Changes in Cytokinin Concentrations in Root Eycudate during the Development of Sweet Protato Plants

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Changes in Cytokinin Concentrations in Root Exudate during the Development of Sweet Potato Plants*

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Summary

Presence and levels of cytokinins in the root exudate of sweet potato (*Ipomoea batatas* Lam. cv. Kohkei No. 14) were determined at five different stages during the exponential growth phase by means of mass spectrometry using deuterium-labeled standards. Both trans-zeatin and trans-ribosylzeatin had a predominant share of 92–97% of total cytokinin levels found throughout the plant development, while cis-zeatin, trans-9-glucosylzeatin. N^6-isopentenyladenosine and 9-glucosyl-N^6-isopentenyladenine were at very low levels. No cis-ribosylzeatin and cis-9-glucosylzeatin were detected. The total concentrations of both trans-zeatin and trans-ribosylzeatin increased gradually from 108 nM to 194 nM in correspondence with the plant development. It seems, therefore, that the greater supply of these two substances in root exudate may be responsible for the active growth of adventitious buds and tuberization of tuberous roots of sweet potato plants.

INTRODUCTION

Cytokinins in sweet potato (*Ipomoea batatas* Lam.) have been identified in connection with the development of tuberous roots by means of *Amaranthus* betacyanin bioassay and mass spectrometry.[21,32,33] The major cytokinin species were trans-RZ, trans-Z, iPG and iPA: trans-RZ, iPG and iPA occurred abundantly in the roots and the tuberous roots, while trans-RZ, cis-RZ and iPG were more prevalent in the stem and leaf.[32] Cytokinins, in general, are produced by the roots and transported to the stem and leaf, where they are involved in the regulation of the growth.[4,6,15,19] Transport of cytokinins in the xylem sap has been demonstrated in both herbaceous[2,3,5,7,8,14,15,18,25,26,27,30,37,38] and woody plants.[1,13,31,40,41] However, their presence in the root exudate of sweet potato has not been characterized. By application of mass spectrometry using deuterium-labeled standards,[9] I have characterized the endogenous cytokinin species in the root exudate of sweet potato plant and examined whether or not quantitative and qualitative changes in cytokinins occur there. This communication describes the chracterization of six cytokinin species as well as the changes in their concentration during plant development.

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* Part of this study was made at Tokyo University of Agriculture and Technology
Abbreviations: Z, zeatin; RZ, ribosylzeatin; GZ, 9-glucosyl-zeatin; iPA, N^6-isopentenyladenosine; iPG, 9-glucosyl-N^6-isopentenyladenine; HPLC, high-performance liquid chromatography;

MATERIALS AND METHODS

Root exudate. Vine cuttings of sweet potato (*Ipomoea batatas* Lam, cv. kohkei No. 14) were planted at university farm (Fuchu, Tokyo) on April 30th, 1985. In July and August the plants were cut at about 15 cm above the ground at 5 p.m., and the root exudate was then led into a cylindrical bottle through a rubber cap. The bottle was covered with a polymer sheet. At 9 a.m. at the next day, the exudate was collected. After filtration, the filtrate was mixed with an equal amount of absolute ethanol, and then stored at −20℃ until purification.

Chemicals. Deuterium-labeled standards used for the experiments were prepared as descirbed previously.[9,32,33] Z, RZ and GZ consisted of the trans-isomer (92%) and cis-isomer (8%).

Purification of cytokinins from the exudates. To the exudates summarized in Table I were added Z–d_5, RZ–d_5, GZ–d_5, iPA–d_6 and iPG–d_6 as internal standards. The solution was evaporated to dryness, and the residue was redissolved in distilled water (2 m*l*). The solution was passed through a Sep-Pak C_{18} Cartridge (1 cm × 1 cm i.d., Waters, U.S.A.). The cartridge was washed with 2 m*l* distilled water, and eluted with 16 m*l* of 40% (v/v) methanol. The eluate was evaporated to dryness, and the residue was redissolved in a small volume of 40% (v/v) methanol. When there was any insoluble precipitate, the material was filtered off with a membrane filter. The filtrate was applied to a Radial-Pak Cartridge C_{18} (10 cm × 8 mm i.d., Waters, U.S.A.). The column was eluted with 40% (v/v) methanol to give three fractions, Z+RZ+GZ, iPA and iPG fractions according to the elution volume of the authentic specimen. Each fraction of iPG and iPA was separately purified by a second HPLC using a solvent of 22% (v/v) acetonitrile. The fraction containing Z, RZ and GZ was also purified with 2nd HPLC using 12% (v/v) acetnitrile to give a Z+RZ fraction and GZ fraction. The GZ fraction was finally purified by HPLC with a solvent of 20% (v/v) methanol. The Z and RZ in the fraction of the Z+RZ were each resolved to a single fraction with an Asahipak GS 320 column (Asahikasei Corp., Japan) using a solvent of 60% (v/v) methanol containing 0.4% ammonia. The stereoisomers of Z were separated by HPLC with a solvent of 30% methanol. In the above purification system, the internal standards added to the exudate were recovered together with endogenous cytokinins.

