IDENTIFICATION OF cis-ZEATIN-D-RIBOSIDE FROM THE TOP OF TOBACCO PLANT

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One of the cytokinin-active ribonucleosides, cis-zeatin-D-riboside has been purified and identified from the top of tobacco plant (*Nicotiana tabacum* L. cv. Shiroenshu) by means of gas chromatography-mass spectrometry of the purified extract. The level of the endogenous cytokinin in the top of tobacco plant was estimated to be the order of 10 ug/kg. Furthermore, relatively simple techniques employed in this study are also discussed.

Tobacco plants seem to grow slowly at their early age because the seeds and the seedlings are extremely small. However the growth proceeds rapidly in a certain age after the transplantation. As an usual cultural practice, growing top or crown of the plant is pruned away at an early stage of flowering. The object of this practice is to protect them from producing seed, and thus force the synthesized carbohydrates and nitrogenous compounds to remain in the leaves for further growth and enrichment. This practice called topping thus results in larger, thicker, and darker leaves that

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mature faster and more uniformly than on untopped plants. This practice may suggest that the growth of the plants depends largely upon the level of the plant growth regulators which are present in the top. Nevertheless, essentially no investigations have been made on the endogenous regulators except a water soluble gibberellin.[1]

The endogenous cytokinins of the tobacco plants have also been the subject of only a few investigations. For example, Upper et al[2] attempted to detect and measure the cytokinins in extracts of tobacco plant, but they did not attain a clear results.

Identification and quantitation of the endogenous cytokinin of tobacco plant are significant not only from the standpoint of the growth regulation of tobacco plant but also for the evaluation of cytokinin bioassay method. From an earlier period of cytokinin researches until present time, the presence and levels of cytokinins have been measured by various bioassays. The most widely used is the tobacco callus bioassay, in which the difference between the growth of the callus tissues exposed to the cytokinin, and that of the control tissues, provides a measure of the potency and concentration of the cytokinin-active compounds.

This communication reports on the purification and identification of cis-zeatin-D-riboside from the top of tobacco plant.

The tops of tobacco plants (Nicotiana tabacum cv. Shiroenshu) with some flowers (3.3 kg) cultivated in the field of Nasu area, Tochigi Prefecture, Japan were harvested in July 1977 and dipped immediately into 99% ethanol (8 liters). The sample was stored at 6°C for 30 days. After filtration, the residue was extracted twice with 80% ethanol (10 liters) under similar conditions. The ethanol extracts were combined and fractionated as shown in

