

## Involvement of Ribonuclease and RNA Polymerase Activity in Chill-induced Increases of RNA Concentration in Figleaf Gourd Roots

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### Summary

We previously found a large increase in RNAs in roots of chill-tolerant figleaf gourds (*Cucurbita ficifolia* Bouché) but not in those of less tolerant cucumbers (*Cucumis sativus* L.), when the roots were exposed to 14°C (Kanda et al., 1994). In this study, changes in ribonuclease and RNA polymerase activities in the roots were followed for 6 days after their exposure to 14° and 23°C. The root enzyme activities were assayed at a) the optimal temperatures for the enzymes and b) root-growing temperatures. Neither ribonuclease nor RNA polymerase activity accounted for the characteristic increase of RNA concentrations in chilled roots of figleaf gourds, irrespective of assay temperatures. However, the ratio of RNA polymerase activity to ribonuclease activity closely correlated with RNA concentrations in both species. This correlation was observed only when the enzyme activity was assayed at root-growing temperatures. In this case, ribonuclease activity 2 days after exposure to 14°C was much lower in figleaf gourd roots than that in cucumber roots. However, the activity changed little thereafter in either species, but the RNA polymerase activity in figleaf gourd roots did increase gradually during exposure to 14°C; it was still lower than the activity at 23°C after 6 days. This increase was not observed in the cucumber roots. These results strongly suggest that, 1) root RNA concentrations in figleaf gourds and cucumbers are largely regulated by RNA polymerase activity relative to ribonuclease activity, and 2) the marked increase of RNA concentrations in figleaf gourd roots grown at 14°C was caused mainly by low temperature-induced increases of RNA polymerase activity together with much reduced activity of ribonuclease.

### Introduction

The roots of figleaf gourds are relatively tolerant to low temperature, and thus have been used widely as a root-stock of cucumbers in protected cultivation during winter (Tachibana, 1982). Recently, growers are changing the root-stock species of cucumbers from figleaf gourds to "bloomless" root-stock species. The latter species have a low capacity to absorb silicon, which leads to a low incidence of bloom symptoms on the skin of the cucumbers (Yamamoto et al., 1989). However, the roots of "bloomless" root-stock species are less tolerant to low soil temperature than are those of figleaf gourds, so that efforts are now being directed towards increasing the chill-tolerance of

these root-stock species (Yamamoto, 1989).

Previously, we reported that RNA concentrations in the roots of figleaf gourds exposed to 14°C were significantly higher than in roots kept above or below 14°C (Kanda et al., 1994). This rise of RNA concentrations at 14°C was considered to have resulted from net accumulation of RNAs in response to the chilling temperature, because root growth was most rapid at 14°C with decreased cell numbers per unit fresh weight, and because by transferring the roots from 14°C to 23°C the RNA concentrations decreased to the pre-chilling level within 2 days.

RNAs usually accumulate in plant tissues of cold resistant species when exposed to chilling temperatures (Guy, 1990). Treatment of plants with inhibitors of RNA and protein synthesis during cold acclimation inhibits the development of

cold hardiness (Hatano et al., 1976). The rise of RNA concentrations, along with a concomitant increase of protein synthesis, has been related to the acquisition of cold tolerance by the plants during cold acclimation (Chen and Li, 1980; Paldi and Devay, 1977; Sarhan and Chevrier, 1985).

To date, the mechanisms by which RNAs accumulate in chilled plant tissues are not fully understood. Factors responsible for the increased RNA synthesis under low temperature regimes may include an increase in RNA polymerase activity as well as a decrease in ribonuclease activity in chilled tissues (Gusta and Weiser, 1972; Kritenko et al., 1986; Sarhan and Chevrier, 1985). According to Sarhan and Chevrier (1985), a large increase of RNA content in winter wheat shoots during cold acclimation was associated with a significant increase in RNA polymerase activity and a decrease in ribonuclease activity in the chromatin. Hormones are also known to regulate the rate of RNA synthesis (Naito et al., 1980; Tomi et al., 1983; Van der Linde et al., 1984). However, only limited information is available on the physiological causes of accumulation of RNAs in chilled plant roots.

