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Senescence of rice leaves XXIX. Ethylene production, polyamine level and polyamine biosynthetic enzyme activity during senescence

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Changes of ethylene production, polyamine level and activities of enzymes related to polyamine biosynthesis during senescence of intact leaves of rice (*Oryza sativa* L.) seedlings were investigated. Ethylene production decreased markedly at the early stage of senescence, but increased slightly at the later stage. Putrescine, spermidine and spermine, but not cadaverine and diaminopropane, were present in rice leaves throughout senescence. The spermine level decreased progressively with increasing age. The putrescine and spermidine levels as well as arginine decarboxylase activity decreased at the early stage of senescence and increased at the later stage. The increase of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities occurred at the time when leaves were fully expanded and at an advanced stage of senescence. These data seem to support the view that polyamines play an important role in the regulation of rice senescence. Results also suggest that endogenous polyamine levels and ethylene production may not be interrelated during senescence of rice leaves.

Key words: *S*-adenosyl-L-methionine decarboxylase; arginine decarboxylase; ethylene; leaf senescence; ornithine decarboxylase; *Oryza sativa*; putrescine; spermidine; spermine.

Introduction

There are several reports indicating that polyamines can retard leaf senescence [1–5] and that endogenous levels of polyamines decrease with advancing leaf age [6,7]. However, in at least one system (alkaloid-bearing *Heliotropium* plants), no significant polyamine decline could be observed during leaf senescence [8]. Previously, we have demonstrated that exogenously applied polyamines or Dap, an oxidation product of naturally occurring polyamines, retard senescence of detached rice leaves [2,9]. Thus, it would be of great interest to determine the changes in polyamine levels and study their biosynthesis during senescence of intact leaves of rice seedlings.

One mechanism by which exogenous polyamines retard leaf senescence might be related to their inhibition of ethylene biosynthesis [3,5,10]. Both ethylene and polyamines share SAM as a common precursor [11]. Thus, changes in ethylene production during rice leaf senescence were also investigated.

Materials and Methods

Plant material

Rice (*Oryza sativa* L. cv. Taichung Native 1) seedlings were grown as previously described [12]. Leaf samples (3 cm from tip) were collected from the third leaves of seedlings at various times from 8 to 23 days after planting. The changes in the content of Chl were used as an index of leaf

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Abbreviations: ADC, arginine decarboxylase; Cad, cadaverine; Chl, chlorophyll; Dap, 1,3-diaminopropane; HPLC, high performance liquid chromatography; ODC, ornithine decarboxylase; PPO, 2,5-diphenyloxazole; Put, putrescine; SAM, *S*-adenosyl-L-methionine; SAMDC, *S*-adenosyl-L-methionine decarboxylase; Spd, spermidine; Spm, spermine.

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senescence. All experiments described here were repeated three times. Similar results and identical trends were obtained each time. The data reported here were from a single experiment.

Chlorophyll determination

Chlorophyll content was determined according to Wintermans and De Mots [13] after extraction in 96% (v/v) ethanol. Chl content is expressed as mg/g fresh weight.

Extraction and determination of polyamines

Leaves weighing about 125 mg were homogenized in 5 ml of cold perchloric acid (5%, v/v) and the extracts were kept at 4 °C for 24 h. The homogenate was centrifuged for 20 min at 17 000 × *g* and the clear supernatant fraction was used for benzoylation following the method of Flores and Galston [14]. One milliliter of 2 N NaOH was mixed with 1 ml of supernatant fluid. After addition of 15 μl of benzoyl chloride, vortexing for 10 s, and incubation for 20 min at room temperature, 2.5 ml of saturated NaCl was added. Benzoyl-polyamines were extracted in 2.5 ml of diethyl ether. After centrifugation at 3000 × *g* for 15 min, 1.5 ml of the ether phase was collected, evaporated to dryness by vacuum concentrator and redissolved in 100 μl of methanol. Polyamine standards were benzoylated in a similar way. Ten microliter aliquots of each redissolved sample were injected into a Waters M-6 UK Universal Liquid Chromatograph with a 4 × 250 mm, 5 μm particle size C18 reverse-phase column. They were eluted at a flow rate of 1 ml/min using a water (solvent A)/methanol (solvent B) stepped gradient program (50–65% B (v/v) in 7 min/65–80% B (v/v) in 10.5 min/80–100% B (v/v) in 2 min) followed by a column cleaning (100% B (v/v) 1 min)/regeneration (50% B (v/v) 5 min) cycle. Detection was accomplished with a Waters M481 absorbance detector at 254 nm. Results were recorded on a Waters M740 data module and plotted as nmol/g fresh weight.

