Effects of Gibberellic Acid and Abscisic Acid on Proteolysis of Senescing Leaves from Rice Seedlings*

S. M. Kang · N. J. Kang · J. L. Cho · Z. H. Kim and Y. W. Kwon

老化 水稻幼苗葉의 蛋白質分解에 미치는 GA₃과 ABA의 影響

姜性模** · 姜南埈** · 趙丁來** · 金周玹** · 樸容雄***

ABSTRACT: The effect of gibberellic acid (GAs) and abscisic acid (ABA) on KCl—enhanced proteolysis of senescing leaves of rice (Oryza sativa L. cv. Chilsung) was studied. Emphasis was given to their effects on KCl—enhanced efflux of amino acids and proteinase activity. When treated singly, GAs affected leaf proteolysis little, while ABA increased proteolysis, the rate of amino acid efflux, and ribulose—1,5—bisphosphate carboxylase/oxygenase (Rubisco) —degrading endoproteinase activity. An additive increase in all three parameters mentioned above was observed when leaves were treated with ABA and KCl. No such an additive effect was found when GAs was treated with KCl. Both GAs and ABA helped to alleviate the KCl—suppressed activity of Rubisco-degrading exoproteinases. The additive increase in proteolysis of rice leaves in the presence of both ABA and KCl could thus be ascribed to a further increase in the efflux of protein hydrolyzates and Rubisco-degrading endoproteinase activity.

An increase in proteolysis was accompanied by a decrease in water absorption, and the combined treatment of ABA with KCl resulted in a further reduction of water absorption.

Key word: Rice, Senescing leaf, Proteolysis, GAs, ABA

Leaf senescence, as most commonly measured by the changes in chlorophylls and proteins, is affected by a variety of internal and external conditions, the most notable of the former being the level and/or activity of plant hormones. Generally, cytokinin acts as a retardant and abscisic acid (ABA) as a promoter of rice leaf senescence⁴,⁷,¹²,¹³,¹⁵,¹⁶,²²,²³. The role of gibberellic acid (GAs) in leaf senescence is less clear⁶,⁸,¹⁴,¹⁹. Changes in chlorophylls and proteins have been measured after applying GAs and ABA to intact¹,⁸,²² and detached¹³,¹⁵,¹⁶,²³ rice leaves. However, the results differ between different developmental

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stages and environmental conditions. Few workers reported changes in the activity of proteolytic enzymes to account for the measured changes in proteins\textsuperscript{2,20}.

We have previously reported that NaCl and KCl increase chlorophyll breakdown and proteolysis of senescing rice leaves\textsuperscript{10,11}. The mechanisms involved in the increase in proteolysis include the increased synthesis of proteolytic enzymes and their activity. One of the important regulatory mechanisms which increased proteinase activity was the increased efflux of amino acids from senescing leaves to the incubation media\textsuperscript{10}. The increase in amino acid efflux in the presence of the salts is similar to the effect of ABA on senescing leaves; ABA also increases the leakage and/or exudation of solutes from senescing leaves\textsuperscript{5,15,24,25,26,27}. Since the level of ABA increases in senescing leaves of rice\textsuperscript{2} and oat\textsuperscript{7}, it was our interest to understand how hormones, especially ABA, affect the salt-enhanced proteolysis in senescing rice leaves.

The purpose of this study was to monitor the effect of GA\textsubscript{3} and ABA on proteolysis of senescing leaves, and especially, their effect on KCl-enhanced proteolysis at the enzymatic level. Results show that, while GA\textsubscript{3} does not affect segment senescence significantly, the effect of ABA is additive to that of KCl in promoting proteolysis. The additive effect was confirmed in leaf protein decline, amino acid efflux, water absorption and Rubisco-degrading endoproteinase (R-endo) activity. The results on amino acid efflux further strengthens our previous conclusion\textsuperscript{10,11} i.e., it can be an important mechanism in regulating proteinase activity of senescing rice leaves.

