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STUDIES ON SHOOT REDIFFERENTIATION
FROM
CULTURED TOBACCO CELLS

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CONTENTS

INTRODUCTION

Differentiation in organisms is one of the basic problems in physiology and biochemistry. The most remarkable difference between animals and plants is that the direction of differentiation for each cell is determined: a) in the early stage of development in animals, b) at a certain time in appropriate environment in plants. This characteristic of plant cell differentiation reflects its great capacity to adapt to the environment. Research on differentiation in microorganisms and animals has made great progress. However, research on higher plants has been insufficient.

Techniques for plant tissue and cell culture have, however, advanced rapidly, which has given us an advantageous experimental system for differentiation: differentiated cells → dedifferentiated cultured cells → redifferentiated cells. It has now been demonstrated that many species of living cells from mature plant organs retain their capacity to divide after being excised and cultured. Since it is generally held that these cultured cells are totipotent, it should be possible to direct them into a variety of developmental pathways by placing them in appropriate environments, in particular hormonal environments. This is the basic assumption which underlies differentiation studies with cultured plant cells.

Plant tissue and cell culture is believed to have originated with the work of Haberlandt in 1902[1]. He stated clearly the desirability of culturing isolated vegetative cells of higher plants,

"To my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants in simple nutrient solutions have been made. Yet the results of such culture experiments should give some interesting insight into the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within the multicellular whole organism are exposed. - - -, if we could culture isolated plant cells, then we could demonstrate experimentally the suspected totipotency of all the living cells of higher plants and, in so doing, the way might be opened to direct and reverse experimentally the processes of cellular differentiation."

While he was the first to express the idea of plant tissue and cell culture, he was not able to succesfully carry out his hypothesis, which was probably due to the fact that he attempted to culture mature cells and that the nutrients used may not have been adequate[2-5]. In spite of his failure, his idea continued to live, and many experiments were done during the following 30 years.

In 1934, White[6] succeeded in the continuous culture of tomato root tip on artificial media. Gautheret[7] also reported that pieces of cambial tissue continued to proliferate for some months.

In 1939, White[8], Nobécourt[9] and Gautheret[10] independently reported their successes in the first unlimited culture of callus tissue composed of unorganized and dedifferentiated plant cells. The reasons for their successes may be numerous but probably use of a favorable type of tissue coupled with a favorable medium (vitamins[11] and indole-3-acetic acid (IAA)[12,13] etc.) were the

major contributing factors.   Since 1939, many significant contributions have been made: the use of coconut milk as a growth promotor[14], the successful culture of single cells[15], the discovery of kinetin as a promotor of cell division[16] and mass culture[17] to name a few.   In the past ten years, the techniques for tissue and cell culture have greatly progressed and have been used in many areas of the plant sciences[5,18].

Nobécourt[9] showed in his pioneer paper that cultured carrot cells could differentiate roots and White[19] described the differentiation of leafy buds when his cultured cells of Nicotiana were transferred to a liquid medium.   Thus, it was seen that as cultured cells aged they showed an increasing degree of organization and subsequent differentiation.   However, no immediate progress was made towards identifying the factor controlling this organogenesis[20].

Late in the 1950s Steward et al[21] showed that the whole carrot plant could be regenerated from cultured somatic cells through induction and the development of the embryoid (embryogenesis), using a medium containing coconut milk and growth regulators.   Reinert also reported the same results[22,23].   In contrast Skoog et al[24] demonstrated that in cultured tobacco cells, roots or shoots could be induced separately by varying the ratio of kinetin/IAA in a completely synthetic medium (organogenesis).   These two types of differentiation (embryogenesis and organogenesis) showed that cultured cells are totipotent and that the direction of differentiation can be mainly controlled by growth regulators.   In particular,

organogenesis is regulated by the combination of auxins and cyto-kinins. Thus, a system of differentiation has been established using cultured plant cells. Table I shows the typical organo-genesis in cultured cells in a completely synthetic medium.

Growth regulators are key materials which control differenti-ation. For callus induction, auxin as the growth regulator is required. Auxin(2,4-D) has been reported to be incorporated into cells and to bind with proteins, particularly nucleoproteins, to induce DNA replication and subsequent cell division[33-38]. Cytokinins play an important role in organogenesis in cultured plant cells, especially in tobacco cultured cells(Table I). The molecular basis for this cytokinin control during organogenesis is unknown, although cytokinins are defined as compounds which promote cell division, and have many physiological effects on intact plants, excised plants and cultured cells[39.40].

The differentiation system in a cell culture has another important problem in regard to totipotency. When callus(cultured cells) is induced and an organ is regenerated, the interesting phenomenon of chromosomal variability occurs. Cultured dividing cells very often show varied euploidy and/or aneuploidy[41-45]. It is very curious that higher polyploid or aneuploid cells can divide. Moreover, whole plants with various ploidies have been reported to be regenerated from cultured cells[46-49]. Aneuploid regenerated plants have also been obtained[49]. And cultured cells preserving their diploidy are also often reported. Clarifying

Table I. Organogenesis in Cultured Cells.

Plant Species	Conditions for Organogenesis	Organ	Ref. No.
Daucus carota	IAA(5,10,20 ppm) +Kin(0.05ppm)	Root	25
	IAA(2.5ppm) +Kin(0.1,0.2,0.3ppm)	Shoot and Plantlet	
Nicotiana tabacum	Nitsch-H+IAA(0.1ppm) +Kin(0.02ppm)	Bud	26
	Nitsch-K	Root	
	RM-1964+IAA(2ppm) +Kin(0.1-0.2ppm)	Root	27
	RM-1964+IAA(2ppm) +Kin(1ppm)	Bud	
	Modified White +IAA(2ppm)+Kin(0.02ppm)	Root	24
	Modified White +IAA(2ppm)+Kin(0.5, 1.0ppm)	Bud	
	RM-1962+Kin(5ppm)	Bud	28
Oryza sativa	RM-1962+2,4-D(10^{-7}M)	Shoot	29
	RM-1964 only	Shoot and Plantlet	30
Triticum aestivum	White only, +2,4-D(0.1-0.2ppm), or +Kin(0.2ppm)	Shoot	31
	White+IAA(2ppm) +Kin(0.2ppm)	Root	
Avena sativa	RM-1964 only, or +Kin(0.46uM)	Shoot and Slight Root	32

these features of cultured cells is a most difficult problem,

but it represents one possible way for understanding the nature of

the regulation of differentiation.

Some changes in the metabolic patterns observed in dedifferen-

tiated cultured cells are associated with the beginning of morpho-

genesis.   These changes have been studied with the hope of deter-

mining the relationship between metabolic differentiation and

morphogenesis.   Changes in nucleic acid metabolism[50-52], protein

metabolism[51,53-55], enzyme activity[56,57], starch metabolism[58,59]

and alkaloid production[60,61] have been reported in relation to

organogenesis.   But there has been no inclusive biochemical and

molecular explanation of organogenesis.   The following appears

to be the key to the most basic mechanism of organogenesis; cell

differentiation is based almost certainly on the regulation of

gene activity[62].   The regulation of gene activity involves the

regulation of DNA replication and transcription etc.   However, to

date very few studies from this point have been made in relation

to organogenesis in cell culture[63].

To understand organogenesis in cultured plant cell systems

the following four points must be clarified: 1) the properties of

cultured dedifferentiated cells; 2) the mode of action of growth

regulators; auxins and cytokinins; 3) the gene activation and in-

activation which directs differentiation; and 4) the biochemical

changes associated with morphological development.

The purpose of the present study was to obtain information on the biochemical and molecular processes of organogenesis in a cultured tobacco cell system and on the properties of cultured cells, themselves. First the system of shoot redifferentiation by cytokinins was established for cultured tobacco cells. Then the relationships of the structure of cytokinins to shoot formation and the metabolism of ^{14}C-labeled kinetin were described. The following biochemical changes accompanying shoot redifferentiation were studied: changes in protein synthesis and the properties of RNA polymerase as one participant in transcription during shoot redifferentiation. Based on the results of this investigation, the mechanism of shoot redifferentiation by cytokinins in cultured cells is discussed.

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CHAPTER I

SHOOT REDIFFERENTIATION FROM CULTURED TOBACCO CELLS BY CYTOKININS IN VITRO[1,2].

INTRODUCTION

Cultured plant cells(callus) are believed to have totipotency and this is thought to be influenced by environmental conditions, in particular by the presence of plant growth regulators, which developes embryogenesis or organogenesis(organ formation).

Experimental studies of organogenesis took a modest leap forward during the 1950s as a result of the discovery of cytokinins. Skoog et al[3] demonstrated that in cultured tobacco cells a high cytokinin/auxin ratio induced shoot formation and a low ratio induced root formation. Others have also reported organ formation from cultured cells(dedifferentiated cells)[4,5].

Organ induction is divided into two types, and is mainly dependent on the plant species. A low concentration of auxin or its absence in a medium induces organ formation in such cultures as rice cells[6]. Cytokinins are required for organ formation with or without auxins in such cultures as tobacco cells[3].

Some naturally occurring cytokinins and many synthetic ones have been reported[5], since the synthetic kinetin was found to possess cytokinin activity[8,9]. These cytokinins can promote cell growth and induce organ formation from cultured cells as described above.

Many morphological and physiological changes occur with cell division during organogenesis[10-13]. In many cases, the differentiation of tracheid-like cells and vascular bundle cells is observed with the organization of cells during the early stages of shoot formation.

This chapter describes the establishment of a system for shoot formation by cytokinins and some effects of cytokinins on shoot formation, using cultured tobacco cells. Morphological changes during the early stages of shoot formation by cytokinin and some physiological properties of tobacco cells cultured with cytokinin are also described.

MATERIALS AND METHODS

Chemicals.

Zeatin and (±)-dihydrozeatin(Fig. 2) were provided by Dr. K. Koshimizu of the Department of Food Science and Technology, Kyoto Univ. Auxin analogues(Fig. 1), IPA(indole-3-propionic acid), 2,4-DG(N-(2,4-dichlorophenyl)glycine) and MPG(N-methyl-N-phenyl-glycine) were provided by Dr. S. Yamada of the Faculty of Pharmaceutical Sciences, the Univ. of Tokyo. All other chemicals were of reagent grade.

Callus induction and cell culture.

Pith tissue from the tobacco plant(Nicotiana tabacum var. Bright Yellow) was used as the plant material to induce cultured cells (callus). The synthetic medium described by Linsmaier and Skoog[14]

Table I. Composition of Medium

Basal Medium					
NH_4NO_3	1650	mg/l	H_3BO_3	6.2	mg/l
KNO_3	1900		$MnSO_4 \cdot 4H_2O$	22.3	
$CaCl_2 \cdot 2H_2O$	440		$ZnSO_4 \cdot 4H_2O$	8.6	
$MgSO_4 \cdot 7H_2O$	370		KI	0.83	
KH_2PO_4	170		$Na_2MoO_4 \cdot 2H_2O$	0.25	
Na_2EDTA	37.3		$CuSO_4 \cdot 5H_2O$	0.025	
$FeSO_4 \cdot 7H_2O$	27.8		$CoCl_2 \cdot 6H_2O$	0.025	

Sucrose	30	g/l
Thiamine·HCl	0.4	mg/l
myo-Inositol	100	mg/l
Agar	9	g/l
Plant growth substance(s)		
pH	5.7	

was used as the basal medium in the following experiments(Table I).
For the suspension culture, the same mediun with agar omitted was
used.

Segments of sterilized tobacco pith were inoculated on the agar
medium with 10^{-5} M 2,4-D.   The resulting callus (cultured cells)
was subcultured on the agar medium with 10^{-6} M 2,4-D.   Culture
conditions were 25-28°C and darkness.   The cell line obtained in
1968 was designated as strain T5.   Other cultured tobacco cells
were similarly induced and subcultured, using the various auxins
shown in Fig. 1.

To investigate shoot formation(redifferentiation) from cultured
cells(dedifferentiated cells), cells cultured with auxins were
transferred to an agar medium containing cytokinins (Fig. 2), then
cultured at 25-28°C in the dark.   Shoot formation was measured
after 50-70 days of culture.

These cultured cells were designated; 2,4-D cells, IBA cells,
zeatin cells, kinetin cells etc.

Microscopic observation.

