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Occurrence of a Novel Cytokinin in Persimmon Fruitlets

By

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Ethanol extract of persimmon fruitlets (*Diospyros kaki* L. cv. Hiratanenashi) contains cytokinins which stimulate strongly the growth of tobacco callus tissue *in vitro*. One of these cytokinins was purified with *n*-butanol extraction and ion exchange columns, and compared with known natural cytokinins in paper chromatography with several solvent systems. It was revealed that this cytokinin was very closely related to zeatin, but apparently distinguishable from it. Results indicate an occurrence of a novel cytokinin in the persimmon fruitlets.

Introduction

Exogenous supply of cytokinins brings about various noteworthy effects on plant growth[1,2]. These suggest that endogenous cytokinins also participate in the physiological process of tissue and organ developments, and have some important roles in the control of plant growth. In order to understand the roles of endogenous cytokinins, studies on such factors in various species of plant have been carried out in our laboratory[3~9]. In the course of our studies it has been shown that young fruits of *Diospyros kaki*, *Solanum melongena*, and *Vitis vinifera*, young seeds of *Glycine soja* and *Lupinus luteus*[3~5,7], and embryos of *Sechium edule* contain much amount of cytokinins, and a new cytokinin was isolated from *L. luteus*[5] and identified as (−)-6-N-(4-hydroxy-3-methybutylamino)-purine (dihydrozeatin)[7].

The persimmon, another cytokinin-rich plant, is a prevalent fruit-plant and has been investigated extensively in pomology. However, investigation of endogenous cytokinins in relation to the fruit development in this plant was just commenced on[10]. Under such circumstances, informations in regard to properties of cytokinins contained in persimmon are helpful and important. The work described in this paper was undertaken to achieve purification of cytokinins in extracts of persimmon fruitlets and to attempt paper-chromatographic identification of factors in comparison with known natural cytokinins.

Materials and Methods

Persimmon fruitlets (*Diospyros kaki* L. cv. Hiratanenashi) were harvested, when they were 45 day old from full bloom and about 3.1 cm in diameter. They were immediately

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shredded and extracted with double volumes of 80% ethanol three times at room temperature. The ethanolic solution, after suction-filtration with double layers of Toyo filter papers, was evaporated to dryness *in vacuo* at 50°C in a Shibata rotary evaporator. These conditions were used in all subsequent evaporations. The residue was dissolved in water at a concentration equivalent to 100 g fresh fruitlet tissue per ml. This aqueous solution was stocked as an ethanol extract in a freezer for bio-assay and further purification with solvent fractionation, ion exchange column, and paper chromatography. Leaves of this plant were also extracted with 80% ethanol as described above.

Location of cytokinins on paper-chromatograms of extracts was detected as reported previously[9]. An extract to be tested was dissolved in small amount of *n*-butanol, streaked on Whatman No. 1 filter paper and developed ascendingly. The following solvent systems were used; A) water-saturated *n*-butanol, B) 0.02 M boric acid (pH 8.4 with NaOH), C) *n*-butanol : NH_4OH (4 : 1), D) ethylacetate : formic acid : water (60 : 5 : 35), E) water, and F) *n*-butanol : formic acid : water (80 : 15 : 15). A chromatogram was cut into ten segments according to Rf value, and these segments were eluted each with 25 ml water twice. The eluates were tested for their cytokinin activity and growth promotion was plotted against Rf.

Cytokinin activity was determined with tissue culture of tobacco callus (*Nicotiana tabacum* cv. Wisconsin No. 38) which required the presence of a cytokinin for continued cell-division[5,11]. This callus tissue was originally isolated from the stem pith and subcultured as stock cultures routinely on the basal medium[11] supplemented with 0.2 mg/l kinetin except with 0.03 mg/l in the final two transfers. The basal media contained inorganic salts[11], Na_2EDTA (37.3 mg/l), thiamine · HCl (0.4 mg/l), *myo*-inositol (100 mg/l), IAA (2 mg/l), sucrose (30 g/l), and agar (10 g/l). In cytokinin assay, a preparation to be tested was added to the basal medium. The pH of the medium was adjusted to 5.8 with NaOH or HCl before the agar was melted. The medium was divided by 12 ml into test tubes (18×180 mm) and sterilized by autoclaving at 1.0 kg/cm^2 for 15 minutes. More than eight replicate test tubes were prepared for each treatment. As the controls the basal media with and without 0.003 and 0.3 mg/l kinetin were used. Small pieces of tobacco callus (ca. 5 mg in fresh weight) obtained from stock cultures on 0.03 mg/l kinetin medium were implanted each in a test tube. After growth for 30 days at 27°C in darkness the tissues were harvested and their fresh weight was determined. All the experiments were repeated at least twice.