**Materials and Methods**

Growth of rice seedlings (Oryza sativa L. cv. Chilsung) for 16 to 18 days and the preparation of leaf segments from the second true leaves were as described previously\textsuperscript{10,11}. Five 5-cm-long segments were placed, base down, into test tubes containing 2-ml test solutions. Five millimolar sodium phosphate, pH 7.0, served as a control, and KCl (50mM), GA\textsubscript{3} (100μM), and ABA (1μM) were made up in the control buffer. Leaf segments were light-incubated at 28°C for 8 days: one-fourth of them were harvested every other day while the rest were supplied with fresh test solutions. When more than 5 leaf segments were needed for enzyme extractions, segments were pooled from separate tubes containing 5 segments each.

Crude extracts made with 5 mM sodium phosphate, pH 7.0, were passed through a PD-10 column (Pharmacia-LKB), and the first 3. 5-ml protein fraction was used as an enzyme source. In addition to hemoglobin, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein, purified from 8- to 12-day-old seedling leaves, was used as a substrate\textsuperscript{10}. Reaction was linear for at least 4 hours, but was run for 3 hours at 40°C. The ninhydrin-positive compounds (NPCs) were measured from the trichloroacetic acid (TCA) soluble supernatant for the activities of hemoglobin-degrading (H-exo) and Rubisco-degrading exoproteinases (R-exo). The difference in the amount of NPCs present in the TCA-soluble supernatant and its 12 M HCl hydrolyzates was taken to be a measure of R-exo activity\textsuperscript{8,10,11,21}. The NPCs are expressed as the amount equivalent to L-leucine (Leu eq). Proteins and amino acids were measured, using bovine serum albumin and
L-leucine as a standard, respectively. Both fresh weights of leaf samples and volumes of incubation media remained in the tubes were measured when leaf segments were harvested every other day. Differences in water loss between the tubes without leaves and those with leaves were considered to indicate the amount of water absorbed (and transpired) by leaf segments in 2 days. Experiments were repeated at least 5 times with 3 replications each.

Some results were statistically analyzed with the Duncan's multiple range test (DMRT) at 5% significance level. Otherwise, standard deviations were 4 to 8% of the mean, and the averages of the means of the separate experiments are presented.

## Results

When compared with the controls, the presence of GA₃ appeared to affect leaf proteolysis little (Table 1). Amino acids accumulated less in GA₃-treated leaves at day 2 than in the control leaves. Combined treatment of GA₃ with KCl resulted in lower proteolysis and less accumulation of amino acids than in KCl alone.

ABA increased proteolysis and by day 8, the ABA-treated leaves contained 11% less protein than control leaves (Table 1). The initial increase in proteolysis in the presence of ABA was reflected by the increased accumulation of amino acids in leaf samples of days 2 and 4. When leaf segments were exposed to ABA combined with KCl, an additive effect was observed not only in promoting proteolysis, but in accumulating amino acids.

Leaf amino acids increased until day 6, followed by a decline at day 8 when leaves were treated with ABA alone or with ABA and KCl.

A control leaf segment absorbed 231 μl of water in 8 days (Table 2). Addition of KCl reduced water absorption of leaf segments by some 20%. Neither GA₃ in the media affected water absorption significantly, nor did the addition of GA₃ to KCl-treated leaves affect

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### Table 1. Effect of GA₃ and ABA on KCl-increased proteolysis of senescing rice leaves