Assays of polyamine biosynthetic enzymes

Leaves weighing about 125 mg were homogenized in 1.2 ml of extraction medium containing 25 mM Na-phosphate (pH 7.5), 0.1 mM dithiothreitol, 1 mM pyridoxal-5-phosphate and 20 mM Na-ethylenediamine tetraacetic acid (EDTA). The extracts were centrifuged at 17 000 × *g* for 20 min, and the supernatant fraction was used immediately. The optimum pH for ADC (EC 4.1.1.19), ODC (EC 4.1.1.17) and SAMDC (EC 4.1.1.54) of rice leaves was 9.0, 8.5–9.0 and 7.0–8.0, respectively (data not shown). ADC, ODC and SAMDC activities were determined by measuring the release of $^{14}CO_2$ from their respective substrates. The reaction took place in a test tube equipped with a rubber stopper. A filter paper disc, soaked with 30 μl of 2 N KOH was transfixed with a pin through the rubber stopper to trap $^{14}CO_2$. The reaction mixture for ADC in a total volume of 170 μl contained 100 mM Tris–HCl (pH 9.0), 20 μl (5 μCi/ml) of L-[U-^{14}C]arginine (342 mCi/nmol, Amersham) diluted with unlabelled arginine to give a final concentration of 3.7 mM and 50 μl of the supernatant fraction. For ODC, the reaction mixture in a total volume of 220 μl contained 90 mM Tris–HCl (pH 8.5), 20 μl (5 μCi/ml) of L-[1-^{14}C]ornithine (58 mCi/mmol, Amersham) diluted with unlabelled L-ornithine to a final concentration of 21.6 mM, and 100 μl of the supernatant fraction. The reaction mixture of SAMDC in a total volume of 220 μl contained 90 mM Na-phosphate (pH 7.5), 20 μl (1.25 μCi/ml) of SAM-[^{14}C]carboxyl (55 mCi/mmol, Amersham) diluted with unlabelled SAM to a final concentration of 0.57 mM. For all three enzymes, the reactions progressed linearly for 90 min. Enzyme extract denatured by trichloroacetic acid (10%, w/v) was used in the reaction as a control. The tubes were incubated at 37°C for 60 min and the reaction stopped by the addition of 0.2 ml of trichloroacetic acid (10%, w/v). The assay tubes were incubated for an additional 60 min to allow equilibration of $^{14}CO_2$ between the KOH-soaked collecting disc, the gas phase and the acidified reaction mixture in the tube. After equilibration, the KOH discs were removed from the assay tubes and put into 5 ml of scintillation cocktail (4 ml PPO and 1 ml ethylglycomonoethyl ether) in scintillation vials. Three replicates were performed for each extract. The specific activity of the enzyme is expressed as pmol $^{14}CO_2$ released per mg protein per h. The concentration of protein was determined by the

method of Lowry et al. [15] using bovine serum albumin as a reference standard.

Ethylene determination

Leaf segments were placed vertically in test tubes which were closed with rubber stoppers and incubated in darkness at 27°C for 1 h. The ethylene in the gas phase of the closed tubes was determined by analysis of a 1-ml sample withdrawn with a hypodermic syringe as described elsewhere [16].