2,4-D cells and zeatin cells in static cultures were fixed with
acetic acid:alcohol (1:3) for 3 hr.   After fixation cells were
dipped in polyethyleneglycol #1500 (50°C) for 1 hr, then in a
mixture of polyethyleneglycol #1500 and #4000 (1:1, v/v) for 1 hr.
These dehydrated cells were embedded in a mixture of polyethylene-
glycol #1500 and #4000 (1:1) and sectioned into 20-25 u slices.
Each section was placed on a slideglass and the polyethyleneglycol

$$R$$

$$(1) \quad -CH_2COOH$$

$$(2) \quad -(CH_2)_2COOH$$

$$(3) \quad -(CH_2)_3COOH$$
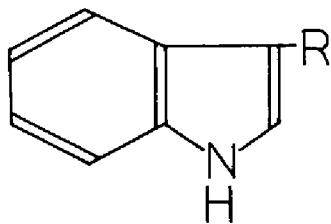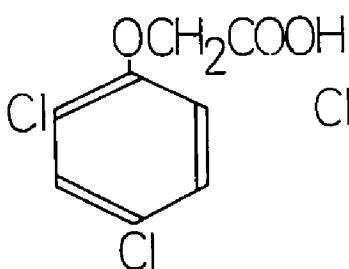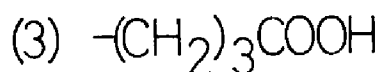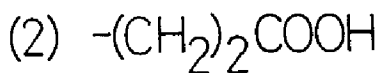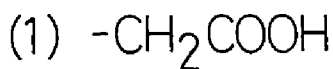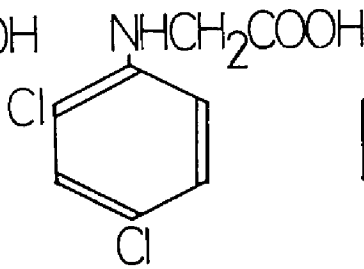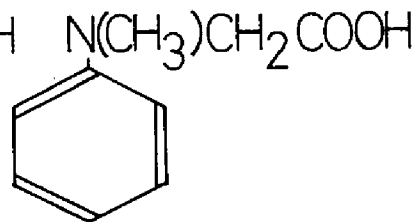
(4)   (5)   (6)

(1)   Indole-3-acetic acid (IAA)
(2)   Indole-3-propionic acid (IPA)
(3)   Indole-3-butyric acid (IBA)
(4)   2,4-Dichlorophenoxyacetic acid (2,4-D)
(5)   N-(2,4-Dichlorophenyl)glycine (2,4-DG)
(6)   N-Methyl-N-phenylglycine (MPG)

Fig. 1.   Structures of the Auxins and Their Analogues.
         Abbrebiations are in parentheses.
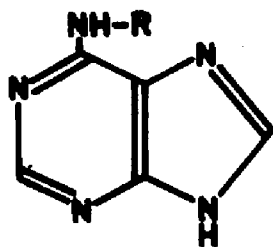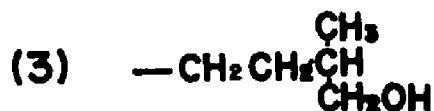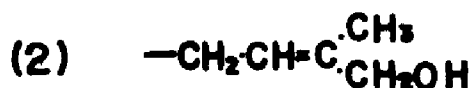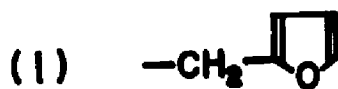
(1) Kinetin: 6-Furfurylaminopurine[8,9]

(2) Zeatin: 6-(<u>trans</u>-4-Hydroxy-3-methylbut-2-eny-
          amino)purine[15]

(3) Dihydrozeatin: ($\pm$)-6-(4-Hydroxy-3-methylbutyl-
          amino)purine[16,17]

Fig. 2. Structure of Cytokinins.

was removed with water. The section covered with glass served as the preparation for microscopic observation. To detect lignified cells, 5 % phloroglucin in 95 % ethanol was dropped on one of these sections, followed by conc. HCl. Lignin was stained purple by this method.

Ribonuclease(RNase) activity.

2,4-D cells and kinetin cells were cultured in suspensions. Cells were homogenized in a chilled mortar with 0.05 M phosphate buffer, pH 6.5, containing Polyclar AT and 1 % Na-ascorbate. The homogenate was filtered through gauze and the filtrate centrifuged at 10,000 g for 20 min. The supernatant obtained was dialyzed by Sephadex G-25 gel filtration. The eluate was used as the enzyme solution. The reaction mixture contained 0.1 M acetate buffer, 4 mg of purified yeast RNA and 2 ml of the enzyme solution (200 ug as protein) in a final volume of 4 ml(pH 5.5). After incubation for 1 hr at 35°C, the reaction was stopped by adding 1 ml of 25 % trichloroacetic acid(TCA) containing uranium acetate. After cooling it in an ice bath, the supernatant was separated by centrifugation and the decomposed products were determined from the absorbance at 260 nm. A non-incubated mixture undergoing the above procedures served as the reference. One enzyme unit was defined as the enzyme activity causing changes in the absorbance at 260 nm of 1 for 1 hr.

RESULTS AND DISCUSSION

(1) Conditions for shoot formation from cultured tobacco cells by cytokinins.

Table II shows the conditions for shoot formation from cultured tobacco cells. Tobacco cells cultured with 10^{-6} M 2,4-D (2,4-D cells) were transferred to an agar media containing a combination of 2,4-D and zeatin. Shoot formation was observed 6-8 weeks after inoculation at a concentration of 5×10^{-5} M zeatin in the presence of a low concentration of 2,4-D ($0 - 10^{-6}$ M), while no shoot formation was found in the presence of 10^{-5} M 2,4-D(Fig. 3). As the concentration of 2,4-D decreased, increased shoot formation was observed. In contrast, cell proliferation occurred in the presence of both 2,4-D and zeatin. A combination of a high concentration of 2,4-D ($10^{-5} - 10^{-6}$ M) and a low concentration of zeatin ($0 - 10^{-7}$ M) gave particularly good cell proliferation. At a high concentration of zeatin, cell proliferation was repressed. Consequently, it is presumed that, when shoot formation occurs, cell proliferation is reduced. Skoog et al[3] reported that 0.02, 0.2, and 0.5 mg/l of kinetin with 2 mg/l of IAA induced root formation, cell proliferation and shoot formation, respectively. Others have also reported similar results[14,18]. In the strain T5 cell line which required only 2,4-D for cell growth, shoot formaton could be induced and developed with zeatin(5×10^{-5} M). The presence of 2,4-D had an inhibitory effect on shoot formation, thus no exogenous 2,4-D was required.
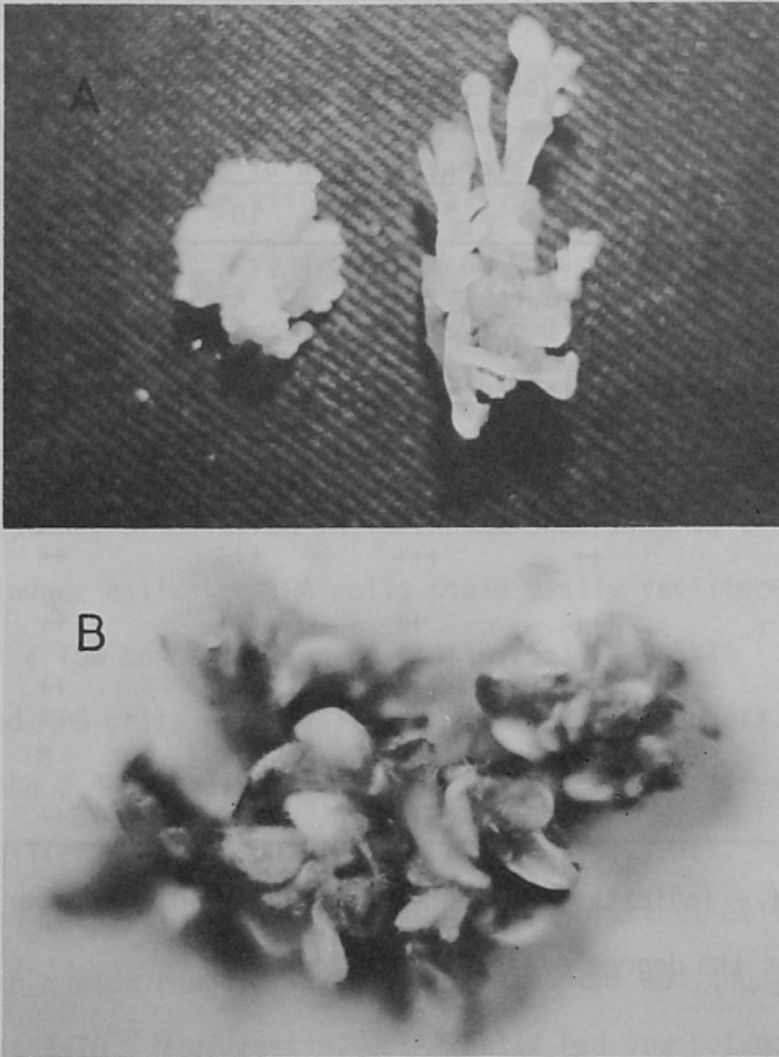
Fig. 3. Redifferentiated Shoots from Cultured Tobacco Cells.
(A): Dedifferentiated 2,4-D cells (left), redifferentiated shoots
(right).
(B): Subcultured redifferentiated-shoots.

Table II. Effects of 2,4-D and Zeatin on Cell Growth and Shoot Formation in Cultured Tobacco Cells.

Concn. of Zeatin,(M)	Concentration of 2,4-D, M				
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	0
0		+++	++	++	++
5×10^{-10}	+++	+++	++	++	+++
5×10^{-9}	+++	+++	++	++	+++
5×10^{-8}	+++	++	++	++	++
10^{-7}	++	+++	+++	++	++
5×10^{-7}	+++	++	++	++	++
10^{-6}	+++	++	++	++	++
5×10^{-6}	++	++	++	+	++
5×10^{-5}	+	S++	S++	S++	S+++

Number of + indicates the degree of cell growth. Number of S+ indicates the degree of shoot formation.

The zeatin requirements shown in Table III indicate that short-term treatment with zeatin does not induce shoot formation. Therefore, zeatin(cytokinin) is required not only for triggering redifferentiation, but also for the development of the shoot during the culture period.

Table IV shows the effects of callus-inducing auxins on organ formation. Calluses were induced from tobacco pith by various auxins and their analogues(10^{-5} M) as sho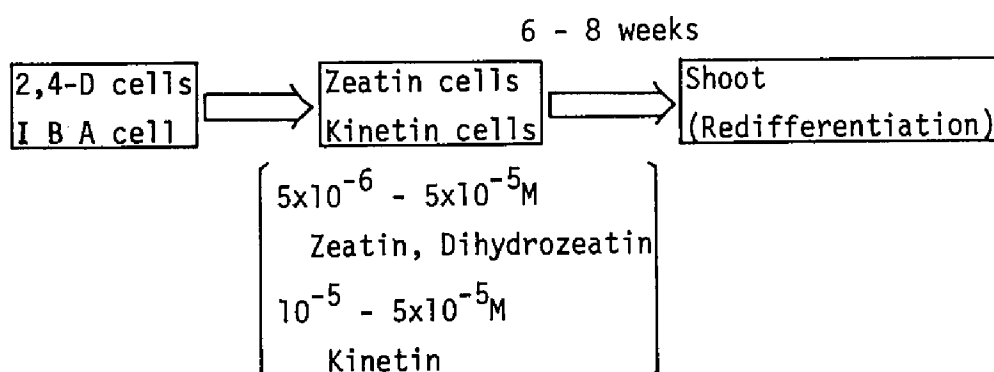wn in Fig. 1. These were transferred to an agar media, containing the indicated concentration of zeatin (no auxins) to test organ formation. The shoot-forming potency of cultured cells varied according to the auxin used to induce callus. IPA cells could easily redifferentiate shoots at a low concentration of zeatin (5×10^{-9} - 5×10^{-7} M). IBA, 2,4-DG and MPG cells also easily redifferentiated shoots. While IPA and the 2,4-DG cells showed only slight cell proliferation, IBA cells proliferated and could be subcultured for years. Among the tested cells, the 2,4-D ones showed the greatest cell proliferation over a wide range of 2,4-D concentrations and shoots were induced at 5×10^{-5} M of zeatin. IAA cells had low potencies both for cell growth and shoot formation. Only root formation was found in IPA cell and 2,4-DG cells in these systems. Results shown in Table IV suggest that the degree of dedifferentiation varies according to the auxin used and that this reflects the potency for redifferentiating shoots as the reverse phenomenon of dedifferentiation.

Table III.   Effect of Short-Term Treatment with Zeatin on Shoot Formation from Cultured Tobacco Cells.

2,4-D cells were transferred to a liquid medium containing 5×10^{-5} M zeatin and cultured for the given period.   Then, cells were transferred to an agar base medium(containing no plant growth regulators).   Shoot formation was measured 55 days after the first transfer.   Number of + indicates the degrees fo cell growth and shoot formation.