High-performance liquid chromatograph. A Shimadzu model LC–3A equipped with a UV detector (270 nm) was used.

Trimethylsilylation. Each fraction of RZ, GZ, iPA and iPG in a mini-vial was dried in *vacuo* over phosphorous pentoxide at 90℃ for 30 min.. To the dry residues were added pyridine (35 μ*l*), hexamethyldisilazane (10 μ*l*) and trimethylchlorosilane (5 μ*l*). The mixtures were heated at 120℃ for 1 hr.. Fractions of cis-Z and trans-Z in mini-vials were dried over phosphorous pentoxide and silylated with a mixture of acetonitrile, bis(trimethylsilyl)acetamide and trimethyl-

Table 1 ROOT EXUDATE PRODUCED BY SWEET POTATO OVER A PERIOD OF 15 HOURS AT DIFFERENT STAGES OF GROWTH

Age at sampling (days)	65	81	88	94	100
Number of plants	10	7	8	6	6
Root exudate (m*l*)	49	210	165	158	72

Table 2 AMOUNTS OF DEUTERIUM-LABELED STANDARDS ADDED TO THE ROOT EXUDATE OF SWEET POTATO, AND THE IONS USED FOR MASS SPECTRAL QUANTIFICATION

Cytokinin	Amount (μg) Exudate	Identity	Monitoring ions m/z Labeled	Unlabeled
Z-d_5	6.8	Z-3TMS (M^+)	440	435
RZ-d_5	7.8	RZ-4TMS ($[M-15]^+$)	629	624
GZ-d_5	3.7	GZ-5TMS ($[M-90]^+$)	656	651
iPA-d_6	2.7	iPA-3TMS (M^+)	557	551
iPG-d_6	2.4	iPG-4TMS (M^+)	659	653