Results

As a preliminary experiment, cytokinin activity of the ethanol extracts of persimmon fruitlets and leaves was tested at the concentrations equivalent to 1, 10, and 100 g fresh tissue per liter of medium. As shown in Figure 1 and Table 1, the ethanol extract of fruitlets stimulated strongly the growth of tobacco callus tissue. The maximum growth of the tissue was observed at 10 g equivalents per liter. This activity was stronger than 0.03 mg/l kinetin which was an optimal concentration. In higher concentration, 100 g equiva-

lents/l, the effectiveness was reduced and tissues turned brown. On the other hand the ethanol extract of persimmon leaves was much less effective in all concentrations. Leaf may contain less amount of cytokinins, otherwise some inhibitory substances which mask the cytokinin activity may be present.

The ethanol extract of fruitlets was fractionated further with *n*-butanol. The ethanol extract obtained from 200 g of fruitlets was diluted with 50 ml water, adjusted to pH 8.4 with NaOH and layered with three equal volumes of *n*-butanol. The *n*-butanol and the aqueous fractions were evaporated and tested for their cytokinin activity at 10 and 100 g equivalents per liter. As shown in Table 2 the *n*-butanol fraction was more stimulative than the aqueous fraction. The former at 100 g equivalents/l was more effective than 0.03 mg/l kinetin, while the latter at 100 g equivalents/l was nearly as effective as 0.003 mg/l kinetin. Results of this experiment indicate that the ethanol extract contains at least two kinds of cytokinins, one being extractable with *n*-butanol and the other water-

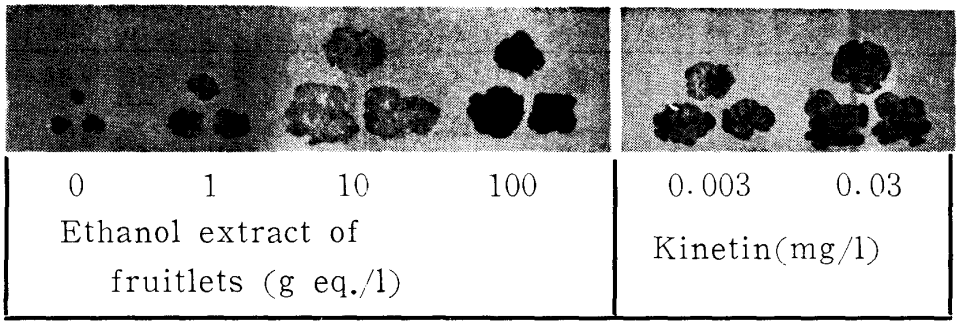

Figure 1. Tobacco callus tissues cultured on the media with ethanol extract of persimmon fruitlets and with kinetin. The extract was tested at the concentrations corresponding to 1, 10, and 100 g fresh fruitlet tissue per liter of medium.

Table 1. Effect of ethanol extracts of persimmon fruitlets and leaves on the growth of tobacco callus tissue. Extracts were tested at the concentrations equivalent to 1, 10, and 100 g fruitlet or leaf tissue per liter of medium. For futher details see text.

Additives	Concentration	Fresh weight per piece (mg)
—	—	21
Fruitlet extract	(g eq./l)	
	1	106
	10	548
	100	260
Leaf extract	1	45
	10	63
	100	6
Kinetin	(mg/l)	
	0.003	217
	0.03	401

soluble.

Miller purified zeatin ribonucleotide by precipitation with the successive additions of barium acetate and then of ethanol[12]. The same procedures were employed for purification of cytokinins in the aqueous fraction. However, precipitate fractions obtained from the aqueous fraction showed no cytokinin activity at 1, 10 and 100 mg/l, while the supernatant was slightly active at 100 mg/l, indicating that the cytokinin present in the aqueous fraction was remained in the supernatant without being precipitated by the treatments of barium acetate and ethanol, and that this factor is unlikely identical zeatin ribonucleotide. The supernatant fraction was insufficient for further purification.

Table 2. Effects of *n*-butanol fraction and aqueous fraction on the growth of tobacco callus tissue. Extracts were tested at the concentrations equivalent to 10 and 100 g fresh fruitlet tissue per liter of medium. For further details see text.