Time of Treatment with Zeatin (Hr)	Cell Growth	Shoot Formation
15	+	-
41	+	-
120	++	-
212	+++	-

6 - 8 weeks

2,4-D cells
I B A cell   ⟹   Zeatin cells
Kinetin cells   ⟹   Shoot
(Redifferentiation)

$$\left[\begin{array}{l} 5 \times 10^{-6} - 5 \times 10^{-5} M \\ \quad \text{Zeatin, Dihydrozeatin} \\ 10^{-5} - 5 \times 10^{-5} M \\ \quad \text{Kinetin} \end{array} \right]$$

Scheme 1.   The Systems of Shoot Redifferentiation from Cultured Tobacco Cells by Cytokinins.

Table IV. Cell Growth and Organ Formation Induced by Various Auxins in Cultured Tobacco Cells.

	Auxin*	Concentration of Zeatin, M				
		5×10^{-9}	5×10^{-8}	5×10^{-7}	5×10^{-6}	5×10^{-5}
Shoot Formation	IAA	-	-	-	+	++
	IPA	++	+++	+++	-	-
	IBA	-	-	-	+++	++
	2,4-D	-	-	-	-	++
	2,4-DG	-	-	++	++	+++
	MPG	-	-	-	+++	+++
Root Formation	IAA	-	-	-	-	-
	IPA	-	++	++	-	-
	IBA	-	-	-	-	-
	2,4-D	-	-	-	-	-
	2,4-DG	-	-	+	-	-
	MPG	-	-	-	-	-
Cell Growth	IAA	-	-	-	+	++
	IPA	-	+	-	-	-
	IBA	-	+	++	+	-
	2,4-D	+++	+++	++	++	++
	2,4-DG	-	-	+	+	-
	MPG	-	+	++	-	-

*) Auxins used to induce callus (cultured cells) from pith tissue of the tobacco plant.

Table V shows that dihydrozeatin (Fig. 2) had activity for producing shoot formation from 2,4-D cells, as did zeatin. The naturally occurring cytokinins, dihydrozeatin and zeatin, were more active than the synthetic kinetin for shoot formation. These cytokinins also enhanced cell growth in 2,4-D cells at low concentrations. Concentrations needed for shoot formation and cell growth differed: 5×10^{-8} M of zeatin and dihydrozeatin and 5×10^{-6} M kinetin for maximum cell growth, and 5×10^{-5} M of zeatin, dihydrozeatin and kinetin for shoot formaiton. Cell growth and shoot formation seem to be opposite effects of cytokinins in cultured tobacco cells.

Due to the results in Tables II, IV and V the following culture systems were used for shoot redifferentiation(Scheme 1). Subcultured 2,4-D cells(dedifferentiated cells) were transferred to a medium with 10^{-5} - 5×10^{-5} M of zeatin or 10^{-5} M kinetin and were cultured in static or suspension cultures. In some experiments IBA cells were used. 2,4-D cells or IBA cells served as dedifferentiated control cells.

(2) <u>Morphological</u> <u>and</u> <u>physiological</u> <u>changes</u> <u>during</u> <u>the</u> <u>early</u> <u>stages</u> <u>of</u> <u>shoot</u> <u>formation</u>.

Growth curves shown in Fig. 4 indicate that over a period of 20 days the 2,4-D cells increased more in fresh weight than did zeatin cells in a static culture. In zeatin cells, the growth rate was slower, reaching a plateau 15 days after inoculation, whereas the 2,4-D cells grew logarithmically for more than 20 days. A

Table V. Effects of Zeatin, Dihydrozeatin and Kinetin on Cell Growth and Shoot Formation in Cultured Tobacco Cells

Concn. (M)	Zeatin		Dihydrozeatin		Kinetin	
	Cell Growth	Shoot Formation	Cell Growth	Shoot Formation	Cell Growth	Shoot Formation
5×10^{-9}	++	-	+++	-	+	-
5×10^{-8}	+++	-	+++	-	+	-
5×10^{-7}	++	-	+++	-	++	-
5×10^{-6}	++	-	++	++	+++	-
5×10^{-5}	+	+++	++	++	++	++

Number of + indicates the degree of cell growth or of shoot fromation.

Table VI. RNase Activity in Tobacco Plants and in Cultured Tobacco Cells.

	RNase Activity	
	U/g fr wt	U/mg protein
Young Green Leaf	15.8	2.8
Pith Tissue	4.4	5.6
2,4-D Cells[*]	17.5	38.8

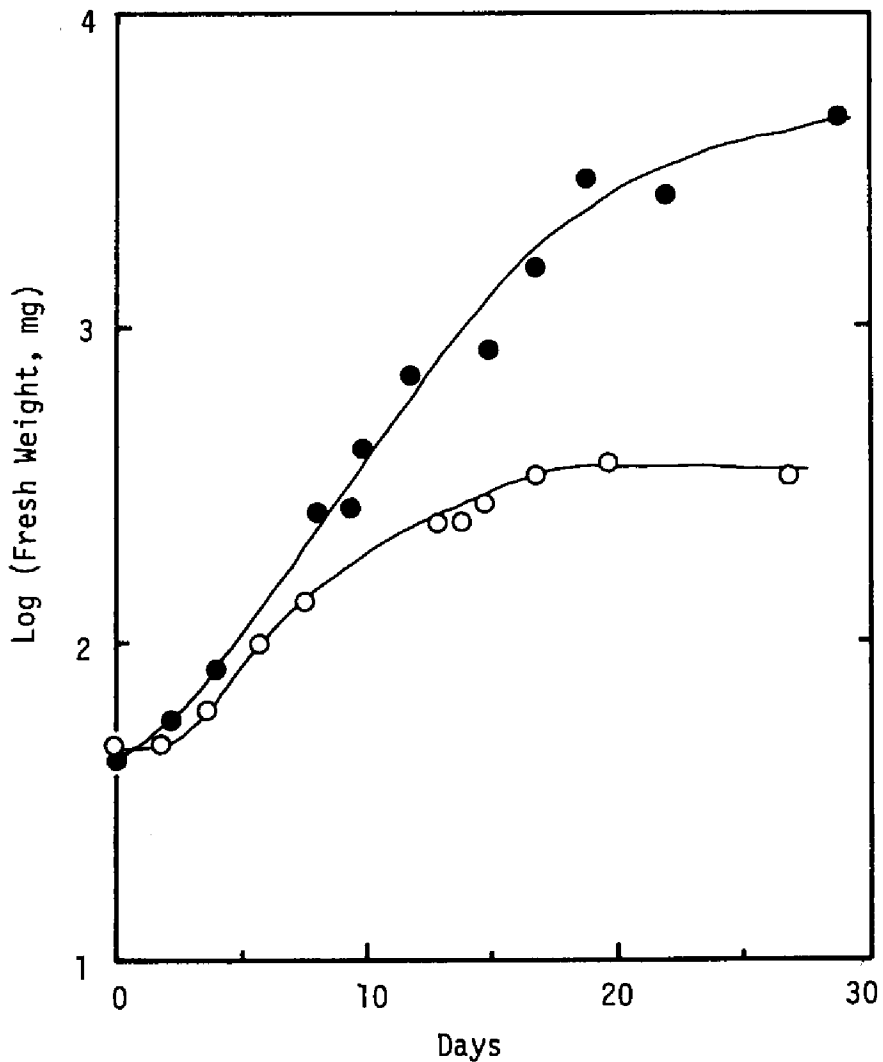*) Cells cultured for 17 days in suspension.

Fig. 4. Growth Curves for Tobacco Cells Cultured on a Medium with 10^{-6} M 2,4-D (-●-) and $5x10^{-5}$ M Zeatin(-O-).

About 50 mg of cells was inoculated in a test tube containing 10 ml of agar medium. Fresh weight was measured at specified intervals. The average fresh weight of 3 replicates is given in the figure.
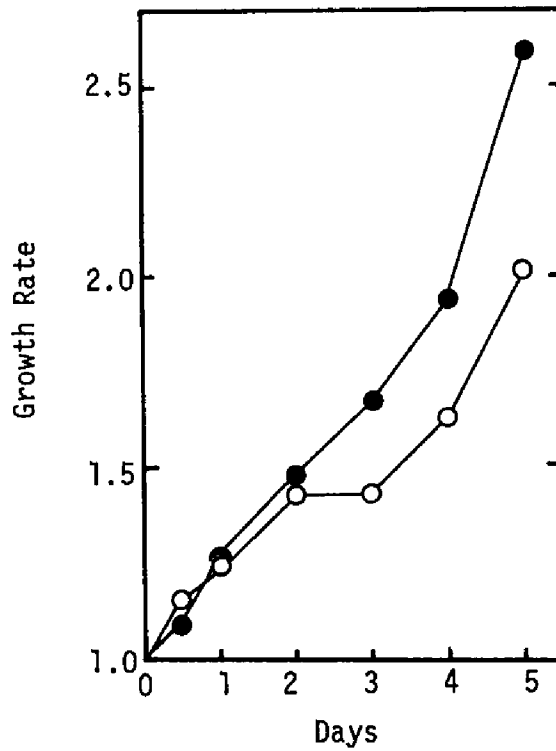
Fig. 5. Growth Rates of Tobacco Cells Cultured with 2,4-D or Kinetin in Suspension.

Cell growth is represented as the growth rate of the initial inoculum as seen from the turbidity at 610 nm.   (-●-) shows the growth rate of 2,4-D cells; (-O-), of kinetin cells.

similar phenomenon was observed in the growth of 2,4-D cells and kinetin cells cultured in suspension(Fig. 5). However, 3 days after inoculation a reduced growth rate was found for kinetin cells. In many cases, an additional low concentration of cytokinin with auxin promotes cell growth[19]. In contrast, Fig. 4 shows that a high concentration(5×10^{-5} M) of zeatin inhibits callus growth. Nudel et al[20] also reported inhibitory effects of a high concentration of kinetin on growth, as well as on the synthetic activity of other components in tobacco cells.

Ratios of dry to fresh weights also changed a few days after inoculation(Fig. 6). 2,4-D cells showed a temporary increase in dry material which later decreased to a constant level (about 2.0 - 2.5 % dry materials, Fig. 6-A). In contrast, zeatin cells had 4 - 5 % dry materials. This indicates that cell components in zeatin cells are enriched. The times of the beginning of the changes in dry materials were consistent with those of the changes in the growth rates in Fig. 4.