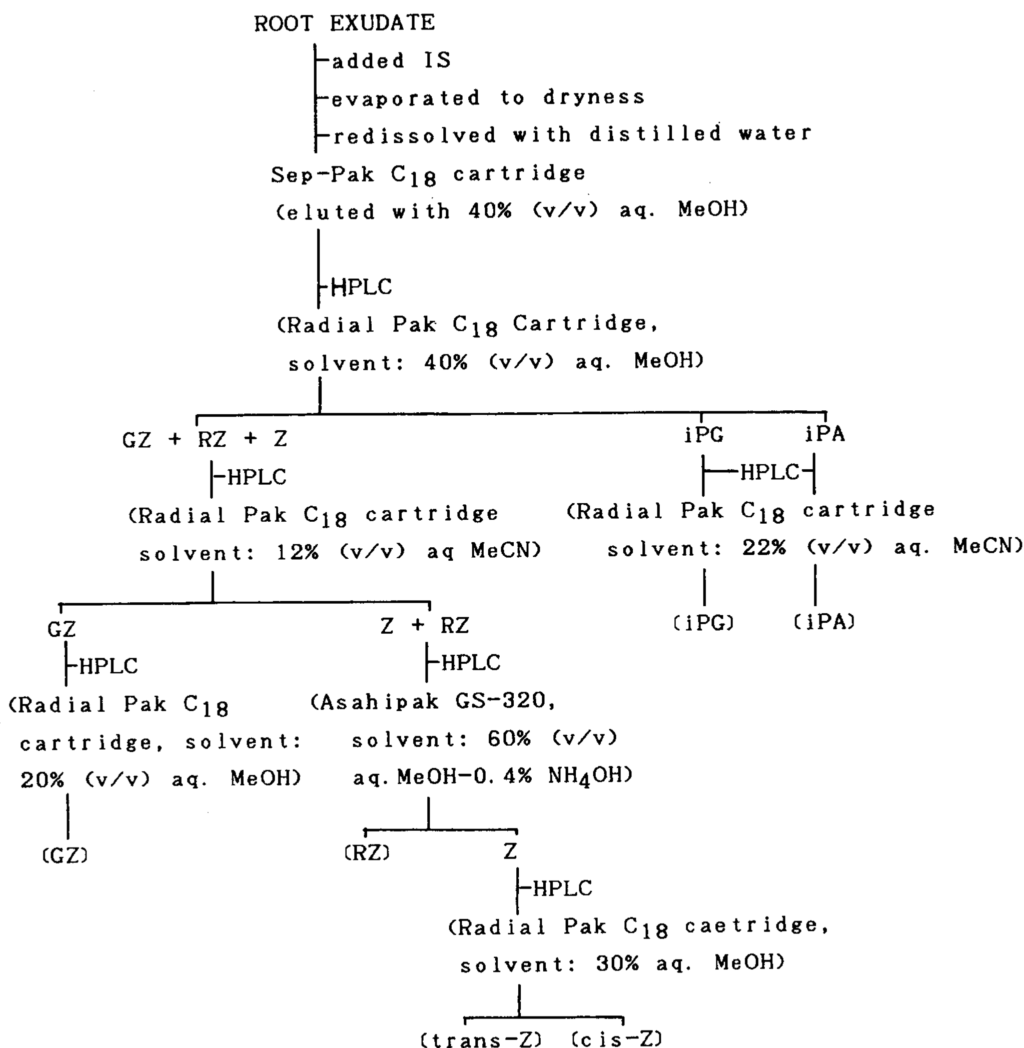

Fig. 1 Procedures used for Purification of Cytokinins from the Root Exudate of Sweet Potato.

chlorosilane (20 : 10 : 1, v/v/v) at 80℃ for 20 min..

Selected ion monitoring. A Shimadzu LKB 9000 gas chromatograph-mass spectrometer equipped with a multiple ion detector was used under the following conditions: ionizing energy, 20 eV: separating port temperature, 280℃: and ion source temperature, 290℃. OV-1 (1.5%) on silylated Chromosorb in a glass column (1 m × 3 mm, i.d.) was used at 30 m*l* of helium per min., The column oven temperatures were 210℃(Z), 250℃(iPA), 260℃(RZ) and 270℃(iPG and GZ).

RESULTS

The exudates shown in Table I were collected at five different tages during the exponential growth phase from July to August in 1985. To each root exudate was added the deuterium-labeled cytokinins as internal standards summarized in Table II. The purification procedures are shown in Fig. 1. Any salt present in the exudate was excluded with a Sep-Pak C_{18} cartridge. A preliminary experiment was demonstrated that cytokinins in aqueous solution were held in the cartridge, and were not eluted even when the cartridge was washed with a small volume of distilled water. This was determined because no cytokinin activity was detected in the passage solution or washed water in an *Amaranthus* betacyanin bioassay. Z and RZ could be separated into a single fraction by a Asahipak GS-320 column using a solvent of 60% (v/v) aqueous methanol containing 0.4% (v/v) ammonia. The steroisomers of Z were also satisfactorily separated by HPLC. The internal standards together with endogenous cytokinins were recovered according to the elution volume of authentic specimen by the above purification system with HPLC.