We expect that an elucidation of mechanism of RNA accumulation in chilled roots of figleaf gourds may contribute to a better understanding of physiological bases of chilling tolerance of plant roots and to the improvement of chilling tolerance of the roots of cucurbits including that of "bloomless" root-stock species. Thus, the objective of this study was to relate the changes in RNA concentrations in the roots of chill-tolerant figleaf gourds and less tolerant cucumbers to the activities of RNA polymerase and ribonuclease in the roots, following exposure to 14° and 23°C root temperatures for up to 6 days.

## Materials and Methods

### 1. Plant materials and root temperature treatments

Seedlings of figleaf gourds and cucumbers cv. Suyo, raised by gravel culture, were planted at the 1-leaf stage in two hydroponic vessels containing a half-strength Hoagland solution. Plants were grown in a climate-control chamber at 26°C day and 20°C night with a photoperiod of 15 hr and a light intensity of 250  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  provided by metal halide lamps. The initial temperature of the

nutrient solution was 23°C in both vessels; the solution temperature in one vessels was lowered to 14°C 6 days after planting. The control vessel was kept at 23°C. Six days later, figleaf gourds grown at 14°C were transferred to the 23°C solution. Root samples of the figleaf gourds and cucumbers were collected in triplicate just before the treatment (day 0; solution temperature at 23°C) and after 2, 4, and 6 days at 14°C and 6 days at 23°C. Roots of figleaf gourds were also taken from the plants grown 2 days at 23°C following 6 days at 14°C.

### 2. Determination of RNA concentrations in roots

RNAs were extracted and purified as described previously (Kanda et al., 1994). RNA concentrations in purified extracts ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) were calculated by multiplying their OD<sub>260</sub> values by 45.

### 3. Ribonuclease assay

Ribonuclease was extracted from 2 g portions of the roots immediately after harvest according to the method described by Chevrier and Sarhan (1980). Briefly, roots were homogenized in 50 mM Tris buffer (pH 7.5) and the homogenate centrifuged. A 50- $\mu\text{l}$  aliquot of the supernatant (equivalent to 5 mg of fresh root) was mixed with 1 ml of 0.75  $\text{mg}\cdot\text{ml}^{-1}$  RNA in 40 mM sodium cacodylate buffer (pH 5.6), and the mixture incubated for 30 min at 37°C, or for 60 min at temperatures at which the roots were growing when they were sampled (referred to hereafter as root-growing temperatures), to simulate the in situ condition. Reaction was stopped by adding 20 mM lanthanum nitrate dissolved in 0.5 N HCl. After centrifugation, the optical density of the supernatant fluid was read at 260 nm; the reaction mixture without the extract served as the blank. Ribonuclease activity was expressed in the OD<sub>260</sub> values per gram root fresh weight. For detection of ribonuclease isozymes, an aliquot of the extract was loaded on 7.5% disc polyacrylamide gels. After electrophoresis, ribonucleases in the gel were made visible by the method of Wilson (1971).

### 4. RNA polymerase assay

RNA polymerase activity in the roots was determined according to Lin et al. (1974). In brief, 10 g of freshly harvested roots were homogenized in 50 mM Mes buffer (pH 8.0) and the homogenate cen-

trifuged at  $5,000 \times g$  to isolate chromatin. To solubilize RNA polymerases, chromatin was suspended in 500 mM ammonium sulfate solution, stirred, and sonicated. The soluble RNA polymerase (40  $\mu$ l, equivalent to 570 mg of fresh root) was mixed with 200  $\mu$ l of  $370 \text{ kBq} \cdot \text{ml}^{-1}$   $^3\text{H}$ -UTP in 50 mM Tris buffer (pH 8.0), and the mixture incubated for 20 min at  $32^\circ\text{C}$  or at root-growing temperatures. Reaction was terminated by adding 240  $\mu$ l of 10% trichloroacetic acid solution. After centrifugation, the radioactivity of the washed precipitate was counted with a liquid scintillation spectrometer. RNA polymerase I activity was measured as described above in the presence of  $\alpha$ -amanitin in the incubation mixture. RNA polymerase II activity was calculated by subtracting RNA polymerase I activity from total RNA polymerase activity. Activity was expressed in the cpm per gram root fresh weight.