As shown in Fig. 7, organization was found in cells cultured for 15 days with zeatin, while 2,4-D cells were amorphous and difficult to prepare in sections. Organized compact cells of small size were found 10 days after inoculation in the zeatin culture. Simultaneously, the lignified cells and tracheid-like cells shown in Figs. 7 and 8, were observed in these cells. These types of cells were seen 6 days after inoculation, and the number of these cells, as well as lignification,increased during culture.
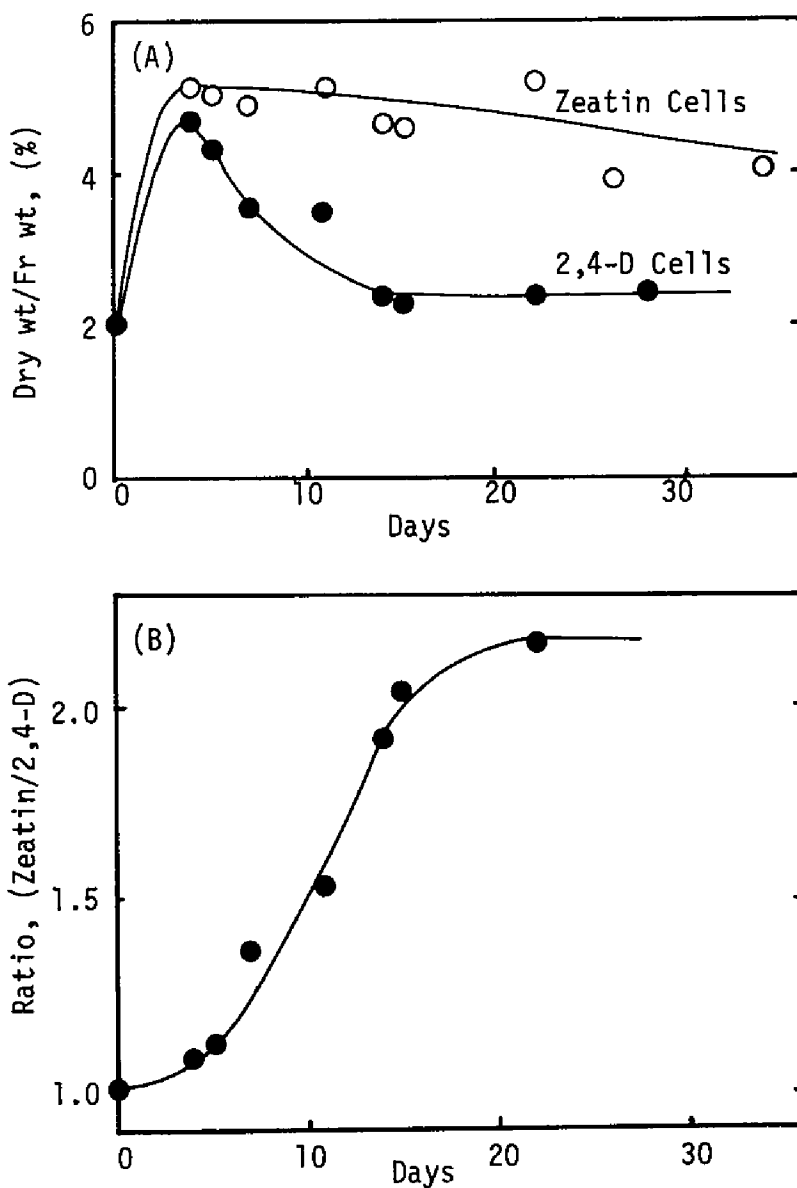
Fig. 6. Changes in Dry Weight to Fresh Weight Percents (A) and
the Ratio for Zeatin Cells as Compared to 2,4-D Cells (B).
Dry weight was measured after about 400 mg (fr wt) of the cells
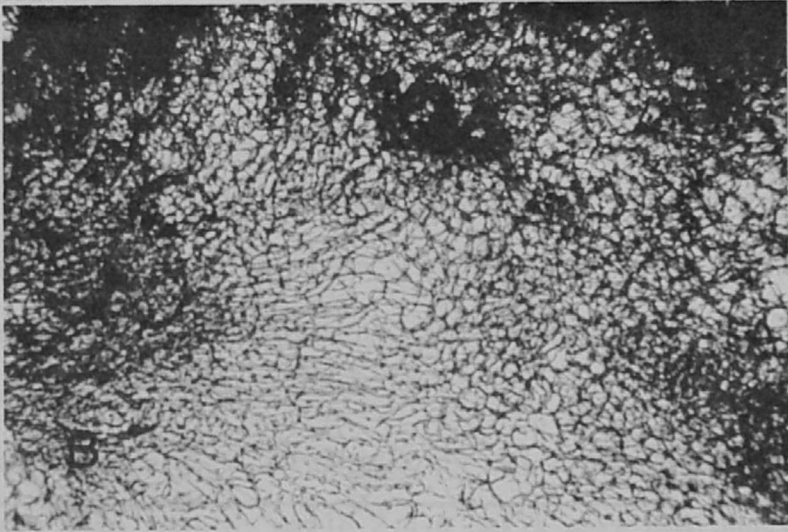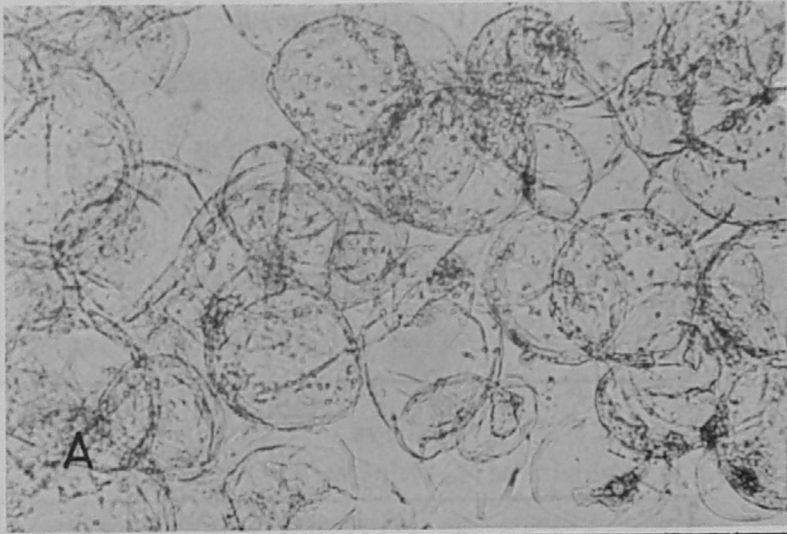was dried at 100°C for 3 hr.

Fig. 7. Microscopic Observations of 2,4-D Cells (A) and Zeatin
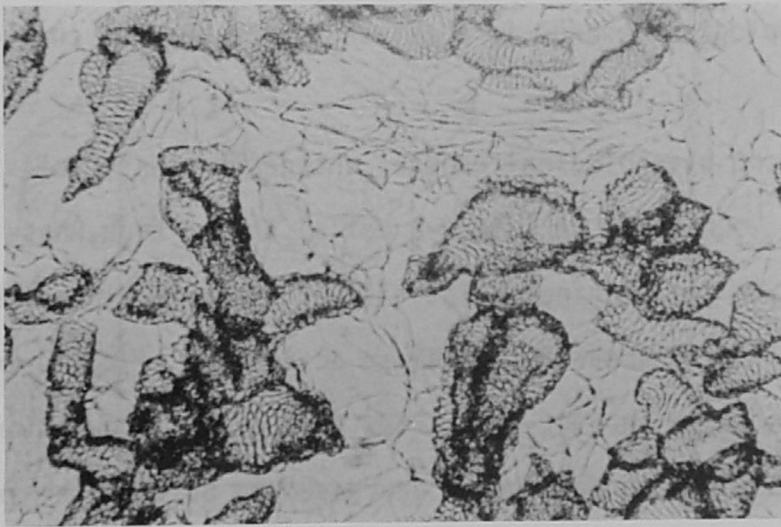Cells (B) Cultured for 15 Days.

Fig. 8. Microscopic Observations of Tracheid-Like Cells in
Zeatin Cells Cultured for 15 Days.

Cell organization in the early stage seemed to occur first around the tracheid-like cells. However, these cells could not develop into vascular bundles[10]. In 2,4-D cells no tracheid-like cells were found. In some reports on tobacco [21] and the soybean[22] kinetin was also shown to promote tracheid-like cell production. In summary, tracheid-like cells first appeared 6 days after inoculation and subsequently cell organization was observed in zeatin cells during the early stages of shoot formation. The beginning of morphological change is consistent with that of changes in cell growth.

As one physiological change, changes in RNase activity were studied. Cultured cells(2,4-D cells) showed high RNase activity compared to the original plant tissue, in particular pith tissue (Table VI). Changes in the RNase activities of 2,4-D and kinetin cells are shown in Fig. 9. A few days after inoculation, RNase activities in both cell types decreased to 50 % of the initial activity. Then a rapid increase was found in 2,4-D cells, while only a gradual one occurred in kinetin cells. Dedifferentiated 2,4-D cells show much higher RNase activity than does the original pith tissue. However, kinetin cells (redifferentiation cells) show lower activity than 2,4-D cells. During the senescence of excised leaves increased RNase activity is repressed by kinetin treatment[23], which repressed the progress of senescence[24].

At the onset of shoot formation early physiological and morphological changes have already occurred a few days after inocu-
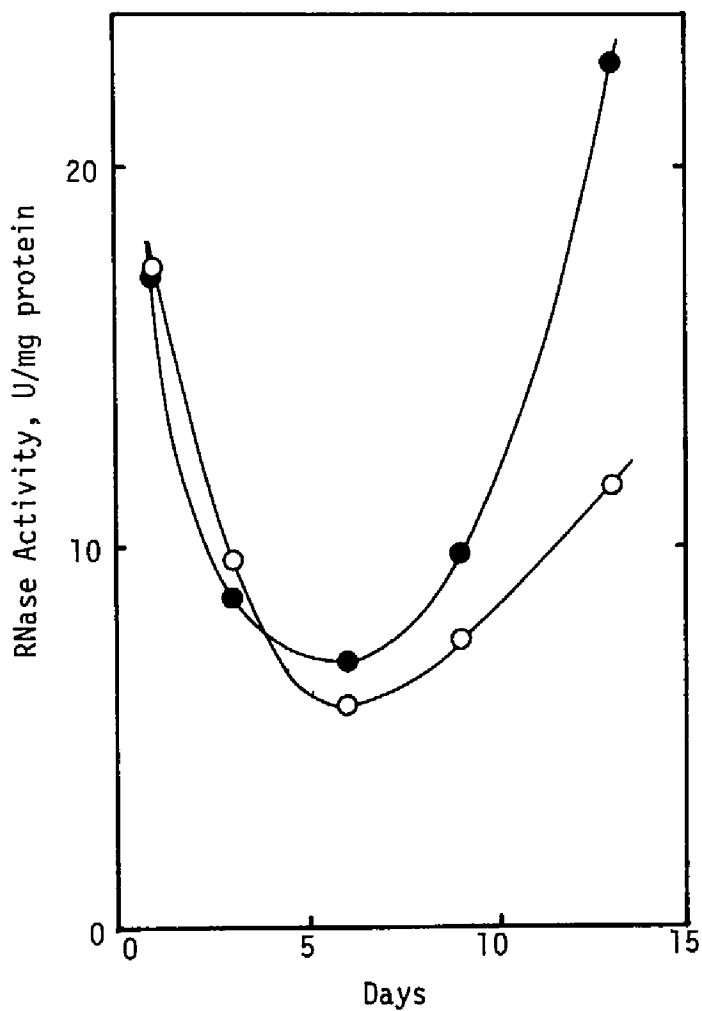
Fig. 9. Changes in the RNase Activity of Cultured Cells with
2,4-D or Kinetin. (-●-) and (-O-) show the respective activities
of 2,4-D cells and kinetin cells in suspension cultures.

lation.　These changes were not found in 2,4-D cells.

SUMMARY

　　Shoot redifferentiation in tobacco cells cultured with auxins (dedifferentiated cells) was examined.　Shoot formation could by induced by the continuous presence of exogenous cytokinin(5×10^{-5} M zeatin, dihydrozeatin and kinetin) as plant growth regulator. The presence of 2,4-D had an inhibitory effect on shoot formation. The concentrations for shoot formation and cell growth differed. The cytokinin concentration causing shoot formation was higher than that giving maximum cell growth.　Zeatin and dihydrozeatin showed higher activities for shoot formation than did kinetin.　Cultured cells induced by various auxins showed different degrees of potency for shoot formation by zeatin.

　　Morphological changes and changes in cell growth, the amount of dry materials and RNase activity were found about 1 week after transfer to the cytokinin medium.　The onset of shoot redifferentiation is believed to occur a few days after transfer to the cytokinin medium and physiological and morphological changes to continue.

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CHAPTER II

SHOOT FORMATION FROM CULTURED TOBACCO CELLS BY OPTICALLY ACTIVE CYTOKININS[1].

INTRODUCTION

After the synthetic compound kinetin[2,3] was found to possess cytokinin activity, the naturally occurring cytokinins zeatin[4] and (-)-dihydrozeatin[5,6] were isolated from _Zea_ _mays_ and _Lupinus_ _luteus_, respectively. These compounds promote cell division in cultured tobacco cells. Kinetin also induces organ formation in cultured cells but it is not known whether the naturally occurring cytokinins affect organogenesis in cultured cell system.

Dihydrozeatin is optically active, although kinetin is not; of the two possible enantiomers, one mirror image may be more active than the other. Unfortunately, both optically active dihydrozeatins are not available for study. Therefore, optically active N^6-substituted adenines[5] with asymmetric carbons adjacent to exocyclic nitrogen atoms(Table I) were tested for their ability to induce organ development in cultured tobacco cells.

MATERIALS AND METHODS

Dihydrozeatin and optically active cytokinins were provided by Dr. K. Koshimizu, the Department of Food Science and Technology, Kyoto Univ. Tobacco(_Nicotiana_ _tabacum_ var. Bright Yellow) cultured

cells were induced from pith tissue with a concentration of 10^{-5} M
of indole-3-butyric acid(IBA) in Linsmaier and Skoog medium[8].
About 40 days after callus induction, this IBA cells were used in
the test of zeatin and (±)-dihydrozeatin and in the test of optically
active cytokinins.   Shoot formation was observed 28 days after
inoculation of cells on Linsmaier and Skoog media containing each
optically active cytokinins, and 53 days after inoculation of cells
with zeatin and (±)-dihydrozeatin.

RESULTS AND DISCUSSION

Letham[9] tested the growth promoting ability of zeatin and
kinetin in a number of axenic systems.   In all systems zeatin
was considerably more effective than kinetin.   Table II shows the
ability of zeatin and (±)-dihydrozeatin to promote cell growth and
to induce shoot formation in vitro from cultured tobacco cells.
For both growth promotion and organ redifferentiation, zeatin
and (±)-dihydrozeatin was equally active.   Hence zeatin and (±)-
dihydrozeatin, like kinetin, have the ability to induce bud form-
ation from cultured tobacco cells.

