and dark on the chlorophyll content and Mg^{2+}-IPPase activity of detached rice leaves. Chlorophyll content and enzyme activity were determined at 4 days after incubation*

Treatment	Chlorophyll (A_{665}/10 segments)	Enzyme units/g FW
Dark	0.44±0.06	31.8±0.3
Light	0.91±0.03	40.8±0.3

senescent detached leaves is due to the occurrence of inactivator.

The effect of benzyladenine (BA), a synthetic cytokinin, on senescence and Mg^{2+}-IPPase activity is presented in Fig. 6. BA effectively retarded senescene and, on the contrary, it elevated the Mg^{2+}-IPPase activity in the dark. This result is consistent with those have been reported by other authors (Kar and Mishra, 1975; Singh and Mishra, 1975). Figure. 7 shows the effect of abscisic acid (ABA) on the senescence and Mg^{2+}-IPPase activity in the light. Senescence was promoted and Mg^{2+}-IPPase activity was decreased significantly as the concentration of ABA increased.

Light is known to delay senescence of detached rice leaves (Hurng *et al.*, 1986). If the decrease in Mg^{2+}-IPPase activity is of functional significance during senescence of detached rice leaves, light is expected to slow down the decling of Mg^{2+}-IPPase activity. As shown in Table 3, this is indeed the case.

Changes of Mg^{2+}-IPPase During Senescence of Intact Leaves

The level of chlorophyll and Mg^{2+}-IPPase activity in the third leaves from 12- to 32-day-old seedlings are shown in Figure 8. Both Mg^{2+}-IPPase activity and chlorophyll content in intact leaves decreased with increasing age. When 12-day-old greenhouse-grown seedlings were transfered to continuous light or darkness, senescence, as judged by chlorophyll content, of intact leaves was retarded by continuous light (Fig. 9). Surprisingly, the enzyme activity of greenhouse-grown leaves did not decline significantly in the dark, but declined drastically in the continuous light over a period of 4 days. Activity under continuous light condition was slightly higher than that in continuous darkness only at 6 days after transfer of seedlings.

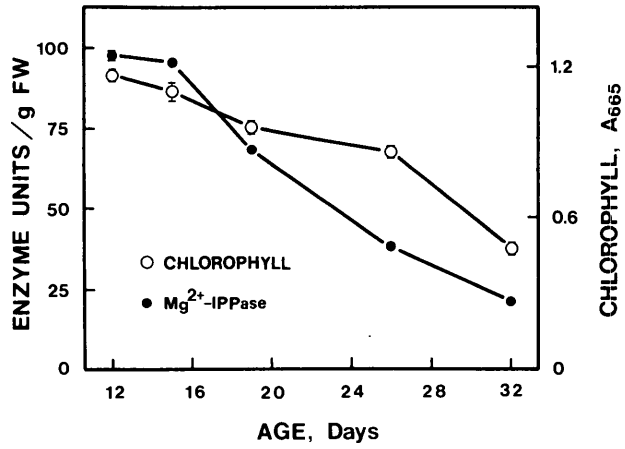

Fig. 8. Chlorophyll content and Mg^{2+}-IPPase activity in intact leaves of rice seedlings during senescence.