## Results

### 1. RNA concentrations in roots

RNA concentrations in figleaf gourd roots exposed to  $14^\circ\text{C}$  increased steadily to 2.5-fold the initial level after day 6 (Fig. 1). In cucumber roots,

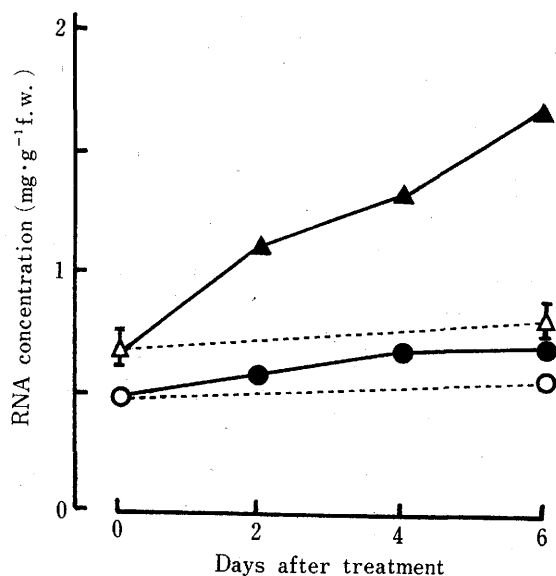


Fig. 1. Changes with time in RNA concentrations in the roots of figleaf gourds ( $\triangle$ ,  $\blacktriangle$ ) and cucumbers ( $\circ$ ,  $\bullet$ ) following exposure to  $14^\circ$  and  $23^\circ\text{C}$  root temperatures. Open and solid symbols represent roots grown at  $23^\circ\text{C}$  and  $14^\circ\text{C}$ , respectively. Vertical bars show  $\pm$  S. E.

however, the RNA concentration increased to only 1.4-fold the initial level during the same period. The roots grown at  $23^\circ\text{C}$  did not show any significant changes in RNA concentrations over the experimental period in both species. These results are essentially the same as those reported earlier (Kanda et al., 1994).

### 2. Ribonuclease activity in roots

Prior to evaluating the effect of root temperature on ribonuclease activity, the subcellular localization of the enzyme in the roots of the two species grown at  $23^\circ\text{C}$  was determined according to the procedure of Chevrier and Sarhan (1980). In both species more than 80% of total ribonuclease activity in the roots was localized in cytosol fraction and very low activity was detected in the chromatin, mitochondrial and microsomal fractions (Table 1). The activity in the cucumber roots was 57% higher than that of the figleaf gourd roots. Based on the above observation, ribonuclease activity in the roots was represented by the activity in cytosol fraction in the subsequent experiments.

Effects of root temperature on the ribonuclease activity in the roots differed markedly with the assay temperature. Figleaf gourd roots grown at  $14^\circ\text{C}$  showed significantly lower activity than did those grown at  $23^\circ\text{C}$  when determined at  $37^\circ\text{C}$ , regardless of root temperatures (Fig. 2A). Contrarily, ribonuclease activity was higher in the cucumber roots grown at  $14^\circ\text{C}$  than it was at  $23^\circ\text{C}$ .