The abilities of optically active N^6-substituted adenines
to induce shoot formation and to promote cell growth are compared
in Table III.   Both R-(-)-6-(α-methyl-α-phenylmethylamino)purine
(R-PE) and S-(-)-6-(α-hydroxymethyl-β-phenylethylamino)purine(S-PA)
induced shoot formation.   R-PE and S-PA were active in inducing
shoot formation at concentration of 4×10^{-5} M and 2×10^{-4} M, respec-

Table I. Optically Active Cytokinins
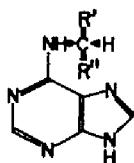
Code Name	Compound	R'	R"
R-PE	R-(-)-6-(α-Methyl-α-phenylmethylamino)purine	$-CH_3$	$-Ph$
S-PE	S-(+)-enantiomer	$-Ph$	$-CH_3$
S-PA	S-(-)-(α-Hydroxymethyl-β-phenylethylamino)purine	$-CH_2OH$	$-CH_2Ph$
R-PA	R-(+)-enantiomer	$-CH_2Ph$	$-CH_2OH$

Table II. Effects of Zeatin and (±)-Dihydrozeatin on Cell Growth and Shoot Formation

Concn,M	Zeatin		(±)-Dihydrozeatin	
	Cell Growth	Shoot Formation	Cell Growth	Shoot Formation
5×10^{-9}	+	-	+	-
5×10^{-8}	++	-	+	-
5×10^{-7}	+++	-	+	-
5×10^{-6}	++	+++	++	+++
5×10^{-5}	+	+++	++	+++

Number of + indicates the degree of cell growth and of shoot formation.

Table III. Effects of N^6-(Optically Active Alkyl and Arylalkyl Substituted) Adenines on Cell Growth and on Redifferentiation of Shoots from Cultured Tobacco Cells.

	Compounds	Concentration, M			
		4×10^{-7}	4×10^{-6}	4×10^{-5}	2×10^{-4}
Cell Growth	R-PE	++	++	++	+++
	S-PE	++	+	±	±
	S-PA	++	++	++	+++
	R-PA	++	++	++	++
Shoot Formation	R-PE	-	-	S++++	-
	S-PE	-	-	-	-
	S-PA	-	-	-	S++
	R-PA	-	-	-	-

1) Number of + indicates the degree of cell growth.
2) Number of S+ indicates the degree of shoot formation.

tively.   At 4×10^{-5}M of R-PE, shoots formed over the surface of the cell aggregate.   S-PA at 2×10^{-4} M also brought about shoot formation on the cell aggregate but it was less than that of R-PE. In contrast, S-PE and R-PA, isomers of R-PE and S-PA, respectively, did not bring about organ development at any of the tested concentrations.   The concentrations at which R-PE and S-PA induced shoot formation were higher than the effective concentrations of zeatin and dihydrozeatin.   Although both dextrorotatory and levorotatory compounds induced cell growth(Table III), levorotatory structures were more active for cell growth and only levorotatory

ones brought about shoot formation. Essentially similar results both for cell growth and shoot formation have been obtained by Matsubara et al[10]. Since configuration markedly influences the ability to induce differentiation, binding of cytokinin N^6 group to a receptor site is probably involved in organogenesis.

SUMMARY

(±)-Dihydrozeatin and optically active cytokinins(asymmetric carbon α to the exocyclic nitrogen) were tested for their ability to induce development of shoots in cultured tobacco cells. (±)-Dihydrozeatin was active to induce shoot formation. The levo-rotatory compounds tested were active in inducing shoot formation but the corresponding dextrorotatory compounds were inactive at all concentrations tested. These findings suggest that the group attached to the N^6 position of cytokinins binds to a stereo-specific receptor site to bring about shoot redifferentiation.

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CHAPTER III

METABOLISM OF KINETIN-8-^{14}C INCORPORATED INTO

CULTURED TOBACCO CELLS DURING THE EARLY STAGES

OF SHOOT REDIFFERENTIATION.

INTRODUCTION

In order to induce the shoot from cultured tobacco cells (dedifferentiated cells), exogenous cytokinins are essentially requisite.

During callus induction, 2,4-D incorporated into cells are accumulated in free form and thereafter forms high molecular weight complexes with proteins (but not with nucleic acids), particularly with lysine-rich histone in pea nucleus presumably to bring about cell division[1,2].

Low molecular weight complexes are also formed for detoxin[1]. After the hydrolysis of these complexes, the radioactivity is recovered as 2,4-D.

Fox et al[3] presented the hypothesis that exogenous cytokinins are incorporated into s-RNA and thereafter give the physiological effects of plant cells[3,4], since the cytokinins are located in s-RNA as one of minor base components[5]. But this hypothesis was denied by Kende et al[6] and Hall et al[7,8]; Hall et al showed that cytokinin, N^6-(Δ^2-isopentenyl)adenine (2iPA), in t-RNA was synthesized by the addition of Δ^2-isopentenylpyrophosphate, derived

from mevalonate, to adenine residue in t-RNA. However, cytokinins in t-RNA are not seem to be active forms since the occurrence in t-RNA are found in wide range of microorganisms, animals and plants. It seems to be one of the source of cytokinins.

On the other hand, cytokinins (zeatin and benzyladenine) are metabolized to a number of metabolites, adenine, adenosine, AMP, riboside and ribotide of cytokinin etc., after incorporated into some kinds of plant cells[9-16].

To date, it has not been clear what kinds of metabolites of cytokinin are actually active forms to induce organogenesis in cultured tobacco system. This chapter describes the incorporation and metabolism of kinetin-8-^{14}C during the early stages of shoot redifferentiation in cultured tobacco cells.

MATERIALS AND METHODS

Cell culture and incorporation of kinetin-8-^{14}C.

2,4-D cells in suspension culture were used. 2,4-D cells were transferred into the medium containing 10^{-5} M kinetin and 0.2 uCi of kinetin-8-^{14}C (sp. act. 15 mCi/mmol, Radiochemical Center, England) and cultured in suspension for given periods at 25°C in the dark.

Extraction of radioactive materials derived from kinetin-8-^{14}C incorporated.

Cultured kinetin cells were harvested at 24 hr intervals and washed with the basal medium. Five grams of each sample was

homogenized with cold 80 % EtOH in a chilled mortar. The homoge-
nate was filtered through Whatman 3MM disc filter paper and the
residue on the filter paper was washed with cold 80 % EtOH.   The
combined 80 % EtOH extract (80 % EtOH soluble materials) was filled
up to 100 ml and taken up 2 ml into a vial tube.   After removing
the solvent (80 % EtOH) and pouring toluene scintillator, radio-
activity of 80 % EtOH soluble materials was determined with
Beckman LS-100 liquid scintillation spectrometer.

The residue on the filter paper (80 % EtOH insoluble materials)
was washed and dried with 100 % EtOH and ether.   Radioactivity of
80 % EtOH insoluble materials was also determined with liquid
scintillation spectrometer.

Enzymatic degradation of 80 % EtOH insoluble materials.

The 80 % EtOH insoluble materials radiolabeled were prepared
from cells cultured with kinetin-8-^{14}C for 24 or 96 hr.   A 20 mg
of EtOH insoluble materials was incubated at 30°C for 60 min with
a following enzyme solution; 1) 1 mg of Pronase E (70,000 PUK/g,
Kaken Kagaku Co.) in 2 ml of 50 mM Tris-HCl buffer (pH 7.8), 2)
1.5 mg of cellulase (type I from Aspergillus niger , Sigma Chemical
Co.) in 2 ml of 50 mM phosphate buffer, pH 6.5, 3) 1 mg of RNase
(type IA from bovine pancreas, Sigma Chemical CO.) in 2 ml of
50 mM phosphate buffer, pH 6.5.   Control was run without enzyme
in each buffer.   Enzyme reaction was stopped by addition of 0.5
ml of cold 20 % trichloroacetic acid(TCA).   Reaction mixture was
filtered through Whatman 3MM disc filter paper and the paper was

washed with 5 % TCA.   After the paper was washed and dried with EtOH and ether, radioactivity remained on the disc paper as high molecular weight components was determined with liquid scintillation spectrometer.

Analysis of 80 % EtOH soluble materials.

Each 80 % EtOH extract (80 % EtOH soluble materials) was concentrated to 0.5 ml and 100 ul of concentrate was spotted on Whatman 3MM cellulose paper.   The paper was developed with n-butanol: acetic acid: water (4:1:1).   After drying, the paper was cut into 1 cm pieces and the radioactivity of each pieces was determined with liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Incorporation of kinetin-8-^{14}C during the early stages of shoot redifferentiation.

Table I shows that increased radioactivies of 80 % EtOH insoluble and soluble fraction were found during the early stages of shoot formation.   In particular, during 0-24 hr and 72-120 hr, the radioactivies rapidly increased.   At 192 hr, the radioactivities of both fractions decreased.   These changes in radioactivities would imply the first incorporation of kinetin-8-^{14}C due to transfer from 2,4-D-medium to kinetin-medium, changes in metabolism of kinetin or other cell components to induce the shoot formation, and the loss (CO_2 release etc.) of radioactivity as a results of metabolism of kinetin, respectively.

Table I. Incorporation of Kinetin-8-^{14}C into Cultured Tobacco Cells.

Time (hr)	Kinetin-8-[14]C Incorporated CPM/g. fr. wt.		
	80 % EtOH Insol.	80 % EtOH Sol.	Total
24	1,443	9,464	10,907
48	1,995	12,166	14,161
72	2,974	12,646	15,620
96	5,024	17,304	22,328
120	5,086	20,866	25,952
192	4,038	14,976	19,014

Table II. Effects of Enzymes on 80 % EtOH Insoluble Materials.

Treatment	pH	Radioactivity Remained, CPM	
		24 Hr[1]	96 Hr[2]
Control	7.8	1093 (100)	1763 (100)
Pronase E	7.8	1060 (97)	1257 (71)
Control	6.5	1250 (100)	2122 (100)
RNase	6.5	709 (57)	953 (45)
Cellulase	6.5	1320 (106)	2569 (121)

1) Culture time with kinetin-8-[14]C.
2) For experimental details, see in "Methods".

High molecular weight components radiolabeled by kinetin-8-[14]C.

Enzymatic degradation of 80 % EtOH insoluble materials were carried out in order to estimate the high molecular weight components with radioactivity derived from kinetin-8-[14]C. RNase treatment caused the 50 % decrease of radioactivies in 80 % EtOH insoluble materials of both 24-hr and 96-hr sample. Pronase E treatment also caused the loss of radioactivity in 96-hr EtOH insoluble materials, while the very little degradation of 24-hr sample by Pronase E was observed. Cellulase gave no effect.

Radioactivities derived from kinetin-8-^{14}C were found in ribonucleic acids degraded by RNase. However, it is presumed that radioactivity of kinetin is incorporated into ribonucleic acids as adenine or guanine, i. e. metabolites of kinetin[6-8, 10,15]. It is of interest that radioactivity of kinetin-8-^{14}C was incorporated into proteins degraded by Pronase E at 96 hr. Matthysse et al have reported the existence of kinin-reactive protein[17]. 2,4-D also binds to some kinds of protein during callus induction[1,2]. However, it is not clear what kind of proteins binds to kinetin, or whether kinetin is incorporated into proteins with the original structure in cultured tobacco cells.

Low molecular weight metabolites of kinetin-8-[14]C.