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment</th>
<th>Incubation (days)</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>85a</td>
<td>61a</td>
<td>38a</td>
<td>29a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>70c</td>
<td>38c</td>
<td>24bc</td>
<td>16b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>GA₃</td>
<td>88a</td>
<td>62a</td>
<td>38a</td>
<td>28a</td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td>79ab</td>
<td>55b</td>
<td>35a</td>
<td>18b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA₃+KCl</td>
<td>72bc</td>
<td>40c</td>
<td>30ab</td>
<td>19b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABA+KCl</td>
<td>68c</td>
<td>35c</td>
<td>25bc</td>
<td>16b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>253b</td>
<td>325b</td>
<td>427b</td>
<td>388bc</td>
</tr>
<tr>
<td>Amino acid</td>
<td>ABA</td>
<td>163c</td>
<td>319bc</td>
<td>453a</td>
<td>460a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA+KCl</td>
<td>256b</td>
<td>359b</td>
<td>466a</td>
<td>425ab</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA+KCl</td>
<td>244b</td>
<td>284ab</td>
<td>442ab</td>
<td>412b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA+KCl</td>
<td>319a</td>
<td>413a</td>
<td>428b</td>
<td>408b</td>
<td></td>
</tr>
</tbody>
</table>

1) Concentrations used : GA₃, 100μM; ABA, 1μM; KCl, 50mM.
2) 100% at day 0 : 239μg proteins and 28μg Leu eq amino acids per leaf segment. Values followed by the same letter within a column of each compound are not significantly different by DMRT at 5% level.

### Table 2. Cumulative amount of water absorbed by a senescing leaf segment of rice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>109a</td>
</tr>
<tr>
<td>KCl</td>
<td>89b</td>
</tr>
<tr>
<td>GA₃</td>
<td>115a</td>
</tr>
<tr>
<td>ABA</td>
<td>90b</td>
</tr>
<tr>
<td>GA₃+KCl</td>
<td>87b</td>
</tr>
<tr>
<td>ABA+KCl</td>
<td>69c</td>
</tr>
</tbody>
</table>

1) See Table 1 for chemical concentrations used.
the KCl-reduced water absorption, ABA reduced water absorption by some 10 to 15%, and the combined treatment of ABA with KCl reduced it by more than 30%.

KCl and ABA increased the efflux of amino acids into the media, whereas GA₃, either with or without KCl, did not affect it (Table 3). On the other hand, the combined treatment of ABA and KCl increased the efflux more than either one alone.

In the presence of GA₃, total activity of

### Table 3. Effect of KCl, GA₃ and ABA on the amount of amino acids effluxed into the incubation media

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation (days)</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg Leu eq/segment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.56c</td>
<td>2.20d</td>
<td>3.28c</td>
<td>1.46d</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td>1.24b</td>
<td>4.84c</td>
<td>7.70b</td>
<td>7.38c</td>
</tr>
<tr>
<td>GA₃</td>
<td></td>
<td>0.52c</td>
<td>2.28d</td>
<td>3.09c</td>
<td>2.01d</td>
</tr>
<tr>
<td>ABA</td>
<td></td>
<td>1.36b</td>
<td>6.38b</td>
<td>8.94a</td>
<td>10.26b</td>
</tr>
<tr>
<td>GA₃+KCl</td>
<td></td>
<td>1.18b</td>
<td>4.77c</td>
<td>7.82b</td>
<td>7.66c</td>
</tr>
<tr>
<td>ABA+KCl</td>
<td></td>
<td>1.92a</td>
<td>7.88a</td>
<td>9.05a</td>
<td>11.82a</td>
</tr>
</tbody>
</table>

1) See Table 1 for chemical concentrations used.

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**Fig. 1.** Relative changes in total (A) and specific (B) activities of H−exo of rice leaves as affected by different incubation media. Leaf segments were light-incubated in the media containing 50mM KCl (⋯⋯⋯⋯), 100µM GA₃ (−−−−−−), and a combination of two (−⋯−⋯). Five mM sodium phosphate buffer at pH 7.0 served as controls (−−−−−−). Day 0 values (100%) of total and specific activities were, in µg Leu equi, 12.0 per leaf segment and 50.2 per mg protein, respectively.
H-exo increased almost 2-fold by day 4, followed by a decline to almost the initial level by day 8 (Fig. 1). Although H-exo activity increased in the presence of GAs, it was lower than in control leaves. KCl lowered total activity gradually to 50% of the initial activity by day 8. Despite that GAs alone decreased H-exo activity slightly, addition of GAs to KCl-treated leaves did not decrease H-exo activity more than KCl alone. On the contrary, GAs slightly counteracted the depressing effect of KCl. Decline in total H-exo activity was evident in the presence of KCl alone or combined with GAs, specific activity nonetheless increased more than 3-fold by day 8 due to a rapid decline in leaf proteins.