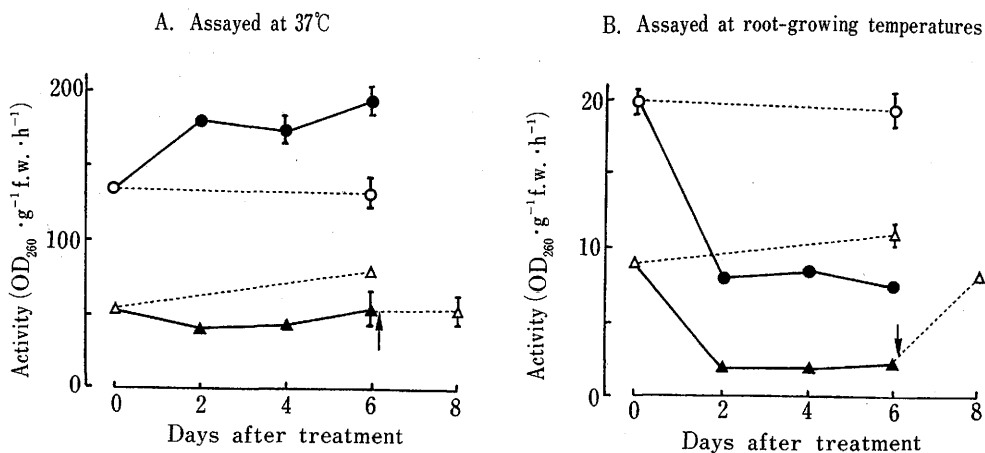
When ribonuclease activity was measured at root-growing temperatures, it was much lower in the roots exposed to  $14^\circ\text{C}$  than in those exposed to  $23^\circ\text{C}$  (Fig. 2B), but the magnitude of the decrease was much greater in figleaf gourd roots than it

Table 1. Subcellular localization of ribonuclease activity ( $\text{OD}_{260} \cdot \text{g}^{-1} \cdot \text{fw} \cdot \text{h}^{-1} \pm \text{S. E.}$ )<sup>z</sup> in the roots of figleaf gourds and cucumbers, grown at a root temperature of  $23^\circ\text{C}$ .

Fractions	Figleaf gourd	Cucumber
Chromatin	$1.71 \pm 0.07$ (5.6) <sup>y</sup>	$3.18 \pm 0.05$ (6.4)
Mitochondria	$1.47 \pm 0.09$ (4.8)	$2.50 \pm 0.10$ (5.0)
Microsome	$1.90 \pm 0.19$ (6.2)	$3.94 \pm 0.11$ (7.9)
Cytosol	$25.49 \pm 2.18$ (83.4)	$39.99 \pm 1.13$ (80.7)

<sup>z</sup> Activity was assayed at  $37^\circ\text{C}$ .

<sup>y</sup> Numerals in parentheses show the percentages of total activity.



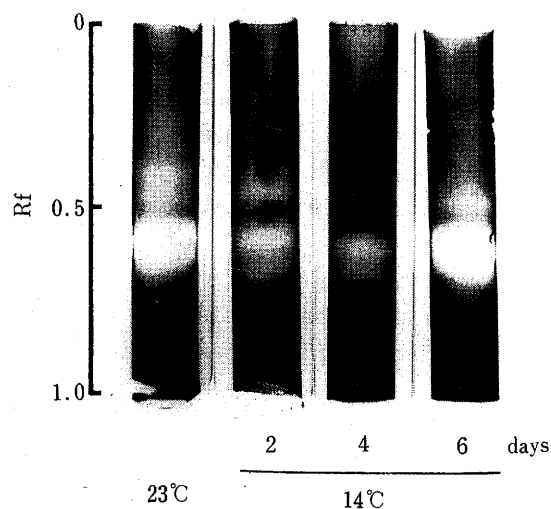
**Fig. 2.** Ribonuclease activity in the roots of figleaf gourds ( $\triangle$ ,  $\blacktriangle$ ) and cucumbers ( $\circ$ ,  $\bullet$ ) grown at 14° and 23°C root temperatures. Figleaf gourds grown at 14°C for 6 days were transferred to 23°C at the time shown by arrows. Enzyme activity was assayed at 37°C (A) and at root-growing temperatures (B). Open and solid symbols represent roots grown at 23°C and 14°C, respectively. Vertical bars show  $\pm$ S. E.

was in cucumber roots. Consequently, ribonuclease activity in figleaf gourd roots grown at 14°C was only about 25% of that in cucumber roots grown at the same temperature, whereas it was about 50% for roots grown at 23°C. The enzyme activity in both species did not change significantly over the period of 6 days after exposure to 14°C. Furthermore, transfer of figleaf gourd roots from 14° to 23°C at day 6 did not cause any changes in the activity when assayed at 37°C (Fig. 2A), whereas the activity reverted to the control level when assayed at the root-growing temperature (Fig. 2B).