Paper chromatography of 80 % EtOH soluble materials shown in Fig. 1 indicate that low molecular weight metabolites of kinetin-8-^{14}C are mainly composed of 5 compounds with following R_f values: peak I, 0.04-0.07; peak II, 0.18-0.21; peak III, 0.29-0.32; peak IV,
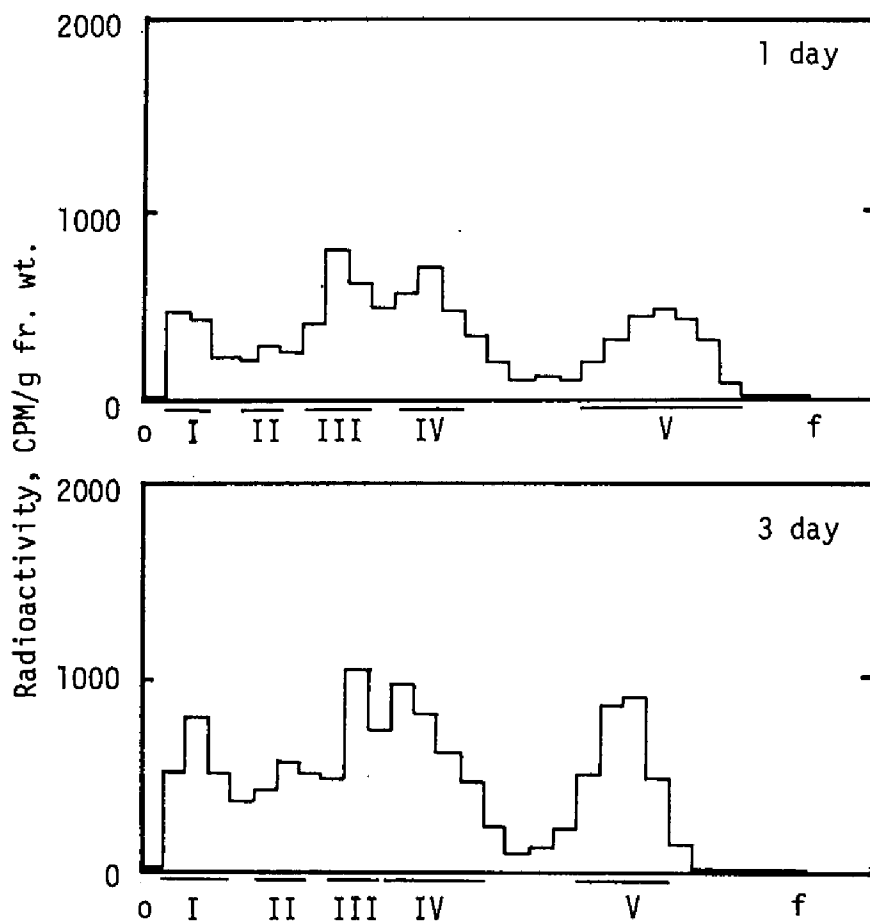
Fig. 1. Paper Chromatographies of 80 % EtOH Soluble Metabolites of Kinetin-8-^{14}C.

o: original point, F: front. Developing solvent: n-butanol, acetic acid, water (4:1:1).
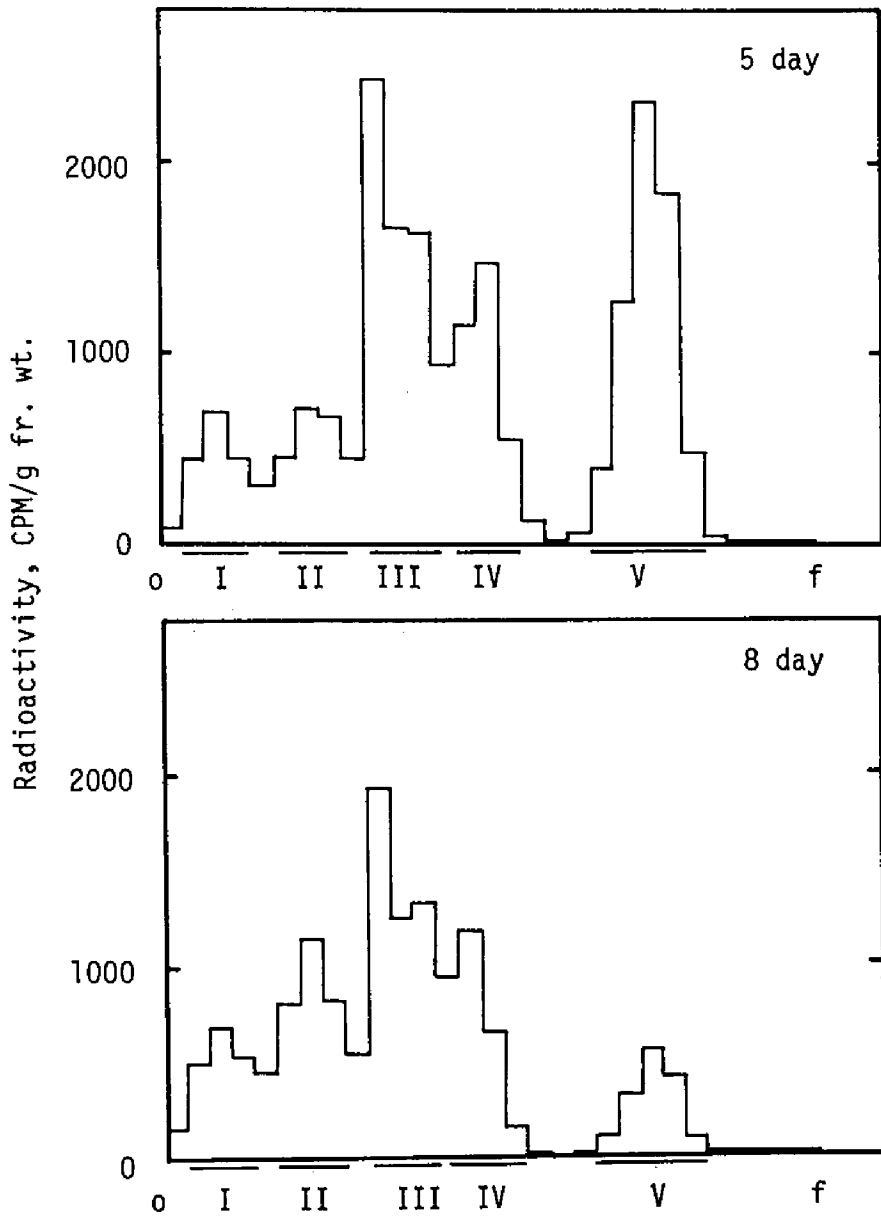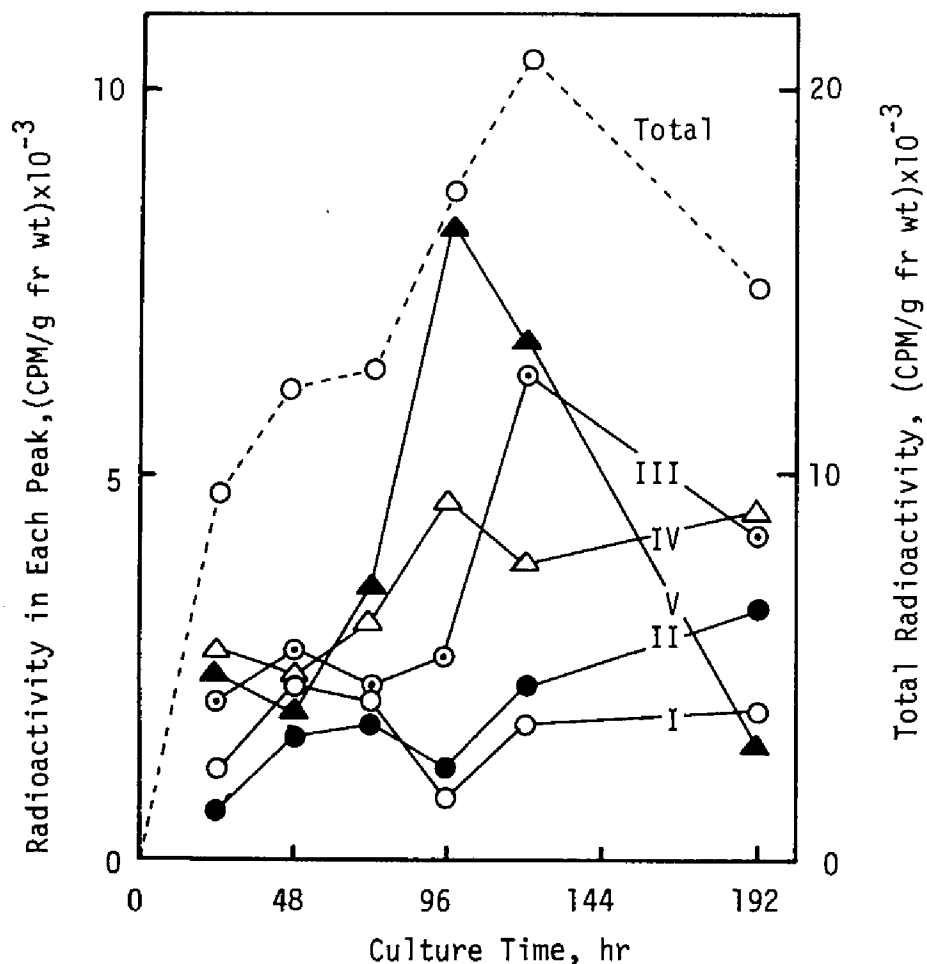
Fig. 1. Continued.

Fig. 2. Changes in 80% EtOH Sol. Metabolites of Kinetin-8-^{14}C. Radioactivity of each peak (peak I, II, III, IV and V) was calculated from Fig. 1. (-O-) shows the radioactivity of peak I; (-●-), peak II; (-⊖-), peak III; (-△-), peak IV; (-▲-), peak V; and (⋯o⋯), total radioactivity, respectively.

0.39-0.50; peak V, 0.75-0.82.   Peak I, IV and V were identified

as AMP, adenine and kinetin, respectively, since the R_f values of

AMP, adenine and kinetin were 0.07, 0.43-0.54 and 0.81-0.86,

respectively.   Peak II (X) and III (Y) were not identified.

Change in radioactivity of each peak is shown in Fig. 2.

Peak IV, V and III increased during 72-120 hr in this order, while

no significant changes were not found in peak I and II.   Therefore,

it is considered that incorporated kinetin is immediately metabolized

to adenine, AMP, (X) and (Y) and a part of kinetin remains in free

form.   Kineitn (peak V), after accumulation to a certain concent-

ration, is seemed to be converted to (Y).   Free form of kinetin

or (Y) is considered to be active form of kinetin.   Details is

not clear.   Recently, 6-benzylamino-7-glucofuranosylpurine[12-14]

and 7-glucosyl zeatin[15] have been reported in addition to ribo-

nucleosides and 5'-ribonucleotides of cytokinins.   These glucosides

are reported to be major and stable metabolites of benzyladenine

and zeatin, and to be active as cytokinin.   Compound (Y) may be

glucoside or riboside of kinetin.   It has also been reported that

cytokinins interact with their action site by loose, probably non-

covalent bonds[18].   And cytokinin, itself, can not directly

affect enzyme activity _etc_. _in vitro_.   Therefore, a possibility

is considered during the shoot redifferentiation: kinetin incorpo-

rated into cells is metabolized rapidly; free kinetin or metabolite

(Y) acts as trigger of shoot redifferentiation with kinetin-reactive

protein rather than directly without mediator.

SUMMARY

Kinetin-8-^{14}C was incorporated rapidly into cultured tobacco cells during the early stages of shoot redifferentiation. About 80 % of radioactivity was located in 80 % EtOH soluble materials. About 50 % of radioactivity in 80 % EtOH insoluble materials was lost by RNase treatment and 30 % was lost by Pronase E treatment. Radioactivity in RNA is presumably due to adenine etc., metabolites of kinetin. Radioactivity in 80 % EtOH soluble materials distributed mainly 5 fractions, AMP, (X), (Y), adenine and kinetin. Metabolite (Y) and free kinetin increased during the culture. It is considered that free kinetin or metabolite (Y) acts as trigger of shoot redifferentiation with kinetin-reactive protein.

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CHAPTER IV

CHANGES IN PROTEIN SYNTHESIS DURING THE EARLY STAGES OF SHOOT REDIFFERENTIATION FROM CULTURED TOBACCO CELLS[1].

INTRODUCTION

Cytokinins appear to regulate many different actions in plant, for example, cell division, enlargement, and differentiation[2]. However, conclusive evidence for specific regulatory functions has yet to be reported. In some studies of cytokinin actions, the investigation has been made into their influence on protein synthesis.

For example, Jouanneau et al[3,4] reported that kinetin promotes protein synthesis in tobacco cell suspensions and that at least one specific difference in protein pattern could be observed before the first cell division occurred. During senescence, it has been reported that cytokinins affect the nucleic acid and protein metabolism[5-8]. In relation to protein metabolism, the cytokinin actions on the expansion of cotyledons[9] and on dormant duckweed[10] have been reported.

During cell culture cytokinins can induce the formation of shoots or roots from cultured tobacco cells[11] but the mechanism is not understood yet. This chapter reports the changes in protein synthesis during shoot formation(redifferentiation) in cultured tobacco cells in vitro.

MATERIALS AND METHODS

Cell culture.

Tobacco callus, strain T5[12], and Linsmaier and Skoog basic medium[13] were used. For shoot formation cultured cells were inoculated in an agar medium containing 5×10^{-5} M zeatin(zeatin cells) or 10^{-5} M kinetin(kinetin cells). 2,4-D cells were served as control cells. All cell cultures were incubated at 25°C in the dark.