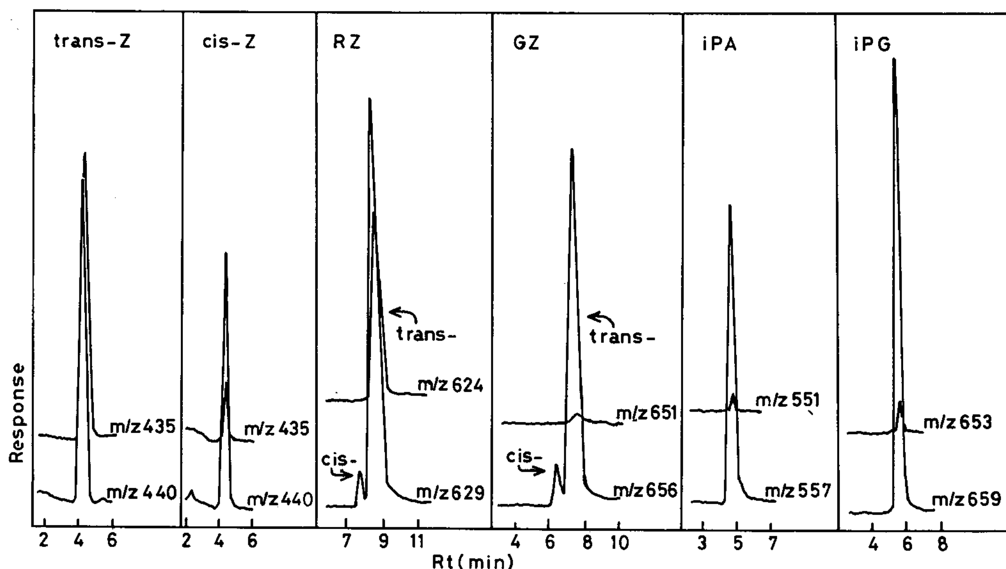

Fig. 2 SIM Chromatograms of Z, RZ, GZ, iPA and iPG Fractions Recovered from the Root Exudate (81 days) GC conditions: 1.5% OV-1, 1 m, 30 m*l*/min. of He, column oven temp. at 210℃ (Z), 260℃ (RZ), 250℃ (iPA) and 270℃ (GZ, iPG).

Selected ion monitoring of the trimethylsilylation products of the purified fraction is shown in Fig. 2. The identity of the peaks was confirmed by their complete mass spectra. Peak height ratios of the endogenous cytokinins to the internal standards were determined, and correction for the unlabeled materials was made from a standard calibration curve. The result was that six cytokinin species: trans-Z, cis-Z, trans-RZ, trans-GZ, iPG and iPA were identified in the root exudates whereas no cis-RZ or cis-GZ was detected in any stage. Figure 3 present cytokinin molar concentrations in the root exudates at five different stages. Trans-Z and trans-RZ were most abundant in the root exudate throughout the development of sweet patato. Others cytokinins chracterizing in the root exudate were in the range of 1–7 nM with little fluctuation during plant development.

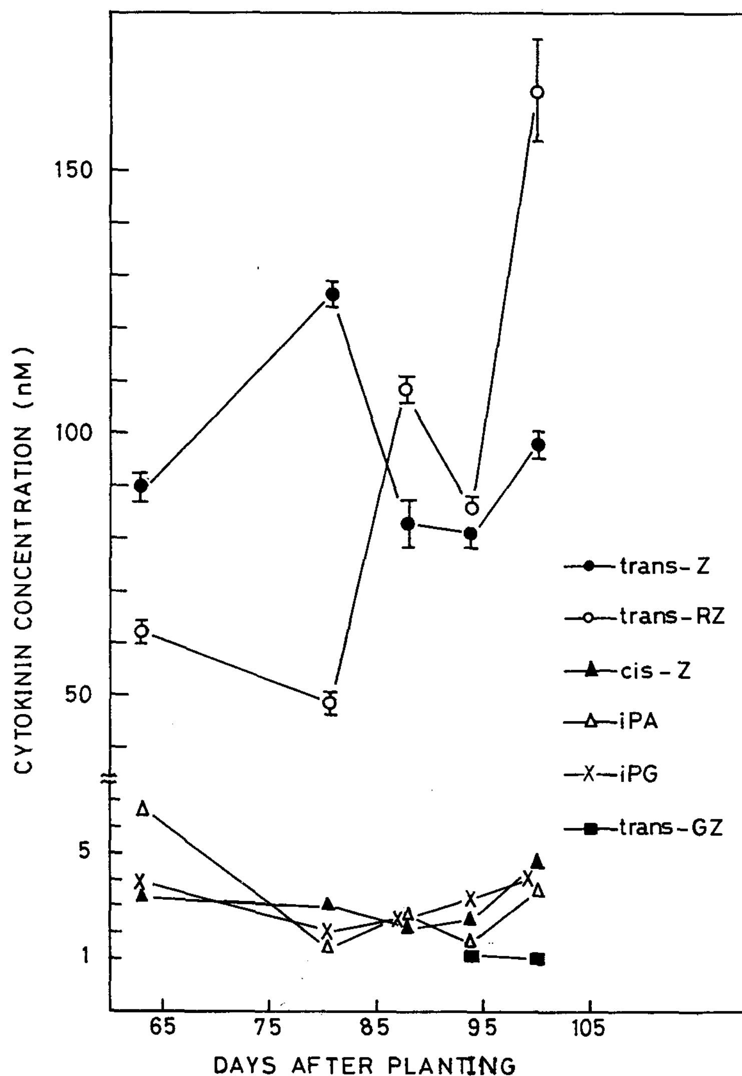

Fig. 3 Cytokinin Concentrations of the Root Exudate of Sweet Potato Determined by Mass Spectrometry. Bars indicate ±SD of three replicates.