Polyacrylamide gel electrophoresis of ribonucleases showed the existence of several isozymes in roots of both species. Isozyme banding patterns did not change with root temperatures in cucumber roots (data not presented), whereas in the figleaf gourd roots, the slowly migrating band disappeared when the roots were exposed to 14°C (Fig. 3). Since the band was very weak, its disappearance may have contributed little to the quantitative change of ribonuclease activity in the roots.

### 3. RNA polymerase activity in roots

RNA polymerase activity in cucumber roots grown at 14°C was higher than that at 23°C when assayed at 32°C (Fig. 4A). It increased slightly during the 6 days exposure to 14°C, but, when



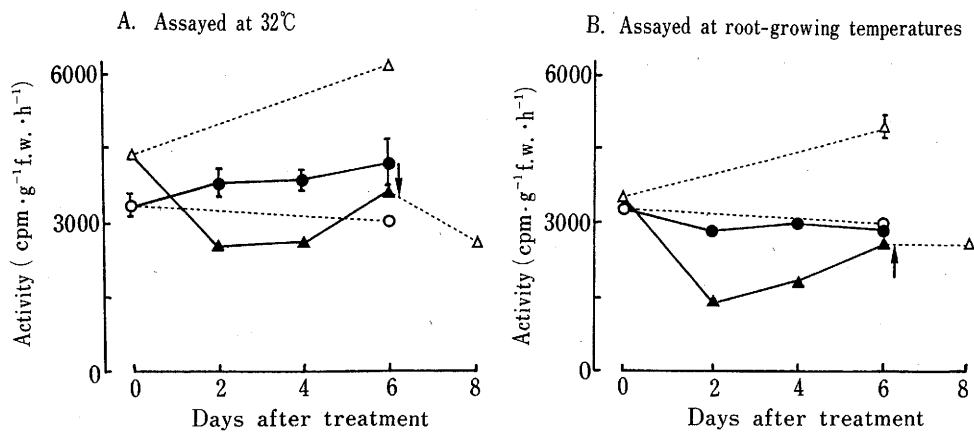
**Fig. 3.** Polyacrylamide gel electrophoresis of ribonuclease isozymes obtained from figleaf gourd roots exposed to 23°C or to 14°C for 2, 4 and 6 days. The banding patterns of ribonuclease isozymes from cucumber roots did not change with growing temperatures (gels not shown).

assayed at the root-growing temperature, the activity did not increase, being identical to that at 23°C (Fig. 4B).

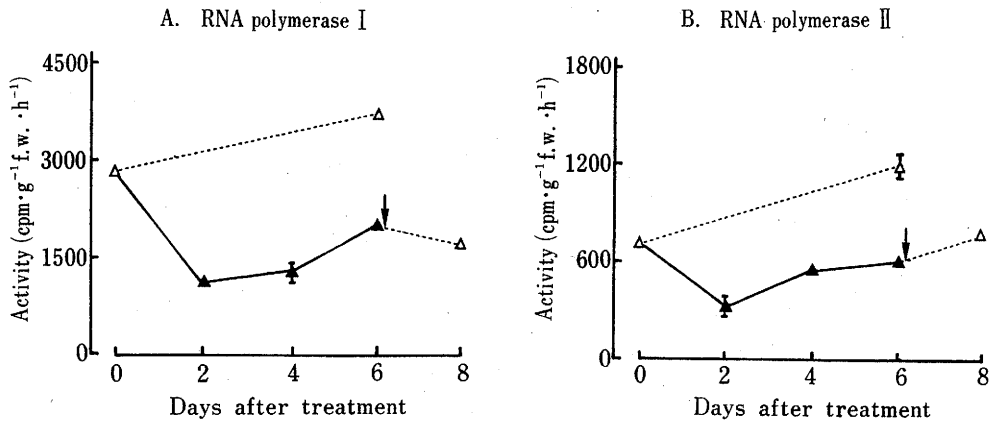
The RNA polymerase activity in figleaf gourds was very low in the roots grown at 14°C, compared to those at 23°C, regardless of assay temperatures (Fig. 4A and B). The activity tended