DNA, RNA and protein determination.

DNA and RNA were extracted according to the method of Schneider[14], except that the acid insoluble material was defatted by heating it at 50°C in ethanol-ether (1:1, v/v) for 15 min before hot trichloroacetic acid(TCA) hydrolysis. DNA content was determined by the diphenylamine method as described by Burton[15]. RNA was determined by the orcinol method as described by Mejbaum[16]. Soluble protein was extracted by homogenizing cells in a glass homogenizer containing 0.1 M phosphate buffer, pH 7.5, centrifuging the homogenate at 10,000 g for 15 min and collecting the supernatant. Protein content was determined by the method described by Lowry et al[17].

[14]C-Leucine incorporation and DEAE cellulose column chromatography.

Cultured cells, incubated with ^{14}C-leucine (L-(U-^{14}C)-leucine, sp. act. 270 mCi/mmol, the Radiochemical Center, England), were homogenized with 0.1 M phosphate buffer (pH 7.5) in a chilled mortar, then the homogenate was centrifuged at 10,000 g for 15 min.

The supernatant was dialysed against 0.01 M phosphate buffer (pH 7.5) overnight. The extract was applied to a DEAE cellulose column and eluted with a combination of linear and step gradients of NaCl. Radioactivity was determined as follows. An equal volume of 10 % TCA was added to part of the fractionated solution and the precipitate was collected on a glass fiber paper (Whatman GF/C) and washed with 5 % TCA, ethanol and ether. Using a toluene base scintillator, the radioactivity was counted with a Beckman LS-100 liquid scintillation spectrometer.

Disc electrophoresis.

Polyacrylamide gel electrophoresis of the fractionated protein was performed according to the method of Davis[18].

Preparation of antigen and antiserum.

Cultured tobacco cells or tobacco leaves grown in a green house for 3 months, were homogenized in a chilled mortar with 1/5 its weight of Polyclar AT and an equal volume of 0.05 M phosphate buffer (pH 7.5) containing 1 % Na-ascorbate. The pH was adjusted to 7.5 with 1 N NaOH. The homogenate was squeezed through gauze, then centrifuged at 10,000 g for 30 min. Protein was precipitated by saturating the mixture with $(NH_4)_2SO_4$. The precipitate was collected by low speed centrifugation, after which it was dissolved in small amounts of and dialysed against 0.05 M phosphate buffer (pH 7.5). All procedures were carried out at 4°C. The protein solution (antigen) was stored in a freezer after the addition of 12.5 % of glucose[19]. Two male rabbits were

immunized with antigen from 15 day old 2,4-D cells and two others
with antigen from tobacco leaf.   Five doses of the same antigen
(the first three, 20 mg as protein and the last two, 10 mg) in
incomplete Freund adjuvant were administered intraperitoneally at
1 week intervals, blood was taken from the carotid artery 1 week
after the last injection.   Sera were prepared by the usual method[20].
Antibody-antigen reactions.

Antibody-antigen reactions were studied using the double
diffusion method in agar gel as described by Ouchterlony[21].

RESULTS AND DISCUSSION

Although 2,4-D cells, strain T5, grow well in the absence
of cytokinins, it will only form shoots when transferred to a
medium with 5×10^{-5} M zeatin as the cytokinin.

Table I shows that in zeatin cells RNA and protein concent-
ration per gram fresh weight began to increase 5 to 6 days after
inoculation, then 15 days later DNA increased, whereas the contents
per fresh weight in 2,4-D cells remained constant.   Increased of
RNA, soluble protein and DNA in this order suggest that protein
synthesis changes during the shoot redifferentiation.   Thorpe et
al[22] have reported that RNA and protein are increased in shoot-
forming regions of tobacco cultured cells, and Werner et al[23] have
also described that some qualitative changes in the proteins of
carrot occur prior to visible morphological onset of root form-
ation.

Table I. Contents of DNA, RNA and Protein.

	Days		
	0	6	15
DNA (mg/g fresh wt)			
2,4-D (A)	0.038	0.065	0.056
Zeatin (B)	-	0.052	0.104
B/A	1.00	0.80	1.86
RNA (mg/g fresh wt)			
2,4-D (C)	2.80	2.72	3.08
Zeatin (D)	-	4.11	4.14
D/C	1.00	1.51	1.34

	Days			
	0	5	10	16
Buffer Soluble Protein (mg/g fresh wt)				
2,4-D (E)	1.34	1.54	1.35	1.33
Zeatin (F)	-	1.83	2.28	1.98
F/E	1.00	1.19	1.69	1.48

Incorporation of ^{14}C-leucine into proteins and DEAE cellulose column chromatography of these proteins were investigated and the results are shown in Figs. 1 and 2.   In Fig. 1, protein peaks for zeatin cells were eluted at a higher concentration of NaCl than were those for 2,4-D cells.   The radioactivity of ^{14}C-leucine, incorporated into protein showed a relatively high level.   A remarkable and characteristic peak at the concentration of 0.3 M NaCl for zeatin cells(B) was obtained as compared to that for 2,4-D cells (A).
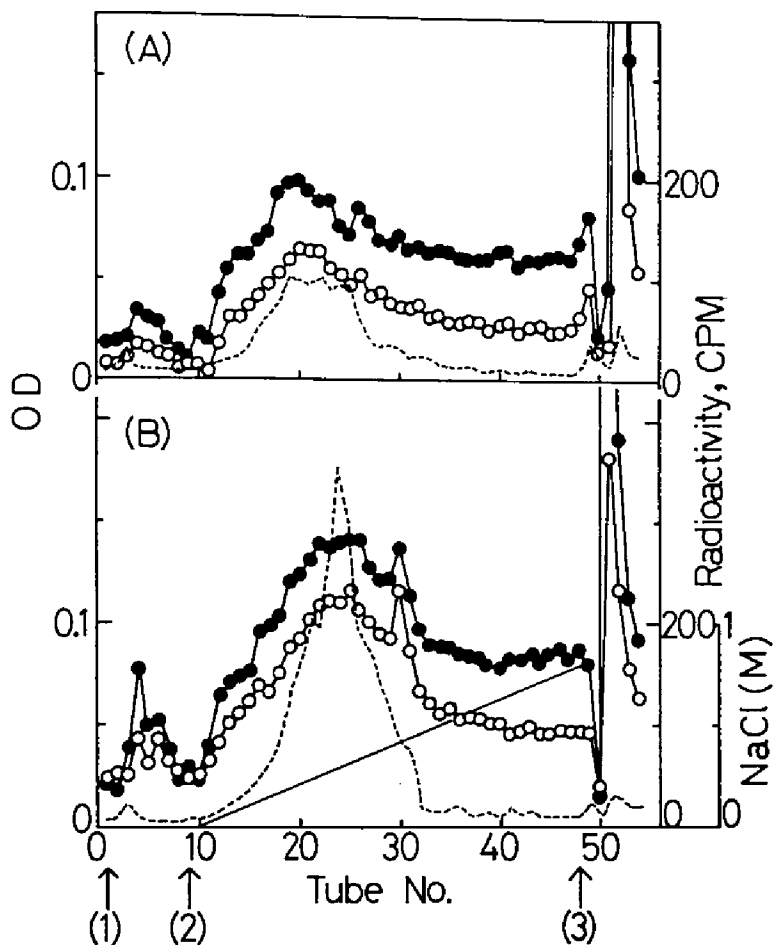
Fig. 1. DEAE Cellulose Column Chromatography after the Incorpo-
ration of ^{14}C-Leucine into Protein Fractions of Tobacco Cells
Cultured for 15 Days with 10^{-6} M 2,4-D (A) and with 5×10^{-5} M
Zeatin (B).

Five grams of 2,4-D cells and 2 g of zeatin cells were separately
incubated in 5 ml of medium containing 0.625 uCi of ^{14}C-leucine
for 5 hr, then the protein solution was prepared by the procedures
described in Methods.   The 3.8 mg (A) and 8.9 mg (B) proteins
were applied on a DEAE cellulose column (1x7 cm) and eluted with:
1) 20 ml of 0.01 M phosphate buffer (pH 7.5), 2) 120 ml of a o to
0.8 M gradient of NaCl in 0.01 M phosphate buffer (pH 7.5), 3)
20 ml of 0.1 N NaOH.   (-●-) shows the absorbance at 260 nm, (-0-)
that at 280 nm and (---) radioactivity.

Fig. 2. DEAE Cellulose Column Chromatography.
(A) and (C) show the percent of total protein in each protein
fraction(omitting fraction 1) in 2 day-old cultured cells. (-●-)
indicates 2,4-D cells and (-O-) zeatin cells.　(B) and (D) show
the percent of radioactivity incorporated into proteins(omitting
fraction 1) at the 2nd and 15th day after the beginning of cell
culture.　Symbols are the same as above.　(E) shows the per-
centage of total protein in each protein fraction in a young
leaf (---●---).　A few grams of 2,4-D cells and zeatin cells,
which had been cultured for 2 or 15 days were separately incubated
in 5 ml of medium containing 0.1 uCi of ^{14}C-leucine for 2 hr.
Extracts were prepared by the procedures described in Methods.
The phosphate buffer soluble fraction from two grams of tobacco
young leaf was extracted using the same methods.　The extract
was applied to a column (1x5 cm) in 0.01 M phosphate buffer(pH 7.5)
and eluted with the following solutions: 0.01 M phosphate buffer
(pH 7.5) (fraction 1), 0.1 M NaCl in 0.01 M phosphate buffer
(pH 7.5) (fraction 2), 0.2 M NaCl in the same buffer (fraction 3),
0.3 M NaCl in the same buffer (fraction 4), 0.5 M NaCl in the
same buffer (fraction 5), and 0.2 N NaOH (fraction 6).　Protein
content was determined by the method of Lowry et al[17].
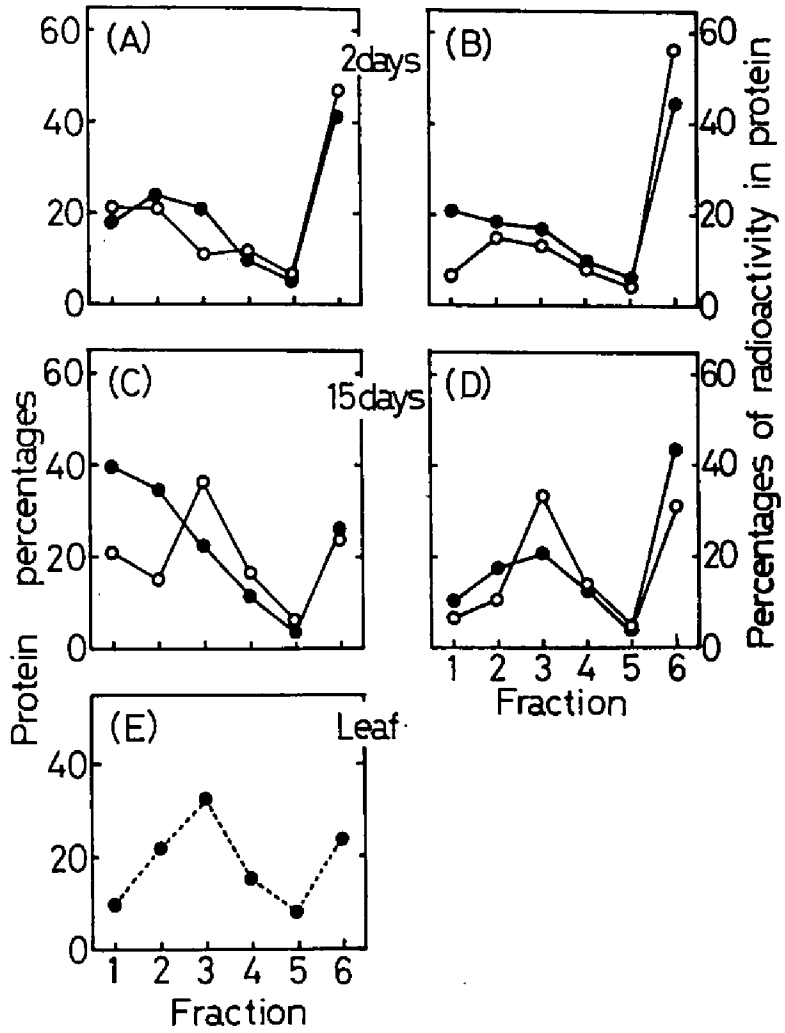
The eluent with 0.1 N NaOH shows a high absorption but much less radioactivity. Phenolics seem to cause the high U. V. absorption at 260 and 280 nm.