Results

The senescence of intact rice leaves was followed by measuring the decrease of Chl. Figure 1 shows the time course of Chl loss and ethylene production rate of leaf segments excised from the third leaf from 8- to 23-day-old rice seedlings. Chl content of leaves decreased with aging. The decrease in Chl was evident in leaves from 12-day-old seedlings. Ethylene production significantly decreased at the early stage of senescence and rose slightly but significantly at the advanced stage. The slight increase in ethylene at advanced stage has been reproduced in other experiments.

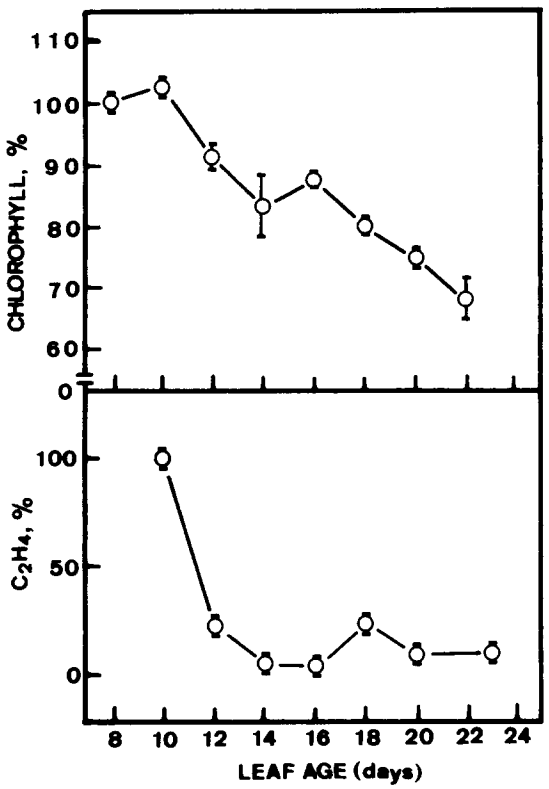

Fig. 1. Changes in Chl content and ethylene production of leaf segments excised from the third leaves of rice seedlings at various ages. Chl content and ethylene production rate are expressed as % of day 8 and 10, respectively. Chl content at day 8 is 3.7 ± 0.2 mg/g fresh wt. whereas ethylene production rate at day 10 is 4.2 ± 0.1 nl/g fresh wt. per h. Values are averages with standard errors (n = 4). Only those standard errors larger than symbol size are shown.

A typical chromatogram of benzoyl polyamines obtained from fully expanded leaves (10 days after planting) in comparison with standards is shown in Fig. 2. Major polyamines in leaf extract were Put, Spd and Spm. Presence of either Cad or Dap was not observed. Spm level decreased with aging and preceded Chl loss (Fig. 3). The Put level in rice leaves during the early phase of senescence was in general similar to that of Spd. Put and Spd levels remained unchanged between days 8 and 10, but decreased between days 12 and 14. An increase in Put occurred at days 16–18 while Spd increased at day 16 and then remained unchanged.

To study the relevance of polyamine biosynthesis in the regulation of senescence of intact rice leaves, we determined changes in polyamine biosynthesis enzyme activities during senescence. Figure 4 shows that the activity of ADC decreased with increasing age up to day 14, increased at day 16 and then decreased again. ODC and SAMDC activities also increase at day 16. In contrast to ADC, a small but significant increase in ODC and SAMDC activities was observed at day 10, when leaves were fully expanded.