to increase over the period of 6 days at 14°C as well as at 23°C; the rate of increase during the last 4 days was faster in roots grown at 14°C than it was in those held at 23°C; i.e., 1.8-fold at 14°C vs. 1.2-fold at 23°C. This comparison is based on the assumption that the increase during the 6-day period at 23°C was linear. The enzyme activity in the roots exposed to 23°C for 2 days after 6 days at 14°C decreased by about 30% when the enzyme activity was assayed at 32°C, whereas it changed little when assayed at root-growing temperature (23°C).

RNA polymerase I activity in figleaf gourd roots assayed at root-growing temperatures was higher than that of RNA polymerase II in figleaf gourd roots at day 0 (Fig. 5A and B). This implies that rRNA is synthesized at a rate higher than that of mRNA. Responses of RNA polymerase I and II activities to root temperatures were essentially the same as those of total RNA polymerase activity, except that RNA polymerase II activity did not decrease during the 2 day readaptation period at 23°C.



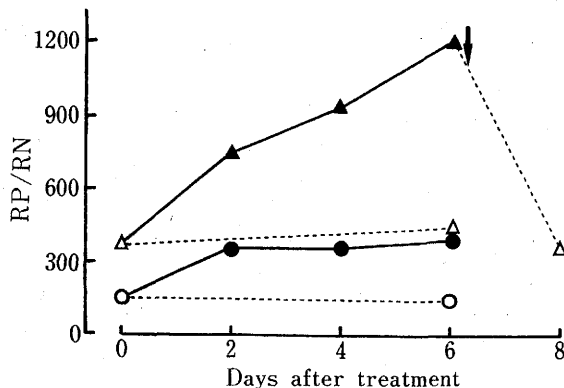
**Fig. 4.** RNA Polymerase activity in the roots of figleaf gourds ( $\triangle$ ,  $\blacktriangle$ ) and cucumbers ( $\circ$ ,  $\bullet$ ) grown at 14°C and 23°C root temperatures. Figleaf gourds grown at 14°C for 6 days were transferred to 23°C at the time shown by arrows. Enzyme activity was assayed at 32°C (A) and at root-growing temperatures (B). Open and solid symbols represent roots grown at 23°C and 14°C, respectively. Vertical bars show  $\pm$ S. E.



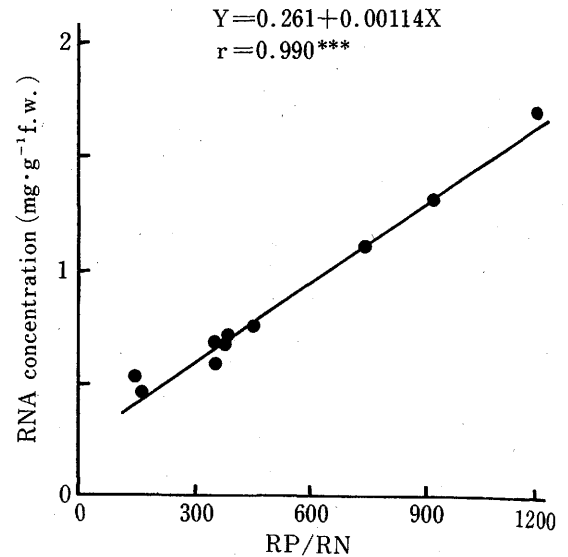
**Fig. 5.** Activity of RNA polymerase I (A) and RNA polymerase II (B) in the roots of figleaf gourds grown at 14°C ( $\blacktriangle$ ) and 23°C ( $\triangle$ ) root temperatures. Plants grown at 14°C for 6 days were transferred to 23°C at the time shown by arrows. Enzyme activity was assayed at root-growing temperatures. Open and solid symbols represent roots grown at 23°C and 14°C, respectively. Vertical bars show  $\pm$ S. E.