Relative changes in total activity of R-exo (Fig. 2) exhibited a similar pattern to those of H-exo. However, the initial day 0 activity of R-exo was lower, the magnitude of the change throughout the incubation was less, and the activity peak is shown 2 days later than H-exo (Fig. 1).

Addition of GAs tended to prevent the

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**Fig. 2.** Relative changes in total(A) and specific(B) activities of R-exo of senescing rice leaves as affected by different incubation media. Leaf segments were light-incubated in the media containing 50 mM KCl(—□—), 100 μM GAs(—○—), and a combination of two(—×—). Five mM phosphate buffer at pH 7.0 served as controls(—○—). Day 0 values(100%) of total and specific activities were, in μg Leu eq. 10.6 per leaf segment and 44.4 per mg protein, respectively.
KCl-induced activity decline from occurring until day 6. KCl increased R-endo activity 39% by day 8 (Fig. 3). GA$_3$ alone did not affect the activity compared with the controls, the activity being increased by 20% in both GA$_3$-treated and control leaves. Addition of GA$_3$ to KCl-treated leaves exhibited about the same level of activity as those treated with KCl alone. Stimulation of R-endo activity by KCl, either with or without GA$_3$, increased specific activity while GA$_3$ alone had no effect.

In the presence of ABA, relative changes in total activity of H-exo (data not shown) were similar to those of the R-exo (Table 4). Unlike GA$_3$, ABA reduced R-exo activity as did KCl. When combined with KCl, ABA counteracted the suppressing effect of KCl on R-exo activity until day 6. ABA alone slightly increased R-exo activity as did KCl. However, the combined treatment of KCl and ABA increased the activity more.

Fig. 3. Relative changes in total (A) and specific (B) activities of R-endo of senescing rice leaves as affected by GA$_3$ and KCl. Leaf segments, taken from the second true leaves of 16-day-old seedlings, were light-incubated in various media containing 50mM KCl (--- o ---), 100μM GA$_3$ ( - - o - ), and a combination of two ( - - x - ). Five mM sodium phosphate buffer at pH 7.0 served as controls ( - - - - ). Purified Rubisco was used as a substrate. Day 0 values (100%) of total and specific activities were, in μg Leu eq, 18.3 per leaf segment and 76.6 per mg protein, respectively.
than either one alone. This increase in total activity by the combined treatment resulted in more than a 9-fold increase in specific activity.

### Discussion

GA₃ has generally been considered a leaf senescence retardant¹⁸,²⁰, although species-specific responses has been observed²⁰.

Little effect of GA₃ was observed in the magnitude of proteolysis (Table 1) and amino acid efflux (Table 3). The activities of H-exo (Fig. 1) and R-exo (Fig. 2) as well as the R-endo (Fig. 3) in the presence of GA₃ never exceeded the levels of control leaves. More than a 50% increase in caseolytic activity by GA₃ was reported in rice¹⁵, however, we could not find such an increase in our system.

When leaves were treated with both GA₃ and KCl, all the measured changes were determined largely by KCl with little GA₃ effect. An exception to this was the apparent alleviation of KCl-suppressed activities of exoproteinases by GA₃ (Figs. 1 and 2), although we consider that this would not have affected overall proteolysis significantly. Unless there is a concomitant increase in endoproteinase activity, which was not the case (Fig. 3), the exoproteinases alone would not do much to increase overall proteolysis. We therefore concluded that GA₃ affects detached rice leaf senescence little, and this conclusion agrees with that of Harada and Nakayama⁸.