Discussion

We previously reported that exogenous application of polyamines and Dap was effective in retarding Chl loss in detached rice leaves [2,9]. The present work shows that Put, Spd and Spm were present in rice leaves throughout senescence. However, Cad and Dap could not be identified in rice leaves. Spm levels in rice leaves decreased with increasing age. Moreover, this decrease occurred prior to Chl loss. The decrease of Put and Spd levels, however, was only observed at the early stage of senescence. It seems that the decrease of

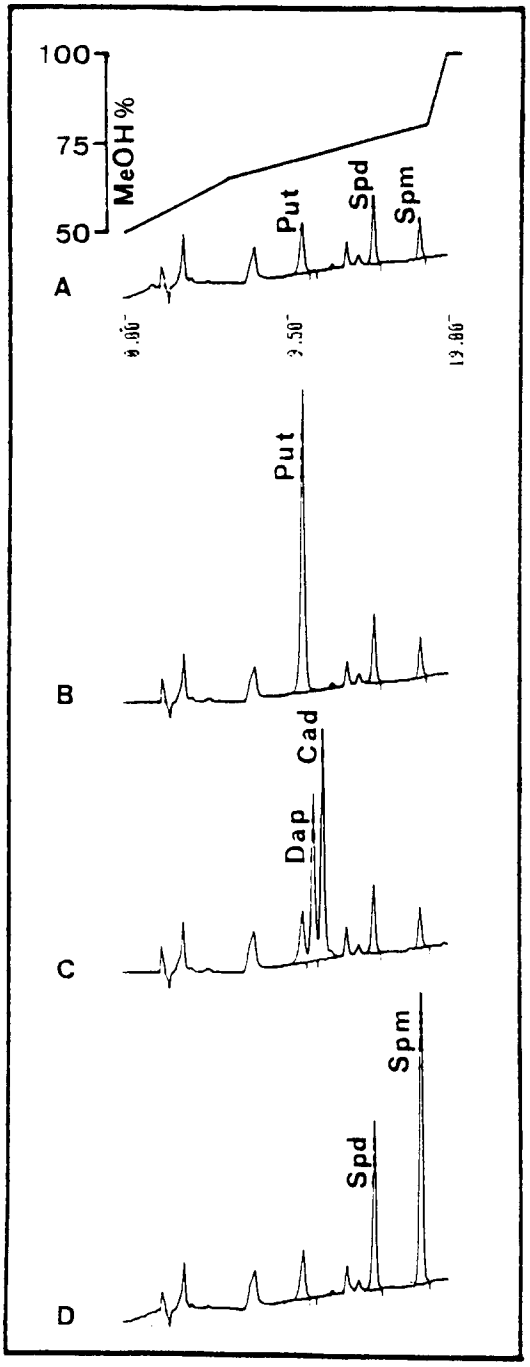

Fig. 2. Separation of benzoylated diamines and polyamines by HPLC. (A) Leaf extract of 10-day-old seedlings; (B) leaf extract with Put standard; (C) leaf extract with Cad and Dap standards; (D) leaf extract with Spd and Spm standards. All standards are 1.5 nmol. Numbers below trace (A) are retention times.

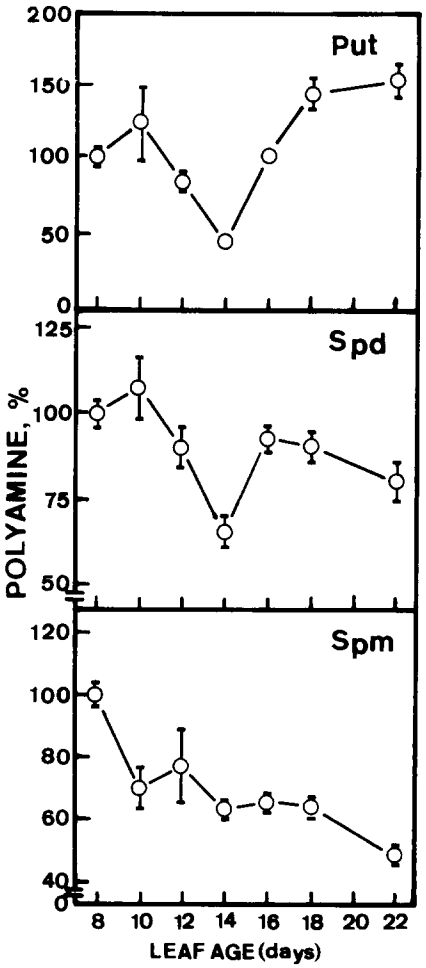

Fig. 3. Changes in free polyamine levels of leaf segments excised from the third leaves of rice seedlings at various ages. Polyamine contents are expressed as % of day 8. Put, Spd and Spm levels at day 8 are 180 ± 5, 204 ± 7 and 155 ± 5 nmol/g fresh wt, respectively. Values are averages with standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