Additives	Concentration	Fresh weight per piece (mg)
—	—	12
	(g eq./l)	
Aqueous fraction	10	32
	100	115
n-Butanol fraction	10	136
	100	520
	(mg/l)	
	0.003	174
Kinetin	0.03	395

The *n*-butanol fraction which was prepared as described above from 2 kg fruitlets was purified as follows. 50 ml aqueous solution of the *n*-butanol extract was acidified to pH 3.0 with HCl, and passed through a 2×18-cm Dowex 50W column (H^+ and 50-100 mesh), which was washed with 150 ml water. The combined effluent and washing were evaporated yielding 2.69 g of dry substance (B-1). The column was eluted with 250 ml of 6 N NH_4OH. The eluate was concentrated to 20 ml, adjusted to pH 7.8, and passed additionally through a 2×18-cm column of Dewex 1 (formate and 100-200 mesh). The column was washed with 150 ml water. Evaporation of the combined effluent and washing yielded 84 mg of residue (B-2). Then the column was eluted with 250 ml of 4 M formic acid. This eluate gave 290 mg of dry residue (B-3).

B-1, B-2, and B-3 were tested at 1, 10, and 100 mg/l. As shown in Table 3, B-1 and B-2 were inactive or rather inhibitory, while B-3 was active considerably at 10 mg/l and highly at 100 mg/l. Thus the cytokinin (or cytokinins) in the *n*-butanol fraction was held on anion- and cation-exchange resins at conditions used.

Miller (1965)[12] identified the factors in corn kernels as zeatin, its ribonucleoside and ribonucleotide with paper chromatography. Such paper-chromatographic identification of cytokinins was employed also by Matsubara et al. (1968)[9] in *Corynebacterium fascians*

Table 3. Effects of partially purified preparations (B-1, B-2, and B-3) obtained from persimmon fruitlets on the growth of tobacco callus tissue. For further details see text.

Additives	Concentration (mg/l)	Fresh weight per piece (mg)
—	—	22
B-1	1	14
	10	16
	100	12
B-2	1	16
	10	14
	100	35
B-3	1	24
	10	122
	100	423
Kinetin	0.03	292

*t*RNA, and proved to be helpful especially in the case of small scale of extraction. In the present study identification of the cytokinin in the B-3 fraction with paper chromatography was attempted.

B-3 (10 mg) was developed on Whatman No. 1 filter paper with solvent A (water-saturated *n*-butanol). Bio-assay of eluates from chromatogram revealed that a peak of cytokinin activity was found at Rf 0.64-0.84, where zeatin, its ribonucleoside, dihydrozeatin and Δ^2-isopentenyladenosine co-chromatographed located (Fig. 2a), but Δ^2-isopentenyladenine did differently. When B-3 was chromatographed likewise with solvents B, C, and D, chromatograms showed a main cytokinin activity at Rf 0.5-0.6 (Fig. 2b), 0.7 (Fig. 2c), and 0.2-0.3 (Fig. 2d) respectively. On these chromatograms, zeatin always located at the position of the persimmon cytokinin, while Δ^2-isopentenyladenine and its ribonucleoside moved quite differently in these solvent systems, zeatin ribonucleoside in solvent B, and dihydrozeatin in solvents B and D. These results indicate that the persimmon cytokinin in B-3 is probably a factor very closely related to zeatin and is not identical to zeatin ribonucleoside, dihydrozeatin, Δ^2-isopentenyladenine or its ribonucleoside.

In order to ascertain whether or not the persimmon cytokinin in B-3 is identical to zeatin, additional studies with paper chromatography by using different solvent systems were carried out. All the B-3 (290 mg) was developed with solvent A, and the eluate from the active zone of the chromatogram was re-chromatographed likewise with solvent B and C successively. The eluate from active zone of the final chromatogram was divided into four parts, and these were re-chromatographed with solvents A, B, E, and F, individually. Bio-assays revealed that all the chromatograms showed one active peak. Chromatograms developed with solvents A and B gave almost same patterns of location of cytokinin activity to Figs. 2a and 2b, except with a slight difference between Rf of the main active peak and that of zeatin. In solvent E (water) the persimmon cytokinin was at about Rf