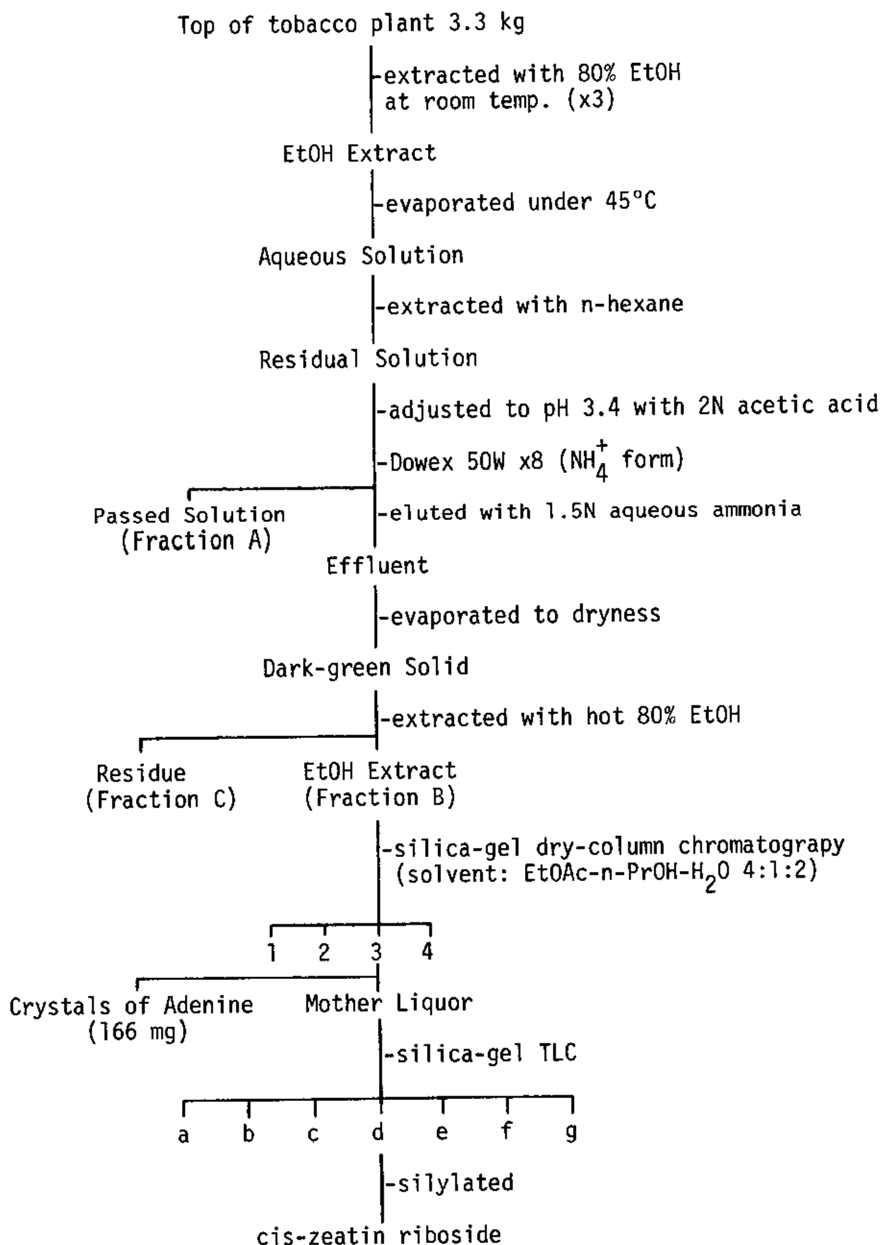

Fig. 1. Procedures for the purification of cis-zeatin-D-riboside from the top of tobacco plant

Fig. 1. The ethanol extracts were evaporated below 45°C, and the residual solution was extracted with 400 ml of n-hexane five times. The aqueous layer was adjusted to pH 3.4 with 2N-acetic acid, and passed through a column of Dowex 50W x 8 (NH_4^+-form, 100-200 mesh, 50 cm x 2 cm i. d.). The column was then washed with distilled water until neutral. The eluate and washings were combined and evaporated (fraction A). The column was subsequently eluted with 4 liters of 1.5N-aqueous ammonia and the eluate was evaporated to dryness below 45°C under reduced pressure. The dark-green solid was extracted with 300 ml of 80% hot ethanol thrice. Cytokinin activity of this ethanol extract (fraction B), the solid residue (fraction C), and the fraction A was assayed by tobacco callus method[3]. The strongest activity was observed in the fraction B. This result was also confirmed by a modified *Amaranthus* betacyanin bioassay, based on the cytokinin-induced formation of betacyanins in the cotyledons and hypocotyls of *Amaranthus caudatus* seedlings[4].

Fraction B was subjected to silica-gel dry-column chromatography (Merck silica gel 60 F_{254} 70-230 mesh; 40cm x 3cm i. d.; solvent, upper layer of ethylacetate-n-propanol-water 4:1:2 v/v). After elution, three UV absorbing bands called tentatively 1, 3, and 4 from the solvent front were observed. The column was then divided into four parts including the three UV absorbing bands and a non-UV absorbing zone between bands 1 and 3. Each of four parts was eluted with 80% ethanol and the eluate was evaporated to dryness. An aliquot of each residue was dried over phosphoric anhydride at 90°C in vacuo (2mm Hg) for 30 min, and silylated according to the procedure reported earlier[5]. An aliquot of the silylated product was injected into a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer with ionizing electron energy of 70 eV and 20 eV. The column was 1.5% OV-1 on silylated Celite 545, and temperature was programmed from 180°C to 270° at a rate of 6°C per minute. Since an indication of