#### 4. Ratios of RNA polymerase activity to ribonuclease activity in roots

RNA concentrations in the tissue could be affected by the activity of either ribonuclease or RNA polymerase, alone in some cases but in combination in most cases. Our examination of the relationship between RNA concentrations in both species roots grown at 14° and 23°C root temperatures and the ratio of RNA polymerase activity to ribonuclease activity (RP/RN) revealed that when the enzyme activity was assayed at root-growing temperatures, RP/RN values in figleaf gourd roots increased 3.3-fold during the 6-day growth period at 14°C (Fig. 6). They decreased rapidly to the pre-chill value within 2 days after retransfer to 23°C. In cucumber roots, the ratio doubled during the first 2 days at 14°C, but remained unchanged thereafter. RP/RN values in roots of both species grown at 23°C remained constant throughout the experimental period. These changes in RP/RN values closely resemble the trends of RNA concentrations in the roots as affected by root temperature (Fig. 1). Thus, the correlation between RNA concentrations in the roots and their RP/RN values is highly significant (Fig. 7). But, the rate



**Fig. 6.** Changes with time in the ratio of RNA polymerase activity to ribonuclease activity (RP/RN) in the roots of figleaf gourds ( $\triangle$ ,  $\blacktriangle$ ) and cucumbers ( $\circ$ ,  $\bullet$ ) following exposure to 14°C and 23°C root temperatures. Figleaf gourds grown at 14°C for 6 days were transferred to 23°C at the time shown by an arrow. Enzyme activities of ribonuclease and RNA polymerase were assayed at root-growing temperatures. Open and solid symbols represent roots grown at 23°C and 14°C, respectively.



**Fig. 7.** Relationships between RNA concentrations and the ratio of RNA polymerase activity to ribonuclease activity (RP/RN) in figleaf gourd and cucumber roots grown at 14°C and 23°C for up to 6 days. Activities of both enzymes assayed at root-growing temperatures were used for the calculation.

of increases of RP/RN values in both species during growth at 14°C was relatively fast, compared to the rate of the RNA increase. When the activity of the enzymes was assayed at their optimal temperatures, no correlation was established between RP/RN and RNA concentrations (data not presented).

#### Discussion

The close correlation between RNA concentrations in the roots and their RP/RN values assayed at root-growing temperatures strongly suggests that RNA concentrations in figleaf gourd and cucumber roots are largely regulated by RNA polymerase activity relative to ribonuclease activity. The lack of correlation when the activity of the enzymes was assayed at their optimum temperatures indicates that the ambient temperature when samples are taken should be used for the enzyme assay, particularly when the enzyme activity is to be related to the metabolic changes caused by temperature stresses. A similar suggestion has been proposed by Burke and Hatfield (1987).

Ribonuclease activity in chilled cucumber roots increased when assayed at 37°C, whereas it decreased in roots of figleaf gourds. The same trend

was noted for RNA polymerase activity in the roots assayed at 32°C. Although these differences in responses of the enzymes to growing temperatures between the two plant species are difficult to explain, the higher RP/RN values in chilled roots of figleaf gourds over those in cucumbers may be ascribed to a greater reduction in ribonuclease activity in the figleaf gourd roots. Likewise, the RNA polymerase activity of figleaf gourd roots was much lower than was that in chilled cucumber roots throughout the experimental period.

The marked reduction of ribonuclease activity in the chilled figleaf gourd roots agrees with the observation made by Dzhokhadze and Tabatadze (1984) that ribonuclease activity was lower in leaves of cold resistant than it was in cold susceptible citrus varieties. Brown and Bixby (1973) opine that the decrease of ribonuclease activity during induction of cold hardiness in mimosa is caused by the inactivation or degradation of enzyme proteins rather than by the decreased synthesis induced by the low temperature.