Spm level is an important step in triggering rice leaf senescence. However, the importance of the decrease in Put and Spd levels at the early stage of senescence in controlling senescence cannot be excluded. There seems to be a direct correlation between the decrease of Put level and ADC activity at the early stage of leaf senescence. Thus, decline of ADC activity at the early stage of senescence may also be involved in the control of rice leaf senescence.

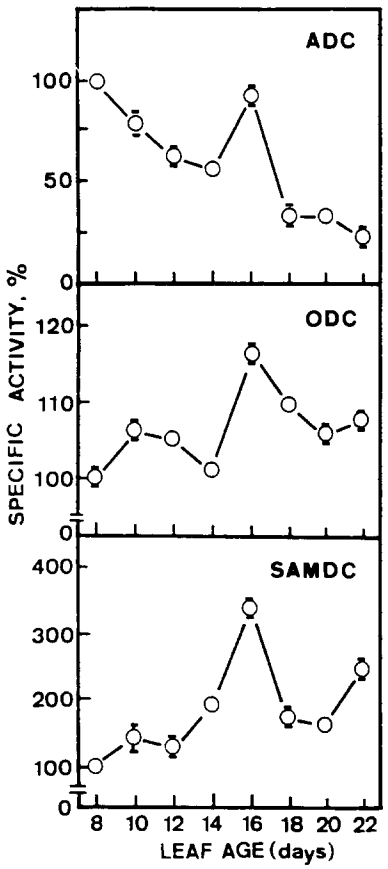

Fig. 4. Changes in activities of ADC, ODC and SAMDC of leaf segments excised from the third leaves of rice seedlings at various ages. Specific activities of enzymes are expressed as % of day 8. Specific activites of ADC, ODC and SAMDC are 18.0 ± 0.2, 4.1 ± 0.1 and 3.7 ± 0.1 pmol $^{14}CO_2$/mg protein per h, respectively. Values are averages with standard errors (n = 3). Only those standard errors larger than the symbol size are shown.

After comparing with the pattern of Chl loss, it is apparent that the climacteric-like rise in levels of Put and Spd and activities of polyamine biosynthetic enzymes occurs when leaves are in an advanced stage of senescence, and is probably caused by earlier events taking place in the course of rice leaf senescence.

No correlation between SAMDC activity and polyamine levels around days 12–14 and between ADC or ODC activity and Put level from days 14 to 22 was observed, suggesting that polyamine levels are not mainly regulated by their biosynthesis. Polyamine oxidation and/or conjugation may be involved in regulating polyamine levels during senescence of rice leaves.

Both ethylene and polyamines share SAM as a common precursor [11]. Furthermore, the effect of polyamines in retarding leaf senescence is generally thought to be mediated through inhibition of ethylene production [3,5,10]. Since the pattern of ethylene production during senescence was in general similar to that of polyamine levels, these data raise the possibility that the mechanism by which polyamines regulate leaf senescence in intact rice plants may not involve inhibition of ethylene production. This conclusion is further supported by our recent finding that polyamines promote biosynthesis of ethylene in rice leaves [17].

A dramatic decrease in ethylene production was observed during the early stage of senescence of rice leaves. A slight but consistent increase in ethylene production only occurred at an advanced stage of senescence. Furthermore, the increase in ethylene production was not considerable. It is possible that the link of senescence of detached rice leaves to ethylene production [16] may not hold for senescence of intact rice leaves.

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