DISCUSSION

It has recently been reported that Z and RZ are the major cytokinin species in exudates;[3,7,8,13,14,15,25,27,30,40,41] this was determined by radioimmunoassay and GC/MS. Their stereoisomer, however, was unclear. The results demonstrate that trans-isomers of both Z and RZ are the principal forms of cytokinin transported in the root exudate of sweet potato. Both trans-Z and trans-RZ were abundant with a share of 92–97% of total cytokinin levels during plant development. In contrast, the levels of cis-RZ, iPA, iPG and trans-GZ, which were relatively high in the roots, tuberous roots and shoots,[33,34] were very low and sometimes undetectable in the root exudate throughout the development. It is well known that trans-Z and trans-RZ have very high cytokinin activity.[18] Therefore, their contribution to the regulation of the development of sweet potato is considered to be great.

Trans-Z concentrations were higher than those of trans-RZ in early stages, but thereafter trans-RZ increased markedly with decreasing trans-Z from mid to last stages. On the whole, the total concentration of both trans-Z and trans-RZ gradually increased from 108 nM to 194 nM throughout the plant development. The present results suggest that trans-Z and trans-RZ are supplied mutually to retain higher levels in the root exudate. Their relative proportion varied at about 80 days after planting, and the significance of this variation is unclear. It has been reported that total Z and RZ levels were high during vegetative growth, and decreased as flower buds developed,[5,14,41] and also that they were involved in enhancing shoot growth.[4] It seems, therefore, that trans-Z and trans-RZ transported in the root exudate were result in active growth of the stem and leaf with the formation of adventitious buds of sweet potato. Furthermore, cytokinins appear to be involved a sink-source relationships in crops.[24] The greater supply of both trans-Z and trans-RZ transported from roots to stem and leaf in the root exudates may be responsible for the tuberization of tuberous roots.

Sweet-potato shoots had a large amount of cis-RZ as reported previously,[32] whereas this substance was undetectable in the root exudate. Furtheremore, cis-RZ was a major cytokinin species in rice plant,[35] etiolated squash seedling,[17] unfertilized cones of hop,[39] tops of tobacco,[23,32] and tuber of potato.[12,22] These studies show a widespread occurrence of cis-RZ in plants, particurlarly in the shoots and the organs that originate from the stem; but the significance of the presence of cis-RZ is still controversial.[36] Carmi and Van Staden[4] pointed out that cytokinin levels in leaves were not totally suppressed by the removal of a large proportion of the roots. Koda and Okazawa[16] also repoted that the shoot apex produced cytokinin *in vitro*. It seems, therefore, that cis-RZ may be synthesized in the stem and leaf themselves to play some physiological function. Thus the significance of cis-RZ biosynthesis in there should be studied in future.

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サツマイモ成長期の根出液における
サイトカイニン濃度の変動

杉山　民二

要　　約

重水素標識体を内部標準とする質量分析法により，サツマイモの旺盛な成長期（5時点）に集めた根出液中のサイトカイニンを同定，定量した。trans-ゼアチンおよびtrans-リボシルゼアチンが主要サイトカイニンで，全成長期をとおして92%～97%を占めていた。一方cis-ゼアチン，trans-グルコシルゼアチン，N6-イソペンテニルアデノシンおよび9-グルコシル-N6-イソペンテニルアデニンは低レベルであった。cis-リボシルゼアチンおよびcis-グルコシルゼアチンは全く検出されなかった。trans-ゼアチンとtrans-リボシルゼアチンの総濃度はサツマイモの成長につれて108 nMから194 nMに徐々に増加した。根出液中の両化合物の高濃度化が不定芽の旺盛な成長および塊根の肥大化に関与していることが考えられる。