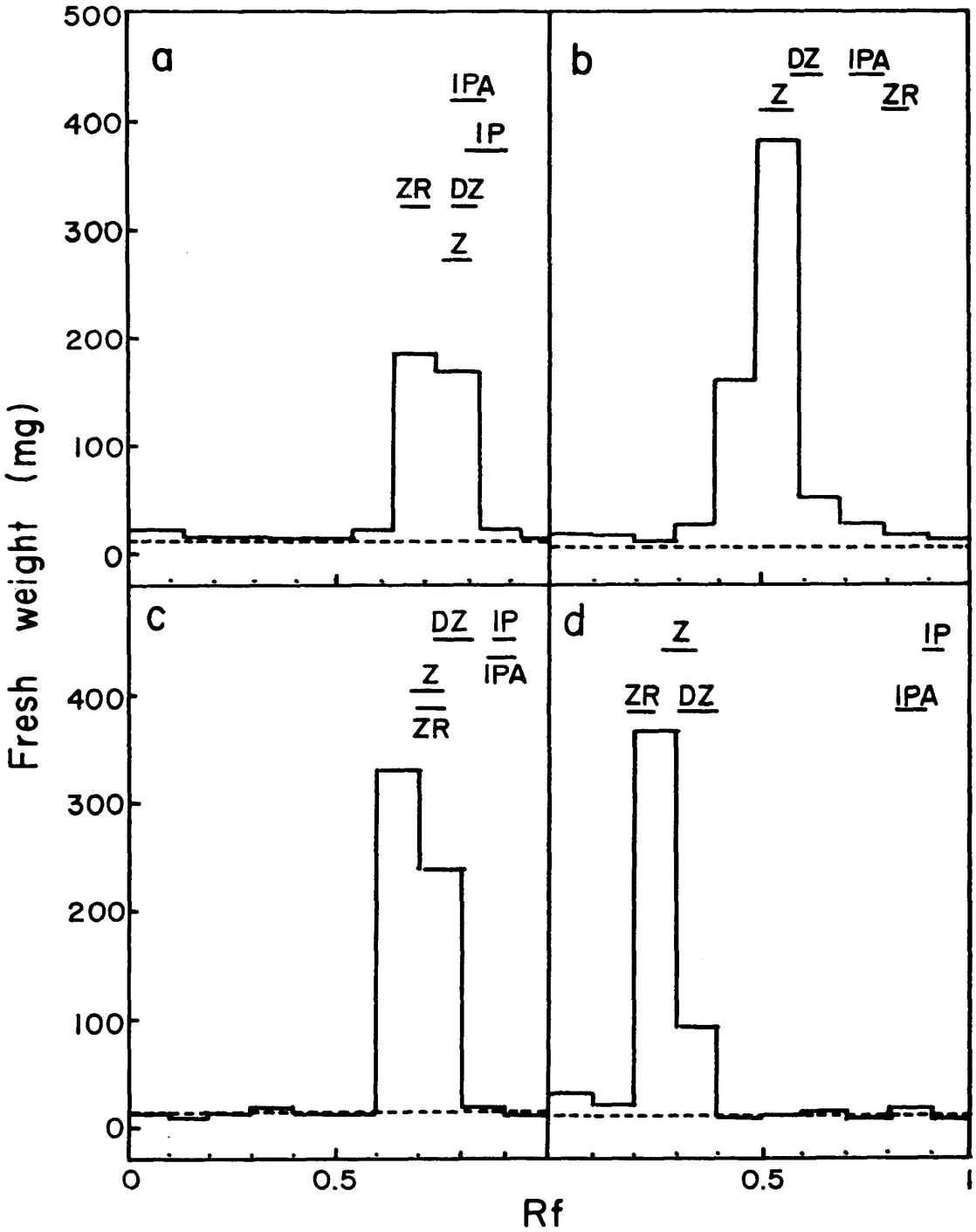

Figure 2. Location of cytokinin activity on paper-chromatograms of partially purified extract of persimmon fruitlets (B-3). Chromatograms were developed with water-saturated *n*-butanol (a), 0.02 M boric acid (pH 8.4 with NaOH) (b), *n*-butanol : NH_4OH (4:1) (c), and ethylacetate : formic acid : water (60:5:35) (d). Lines at the top of the histograms indicate the loci of zeatin (Z), zeatin ribonucleoside (ZR), dihydrozeatin (DZ), Δ^2-isopentenyladenine (IP), and Δ^2-isopentenyladenosine (IPA), when co-chromatographed with the extract.