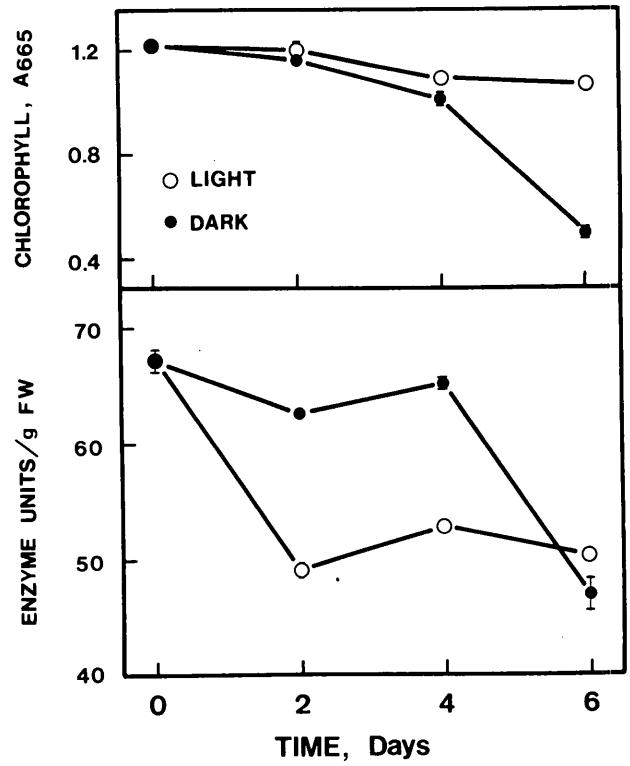

Fig. 9. Effects of light and dark on chlorophyll content and Mg^{2+}-IPPase activity of intact leaves of rice seedlings. Twelve-day-old rice seedlings were transferred to continuous light (16.7 Wm^{-2}) or darkness. Temperature for both light and dark conditons were controlled at 27°C. For enzyme assay and chlorophyll determination, the apical 3-cm portions of the third leaves were collected at 0, 2, 4 and 6 days after transferring seedlings to the light or darkness.

Discussion

Mg^{2+}-IPPase was present in both detached and intact leaves of rice seedlings. The optimum pH and calcium inhibition effect of Mg^{2+}-IPPase in rice-leaf extract are generally in agreement with those from other plant sources (Bucke, 1970; El-Badry and Bassham, 1970; Gavalas and Manetas, 1980; Hara *et al.*, 1980; Krishnan and Gnanam, 1988; Moe and Butler, 1972; Naganna *et al.*, 1955a; Simmons and Butler, 1969). A high specificity for pyrophosphate as substrate has been observed in *Typha* pollens (Hara *et al.*, 1980), sorghum leaves (Krishnan and Gnanam, 1988) and maize leaves (Simmons and Butler, 1969). In addition to pyrophosphate, Mg^{2+}-IPPase in rice-leaf extract also hydrolyzed tripolyphosphate. Of particular interest is the finding that vanadate at the concentration of 50 -100 μM inhibited Mg^{2+}-IPPase activity. Vanadate has been shown to inhibit the plasma membrane ATPase and acid phosphatase activities (Gallagher and Leonard, 1982). Our work further supports the suggestion that vanadate is not a specific inhibitor of the plasma membrane ATPase.

In detached leaf system, the decrease of Mg^{2+} -IPPase activity was found to be associated with the progress of senescence. Treatments such as light and BA application, which retarded senescence, suppressed the decrease of Mg^{2+}-IPPase activity. Furthermore, ABA applied exogenously, which promoted senescence, significantly promoted the decrease of Mg^{2+}-IPPase activity. All these results are in good agreement with those reported by other workers (Kar and Mishra, 1975; Patra and Mishra, 1979; Singh and Mishra, 1975; Rauser, 1971). For intact leaves, Mg^{2+}-IPPase activity also decreased during senescence. However, when greenhouse-grown seedlings were transferred to continuous light or darkness, Mg^{2+}-IPPase activity in intact leaves decreased slightly in darkness but drastically in the light over a period of 4 days, which were not the results we expected if Mg^{2+}-IPPase played a functional role during senescence. Simmons and Butler (1969) also reported that the activity of Mg^{2+}-IPPase of light-grown leaves did not decline in the dark over a period of several days. Clearly, the decrease of Mg^{2+} -IPPase activity may have functional importance during senescence of detached leaves, but not intact leaves, indicating that the senescence of leaves attached to plant is not essentially the same as that of excised leaves. In view of the importance of correlative controls in senescence, it is not surprising that detachment produces some big changes. In conclusion, it seems unlikely that the activity of Mg^{2+}-IPPase can be used to indicate biosynthetic capacity of leaves or as an indicator of leaf senescence as proposed by other workers (Kar and Mishra, 1975; Rauser, 1971).

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水稻葉片老化之研究
(二十一)老化過程鹼性需鎂之焦磷酸鹽水解酵素活性變化

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本文主要探討臺中在來一號水稻幼苗切離與完整葉片老化過程鹼性需鎂之焦磷酸鹽水解酵素(Mg^{2+}-IPPase)活性變化。剛切離之葉片，Mg^{2+}-IPPase 最適 pH 值爲 8-9，而在暗中老化 4 天之葉片，其最適 pH 爲 8.6。Ca^{2+}明顯的抑制 Mg^{2+}-IPPase 活性，焦磷酸鹽與 tripolyphosphate 均可有效的作爲 Mg^{2+}-IPPase 之受質。釩酸鹽(50-100 μM)可顯著抑制 Mg^{2+}-IPPase 活性。不論切離或完整葉片，老化過程中均具有 Mg^{2+}-IPPase 活性。切離葉片老化似乎與 Mg^{2+}-IPPase 活性有明顯之關係，但此種關係並不存在於老化之完整葉片。Mg^{2+}-IPPase 似乎不能作爲葉片老化指標或者用來表示葉片合成反應之能力。