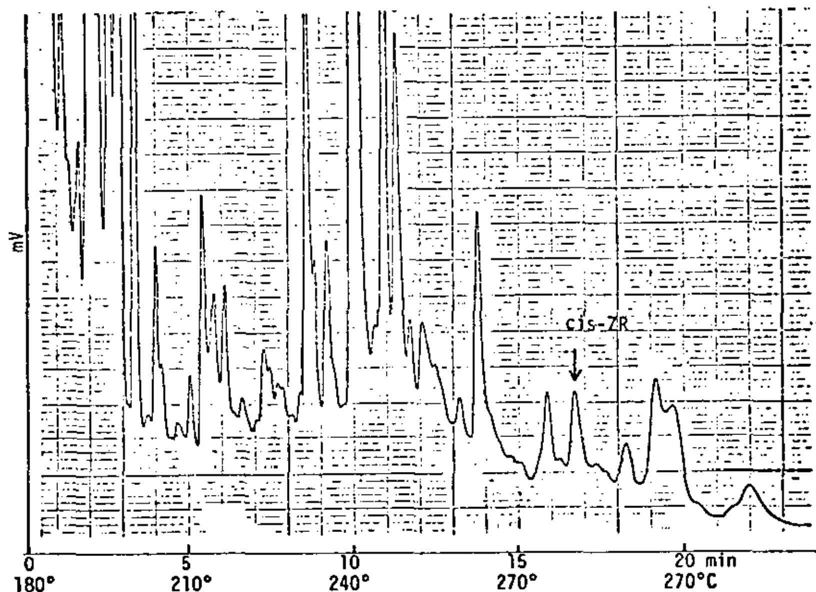

Fig. 2. A Typical TIM chromatogram of TMS derivatives of the fraction d.
Column: 1.5% OV-1 on silylated Celite # 545.
Temperature: programed from 180° to 270°C at a rate of 6°C per min.

Table 1. Relative retention values of cis- and trans-zeatin-riboside

cytokinin	retention time min	relative retention time
ipA-TMS_3	13.4	1.00
cis-ZR-TMS_4	17.0	1.27
trans-ZR-TMS_4	18.0	1.34
sample	16.9	1.26

ZR denotes zeatin-riboside.

the presence of zeatin-riboside in the fraction 3 was recognized by an alternative analysis of the histogram of Amaranthus bioassay, the molecular ion peak (M, m/e 639) and M-15 ion peak of zeatin riboside-4TMS were monitored respectively by using technique of single ion monitoring. The results indicated that the fraction 3 contains a compound in which the m/e of the molecular ion is 639 and that of M-15 ion is 624. The fraction 3 was then evaporated and stored in a refrigerator. After removal of the first crystals (166 mg) identified as adenine, the filtrate was evaporated and the syrup was subjected to preparative TLC (20cm x 20cm, thickness 0.75mm;Merck silica gel GF_{254} type 60; solvent, the same as the above). A zone of the Rf value 0.3-0.4 corresponding to that of zeatin-riboside and zeatin, was scraped and eluted with 80% ethanol. The eluate was evaporated to dryness, silylated, and then subjected to GC-MS under the conditions described before. A typical chromatogram (Fig. 2) and the mass spectrum (20 eV, M^+ m/e 639; M-15 624; M-TMSO 550; M-TMSOH 549; 536; 508; 483; 406; 348; 320; 292; 276; 259; 245; 230; 217; 201; 188; 156) indicated the presence of zeatin-riboside and the retention value corresponded to that of the cis-isomer. Although mass spectrometric differentiation of cis- and trans-isomers of this compound is impossible at the present, the both isomers are readily separated by gas chromatography[6]. Thus assignment of the cis-configuration was made by a comparison of the relative retention values of both synthetic standards and that of the sample(see Table 1).

This compound was originally reported as the constituent nucleoside of sweet corn tRNA[7] and then tobacco callus tRNA[8,9], wheat germ tRNA[10,11], and Pisum shoot tRNA[12], though zeatin was first isolated from young kernels of Zea mays[13]. Contrarily, a mixture of both cis- and trans-isomers was identified from the tRNA of young pea shoots.[14] However the identification of the free

form of cis-zeatin-riboside from plant tissue appear to be one of the rare example[15]. In this connection, it should be noted that trans-zeatin and its riboside were isolated and identified from the spring sap of sycamore(Acer pseudoplatanus L.)[16], the shoot apices of Mercurialis[15], and trans-zeatin from the root of radish (Raphanus sativus L. cv. Sakurajima)[17], whereas cis-zeatin was identified from the culture of Corynebacterium facians,[18] In addition, cis- and trans-unspecified zeatin and its riboside were identified from the cone of hop plant (Humulus lupulus L.).[19] Meanwhile, the geometry of the side chain of this molecule is interesting from the viewpoint of the evolution of tRNA molecules. According to Kaminek's postulate[20] concerning this problem, cis-zeatin-riboside identified here may be interpreted as that of tRNA origin. But, the possibility of the biosynthesis of this compound not involving the degradation of tRNA still remains.

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