0.4 (Fig. 3a), while zeatin migrated at Rf 0.5, and other cytokinins co-chromatographed showed apparently different Rf values. In solvent F, *n*-butanol : formic acid : water (80 :

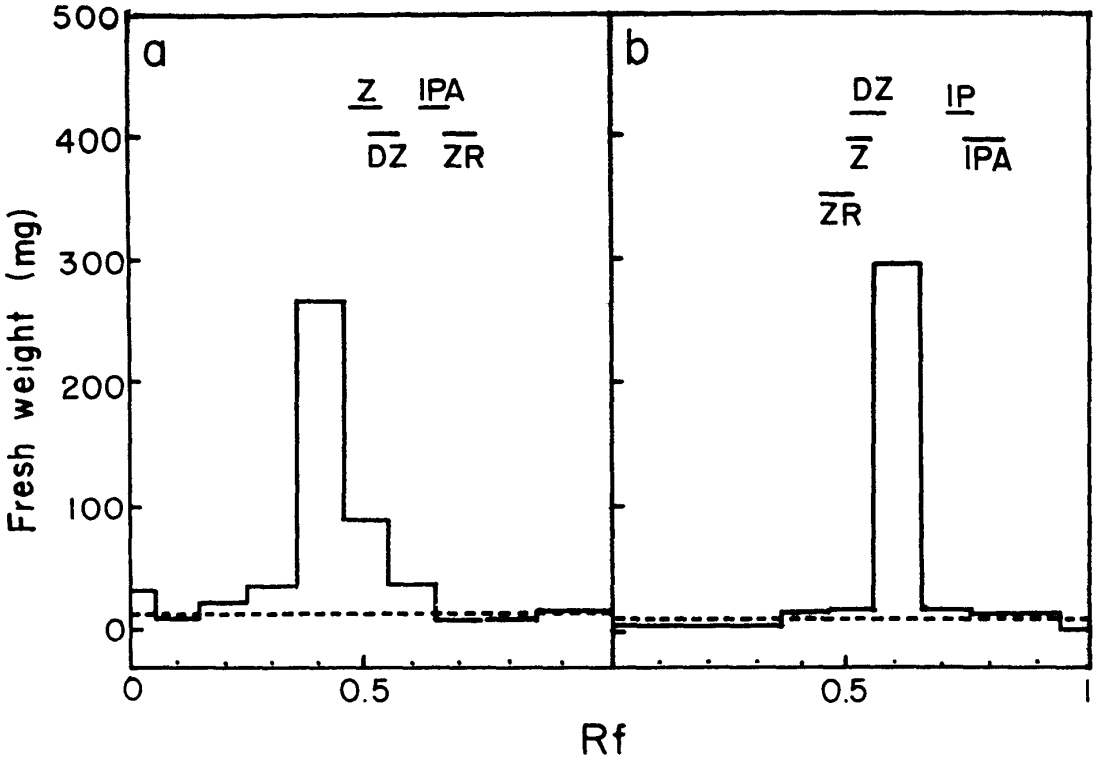

Figure 3. Location of cytokinin activity on paper-chromatograms of the active fraction obtained from B-3. The chromatograms were developed with water (a) and *n*-butanol : formic acid : water (80 : 15 : 15)(b). For further details see text and Figure 2.

15 : 15), the Rf of the persimmon cytokinin was about at 0.6 (Fig. 3b), but that of zeatin at 0.52. Hence, these results support a conclusion that the persimmon cytokinin in B-3 is apparently distinguishable from zeatin, however it is closely related substance, probably a novel cytokinin. Unfortunately there was insufficient for detecting this factor as UV-quenching spot and for spectral characterization.

Discussion

In the present study the ethanol extract of persimmon fruitlets showed a strong cytokinin activity at the concentration equivalent to 10 g fresh fruitlets tissue per liter of medium. Such level of activity was observed generally in the cases of cytokinin-rich materials such as young seeds of *L. luteus*[5] and proved that the persimmon was one of hopeful materials for studies on natural cytokinins. Extraction of the ethanol extract with *n*-butanol showed that the fruitlets contained at least two kinds of factors, one was *n*-butanol soluble and the other water-soluble. Cytokinin activity of the *n*-butanol fraction was considerably stronger than that of the aqueous fraction. Less effectiveness of the aqueous fraction may be caused by less amount or less activity of cytokinins or the presence of some inhibitory substances. Such conditions which control level of cytokinin activity are dependent on the age of fruitlets. Letham showed that the cytokinin activity of apple fruitlet extracts was greatest during the period of intense cell division and that declined markedly about the time of cessation of division[13]. In the case of persimmon

fruitlets the earlier stage seems to contain abundant cytokinins[10]. Hence, serial and systematic studies on contents and kinds of cytokinins as well as on the inhibitory substances are required.