ABA alone increased proteolysis with concomitant increase in leaf amino acids (Table 1) and in amino acid exudation (Table 3). Our results are in agreement with other reports on the promoting effect of ABA on leaf senescence of a variety of plants.⁴,⁵,¹⁸,¹⁹,²⁴,₂⁵,₂⁶. As was the case in senescing oat leaves⁷, ABA levels increase in senescing flag leaves of field-grown rice plants²⁵. ABA may induce a rapid acceleration of cellular degeneration¹⁵,¹⁷, causing the senescing flag leaves of rice⁶ and detached leaves of oat²⁶,²⁷ to leach out the solutes.

Effect of ABA on KCl—increased proteolysis was of great interest. ABA appeared to be additive to KCl in increasing proteolysis, especially during early stages of incubation (Table 1). The additive effect of ABA was
also found in amino acid efflux (Table 3) and in R-endo activity (Table 4). This effect of ABA contrasted to that of GAs in that no additive effect, or negative effect for that matter, was observed between KCl and GAs in proteolysis (Table 1), amino acid exudation (Table 3), and in R-endo activity (Fig. 3). As with ABA (Table 4), GAs also counteracted the suppressing effect of KCl on R-exo activity (Fig. 2). However, the effect of GAs differed from that of ABA in that GAs did not affect KCl-enhanced R-endo activity (Fig. 3) while ABA increased it further (Table 4). Such an additional increase in R-endo activity with ABA and KCl, combined with some recovery from KCl-suppressed R-exo activity with ABA, would have affected the overall increase in proteolysis significantly (Table 1).

It appears that there is a close relationship between protein decline and the extent of water absorption of leaf segments. When proteolysis was promoted by KCl in the media, there was a consistent decrease in water absorption (Table 2) and fresh weight of the segments (data not shown).

Also, the additive effect of ABA to that of KCl in promoting proteolysis (Table 1) was associated with a further reduction in water absorption of the segments (Table 2). However, this is not to suggest that proteolysis requires complete desiccation of the leaves. The degree and the rate of desiccation may be quite important. When cysteine was added to the incubation media, there was a concentration-dependent, rapid increase in proteolysis of rice leaf segments (Kang, unpublished results). Although the increased synthesis and/or activity of cysteine-dependent proteinases may be involved here\(^6\)\(^{13}\), the cysteine-increased proteolysis at the early stage of senescence was followed by no such changes in proteolysis at late senescence, due primarily to severe desiccation of leaf segments.

In summary, the KCl-enhanced proteolysis of senescing leaf segments can further be increased by adding ABA to the KCl-containing media. This additional increase in proteolysis can be explained by a further (a) increase in the activity of R-endo with some recovery from the KCl-suppressed exoproteinase activity, (b) increase in the efflux of protein hydrolyzates, and (c) reduction in water absorption of the segments.

Although ABA is known to alter membrane permeability of senescing leaves\(^5\)\(^{15}\)\(^{17}\)\(^{26}\)\(^{27}\), and the increase in amino acid efflux is indicative of this, it is not known how much the alterations in membrane permeability of the cells affected the additive effect of ABA we observed. Also unknown is the mechanism by which ABA affects de novo synthesis of proteinases, which is known to be increased by KCl\(^10\)\(^{11}\).

**Chinese Summary**

水稻葉切片の蛋白質含量の減少はKCl処理により抑制される。この現象の抑制作用を増加させることが知られている。GAsとABAのKCl処理による増加作用には、GAsがABAと比較して抑制作用を示すことが知られている。この抑制作用は、GAsによりABAの作用が抑制され、ABAの作用がさらに増加することが示唆される。この作用は、蛋白質の分解加速と水の吸収の減少に関与していると考えられる。したがって、蛋白質の分解と水の吸収は相関すると考えられる。