Gradual and steady increases in RP/RN values and in RNA concentrations in figleaf gourd roots after exposure to 14°C may have resulted because of increases in RNA polymerase I and II activities; ribonuclease activity did not decrease correspondingly during the same period. However, the rate of increases in RNA concentrations was slower than that of RP/RN values in figleaf gourd roots grown at 14°C (3.3-fold vs. 2.5-fold), which may indicate that factors other than the ratio of the two enzyme activities are involved in the accumulation of RNAs in chilled roots of figleaf gourds.

We conjecture that the roots of figleaf gourds respond to low temperature by increasing RNA polymerase activity, whereas those of cucumbers do not. Although only the solubilized RNA polymerase activity was determined in the present study, our results do not preclude the possibility that chromatin template activity was also enhanced in the same manner as the solubilized enzyme activity. Similar results have been reported with wheat leaves by Sarhan and Chevrier (1985) and Kritenko et al. (1986), but their results differed from ours in that RNA polymerase activity was far greater in chilled than in unchilled plants. Furthermore, Kritenko et al. (1986) found a marked increase in RNA polymerase activity in cucumber leaves during the course of cold acclima-

tion at 10°C over that of the control plants grown at 25°C. It would be interesting to know whether RNA polymerase in different tissues of the same plant responds differently to low temperatures.

The mechanisms by which RNA polymerase activity increases in chilled plant tissues have not been fully elucidated. Kukina et al. (1985) presented evidence indicating that ABA inhibits rRNA synthesis by blocking the transcription of rRNA precursors in the chromatin. This action of ABA was reversed by treatments with cytokinins. It is also known that magnesium and other metallic ions activate RNA polymerase (Guilfoyle et al., 1976; Wielgat and Kahl, 1979). Cytokinin synthesis in the figleaf gourd roots is markedly stimulated by low temperature (Tachibana, 1988). Whether, 1) this property of figleaf gourd roots is responsible for the increase in RNA polymerase activity and consequently in RNA concentrations in the chilled roots, and 2) the application of cytokinins to the roots can enhance the chilling tolerance in the roots of "bloomless" root-stock species need further explanation.

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# 低温に遭遇したクロダネカボチャの根の RNA 濃度の増大におけるリボヌクレアーゼ活性と RNA ポリメラーゼ活性の関与

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## 摘 要

14℃の低根温遭遇によってクロダネカボチャの根の RNA 濃度が著しく高まる機作を明らかにするため、14℃と23℃の根温で6日間生育している間の、クロダネカボチャとキュウリの根のリボヌクレアーゼ活性と RNA ポリメラーゼ活性の経時変化を調べた。活性の測定は、それぞれの酵素の適温と採取時の根の生育根温で行った。その結果、いずれの酵素活性も単独では、根温14℃で生育したクロダネカボチャの根でみられた RNA 濃度の著しい増大を説明することはできなかった。しかし、リボヌクレアーゼ活性に対する RNA ポリメラーゼの活性の比率は、生育根温で活性を測定した場合にのみ、根の RNA 濃度と高い相関関係を示した。生育根温で活性を測定した場合、リボヌクレアーゼ活性は、両植物とも23℃で生育した根よ

り14℃で生育した根のほうが低かったが、低下の程度はクロダネカボチャのほうが顕著であった。一方、RNA ポリメラーゼ活性は、キュウリでは14℃で生育した根と23℃で生育した根でほぼ同じであったのに対して、クロダネカボチャでは14℃で生育した根のほうが小さかった。しかし、クロダネカボチャの根では14℃で生育している間に次第に活性が増大した。以上の結果から、両植物の根の RNA 濃度は、主として生育根温下での2つの酵素の活性比率に支配されていると考えられる。また、14℃で生育したクロダネカボチャの根における RNA 濃度の著しい増大は、低温によるリボヌクレアーゼ活性の顕著な低下と、RNA ポリメラーゼ活性の低温適応的増大によっていると考えられる。