Following the isolation of zeatin from immature corn kernels[14] related substances have been isolated from various plants[5,7,12]. Some of these cytokinins were found in *t*RNAs of various organisms[9,15,16]. In the present study the persimmon cytokinin was compared with such known natural cytokinins in paper chromatography in order to perform probable identification. When the B-3 fraction was developed with solvents A-D, the active factor was always found at the position of zeatin, suggesting that the persimmon cytokinin was closely related to zeatin. Other natural cytokinins co-chromatographed gave different Rf values from the persimmon cytokinin. However in solvents E and F, this cytokinin was apparently distinguished from zeatin. In this study zeatin ribonucleotide was not used for co-chromatography, because unavailable. However, this cytokinin is not extracted with *n*-butanol from aqueous layer, while the persimmon cytokinin in B-3 was *n*-butanol soluble. In addition paper-chromatographic behavior of the persimmon cytokinin in solvent A, compared with Miller's data[12], revealed that they are different substances.

Ribonucleoside and ribonucleotide of dihydrozeatin should move more rapidly than the persimmon cytokinin in solvents B and E, although they have not been reported to be present. Therefore they are not identical to the persimmon cytokinin. Ribonucleotide of Δ^2-isopentenyladenine which has not been found in unbound form is thought to move more rapidly than Δ^2-isopentenyladenosime in soluent E and of course the persimmon cytokinin. Thus all the evidences obtained above support a conclusion that the persimmon cytokinin in the B-3 fraction is a novel cytokinin.

Recently Burrows et al. (1968)[17] and Hecht et al. (1969)[18] isolated new cytokinins from *t*RNAs of *Escherichia coli* and wheat germ respectively. The presences of these cytokinins in unbound form have not been shown. Nevertheless these new cytokinins and still unknown cytokinins may be distributed widely in different species of plants. The persimmon cytokinin could be one of such new or unknown substances. Isolation of this cytokinin from larger amount of materials and chemical identification are required.

References

1) Miller, C. O., Ann. Rev. Plant Physiol. **12** : 395 (1961).
2) Letham, D. S., ibid **18** : 349 (1967).
3) Matsubara, S., Bot. Mag. Tokyo **77** : 403 (1964).
4) Matsubara, S., Koshimizu, K., and Nakahira, R., Sci. Rep. Kyoto Pref. Univ. No. **17** (A) : 5 (1966).
5) Matsubara, S. and Koshimizu, K., Bot. Mag. Tokyo **79** : 389 (1966).
6) Matsubara, S. and Nakahira, R., ibid **80** : 373 (1967).
7) Koshimizu, K., Matsubara, S., Kusaki, T., and Mitsui, R., Agr. Biol. Chem. **31** : 795 (1967).
8) Matsubara, S., Koshimizu, K., and Nakahira, R., Sci. Rep. Kyoto Pref. Univ. No. **19** (A) : 19 (1968).
9) Matsubara, S., Armstrong, D. J., and Skoog, F., Plant Physiol. **43** : 451 (1968).

10) Personal communication from Prof. Y. Sobajima (Kyoto Pref. Univ.).
11) Linsmaier, E. M. and Skoog, F., Physiol. Planta. **18** : 100 (1965).
12) Miller, C. O., Proc. Natl. Acad. Sci. (US), **54** : 1052 (1965).
13) Letham, D. S., New Zeal. Jour. Bot. 1 : 336 (1963).
14) Letham, D. S., Life Sci. **2** : 569 (1963).
15) Skoog, F., Armstrong, D. J., Cherayil, J. D., Hampel, A. E., and Bock, R. M., Science **154** : 1354 (1966).
16) Hall, R. H., Csonka, L., David, H., and McLennan, B., ibid **156** : 69 (1967).
17) Burrows, W. J., Armstrong, D. J., Skoog, F., Hecht, S. M., Boyle, J. T. A., Leonard, N. J., and Occolowitz, J., ibid **161** : 991 (1968).
18) Hecht, S. M., Leonard, N. J., Burrows, W. J., Armstrong, D. J., Skoog, F., and Occolowitz, J., ibid **166** : 1272 (1969).