To compare protein patterns at different stages, ^{14}C-leucine was incorporated and DEAE cellulose column chromatography was performed. As shown in Fig. 2, patterns for the total protein and the radioactivity incorporated into 2 day-old zeatin cells are not much different from those for the 2 day-old 2,4-D cells (A and B in Fig 2). However, 15 day-old zeatin cells showed quite different patterns from the 15 day-old 2,4-D cells for both total protein and incorporated radioactivity in protein (C and D in Fig. 2). A 15 day culture period on a medium containing zeatin enhanced the synthesis of certain characteristic proteins (fraction 3 on C and D in Fig. 2) which are specific to young leaves of the tobacco plant (E in Fig. 2). Even the protein pattern of incorporated ^{14}C-leucine showed the specific peak at fraction 3 in Fig. 2 (D), the same as in Fig. 2 (E). This means that protein synthesis in zeatin cells differs from that in 2,4-D cells, and that zeatin cells protein content becomes similar to that of leaves after about 15 days of culture. Therefore, 1 investigated, in deteil, the differences in protein patterns between 15 day-old 2,4-D cells and 15 day-old zeatin cells using electrophoresis.

Protein fractions from 15 day-old cells obtained from DEAE cellulose column chromatography (Fig. 2) were electrophoresed with polyacrylamide gel. As shown in Fig. 3 slight discrepancies were
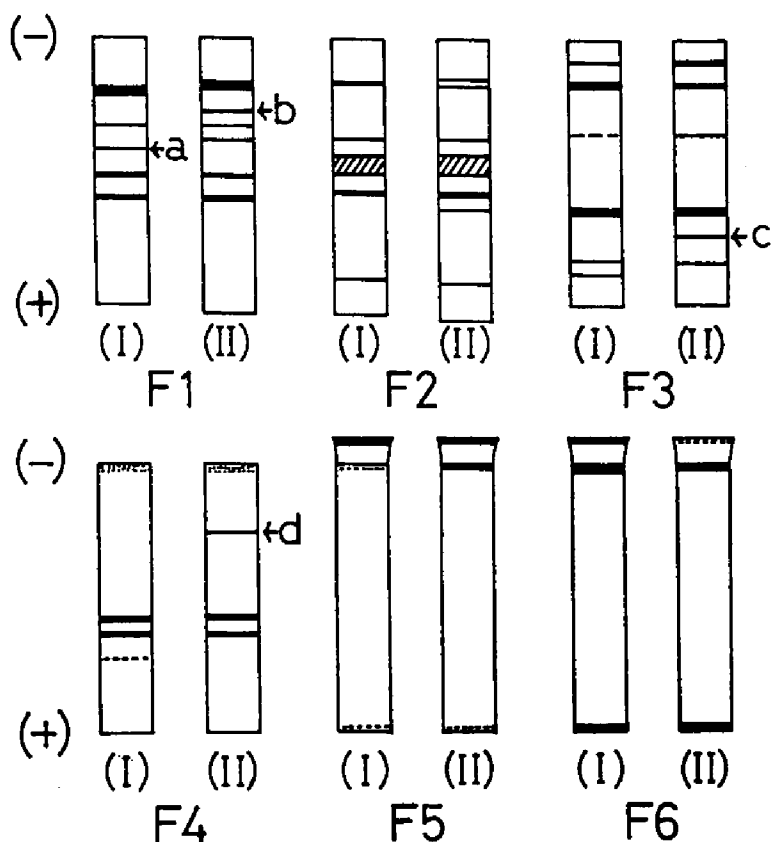
Fig. 3. Polyacrylamide Gel Electrophoresis of Proteins Fractio-
nated in Fig. 2.
FI, F2, F3, F4, F5 and F6 correspond to fraction 1, 2, 3, 4, 5
and 6 in (C) of Fig. 2.   F6 was neutralized with 0.2 N HCl before
electrophoresis.   Samples containing protein ranging from 50 to
150 ug (except F5 which contained 10 to 20 ug of protein) were
layered on gels.   After electrophoresis the gel was stained with
1 % amidoblack 10B, then destained with 7 % acetic acid.   (I) is
from 2,4-D cells and (II) from zeatin cells.

detected between 2,4-D and zeatin cells.   In zeatin cells 3 bands,

(b), (c) and (d) appeared in Fl, F3, and F4, which were not found

in 2,4-D cells.   Band (a) was found only in 2,4-D cells.

Fractions 5 and 6, which were eluted with 0.5 M NaCl and 0.2 N NaOH,

respectively, were not well mapped by electrophoresis.   Jouanneau

et al[3,4] reported the enhancement of ^{14}C-leucine incorporation by

cytokinin in tobacco cultured cells and using disc electrophoresis,

that at least one specific band appeared after the first generation.

The present results showed that at least 4 different bands were

found in cultured tobacco cells treated with and without zeatin

for 15 days.   The characteristic synthesized proteins in zeatin

cells (C and D in Fig. 2) which occurred in quantitative amounts,

did not give quantitative amounts as the very specific protein

bands at F3 in Fig. 3.   However, band (c) is a characteristic

protein band in F3 from zeatin cells and band (c), as well as

band (b) and (d), is believed to contain proteins which are

significantly involved in regenerating leaves from cultured cells.

To confirm and determine qualitatively the protein changes

due to cytokinins, which accompany shoot formation in cultured

cells, we used immunological techniques.   In these experiments,

kinetin cells were used instead of zeatin cells.

To obtain antigen from kinetin cells, cells were inoculated

on a medium containing 10^{-5} M kinetin and cultured for 5, 10, 15

and 32 days.   Kinetin cells cultured for 32 days produced no

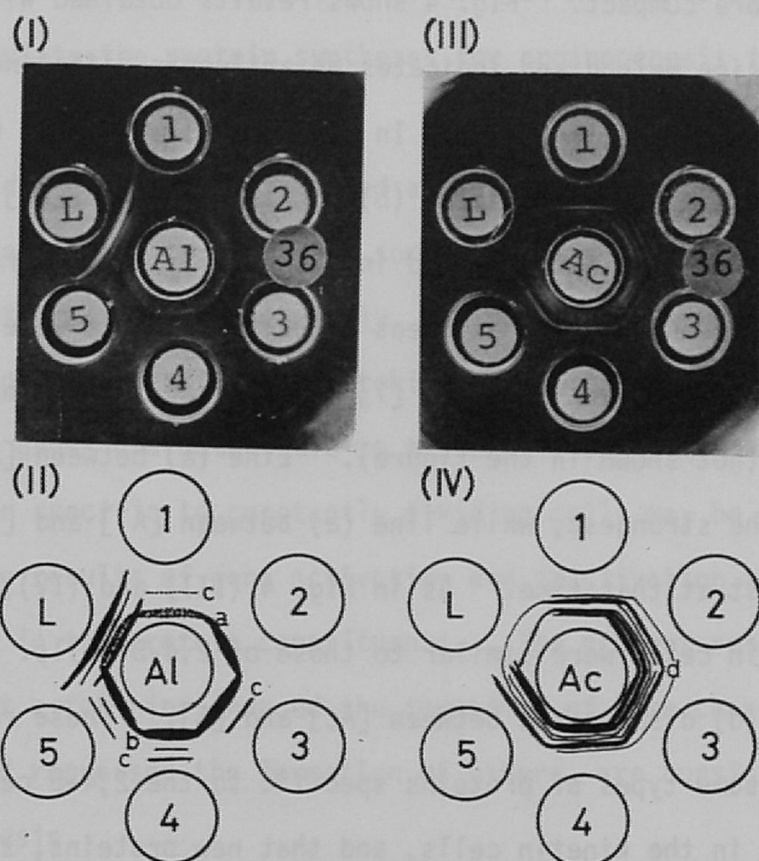visible organogenesis; though the cells, themselves, became harder

Fig. 4. (I) and (III), Photographs: (II) and (IV), Diagramatic
Representation of Immunodiffusion Patterns Observed under the
Microscope.

Fifty ul of antiserum was put into the central hole and 150 ug
(as protein) of antigen into the outer holes.   Agar plates were
incubated for 36 hr at 25°C.   [Ac]; anti-2,4-D cell serum. [Al];
anti-leaf serum. [1]; antigen of 2,4-D cells cultured for 15
days, [2]; antigen of kinetin cells cultured for 5 days, [3];
kinetin cells cultured for 10 days, [4]; kinetin cells cultured
for 15 days, [5]; kinetin cells cultured for 32 days, [L]; antigen
of tobacco leaves grown in a green house.

and more compact.   Fig. 4 shows results obtained with the double

diffusion method and indicates an antigenic difference between

2,4-D and kinetin cells.   In kinetin cells, Fig. 4 (I) and (II),

a specific precipitin line (b) appeared between [A1],and [4] and

[5], which was not observed in 2,4-D cells.   The precipitin line

(a) between [A1] and antigens of cultured cells were observed, but

there was no line [A1] and [1] 12 hr after the beginning of diffu-

sion (not shown in the figure).   Line (a) between [A1] and [5] ·

was the strongest, while line (a) between [A1] and [2] was the

weakest at that time.   As in Fig. 4 (III) and (IV), antigens of

kinetin cells were similar to those of 2,4-D cells.   However,

line (d) disappeared between [Ac] and [5].   These results show

that some types of proteins specific to the 2,4-D cells do not

exist in the kinetin cells, and that new proteins, similar to leaf

proteins, are synthesized.   Furthermore 2,4-D and kinetin cells

retain some common protein constituents which seem differ consider-

ably from those of the tobacco leaf.

These results also indicate that 2,4-D and kinetin cells

retain common protein constituents.   However, it appears that

kinetin cells are synthesizing some characteristic proteins which

cause the formation of or become part of the leaves.

In the cultured tobacco cell line, strain T5, these results

show that cytokinins cause the induction of certain characteristic

proteins, which begin to form leaves from cultured cells in vitro.

Simultaneously, other protein syntheses are repressed by cytokinins

for shoot formation.   However, it is not yet known how cytokinins act and regulate the protein syntheses for organogenesis in cultured cells.

"Cell differentiation is based almost certainly on the regulation of gene activity."[24]   If some genes are activated, RNA and protein syntheses may, subsequently, be induced or repressed.   By adding a high concentration of cytokinin to cultured dedifferentiated cells, genes characteristic to a specific organ may be activated, and a factor specific to constantly dividing cells may be inactivated.   The results of gene activation and inactivation would then appear in the protein constituents.   The present results, showing that cytokinins induced the formation of some characteristic proteins and repressed the formation of others, are consistent with this hypothesis.

Evidence that cytokinin affects RNA synthesis has been reported by Roychoudhury et al[25] and Matthysse et al[26]. Roychoudhury et al reported that kinetin stimulates the synthesis and release of nuclear RNA in coconuts.   Matthysse et al showed that kinetin enhances the rate of RNA synthesis by isolated nuclei of tobacco and soybean calluses and by pea buds.

It would thus seem that cytokinin reacts with certain cell components to regulate gene activity, which is followed by the promotion of RNA synthesis and the effects on protein metabolism shown in the present results.   Thereafter, organ formation should occur morphologically.   However, from the results on callus

induction (dedifferentiation) from differentiated tissue by auxins[27-30], the actions of cytokinins for redifferentiation seem to have conjugated metabolic relationships with auxin action. Dedifferentiation by auxins is the reverse reaction to redifferentiation by cytokinins.

SUMMARY

During tobacco cell culture cytokinins can induce and redifferentiate shoots from cells cultured in vitro.

During the early stages of shoot formation RNA, soluble protein and DNA increased in this order.

Relationships between the synthesis of certain proteins and the disappearance of other proteins during the early stages of shoot redifferentiation, under the specific actions of cytokinins, were sought using DEAE cellulose column chromatography, polyacrylamide gel electrophoresis and immunological methods.

The present results show that cytokinins cause the induction of certain characteristic proteins, when leaves begin to form from cultured cells in vitro. During shoot redifferentiation some proteins synthesized for cell proliferation are repressed. These changes in protein synthesis caused by cytokinins in cultured tobacco cells are discussed in regard to redifferentiation.

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CHAPTER V

DNA-DEPENDENT RNA POLYMERASES DURING SHOOT FORMATION
FROM CULTURED TOBACCO CELLS[1].

INTRODUCTION

Since the first DNA-dependent RNA polymerases (RPases) in the
nuclei of animal cells were solubilized, purified and identified
as multiple forms[2], RPases from many animal cells have been
reported[3]. Recent studies also show that multiple forms occur
in higher plants as well. The RPases which have been purified and
characterized from higher plants[4-17] show in most cases, properties
similar to the RPases from animal cells.

Hormonal control of the activities of the RPases from plant
cells by indole-3-acetic acid (IAA)[10,18,19] and gibberellic acid
(GA)[20] has been reported. However, cultured plant cells (callus)
usually continue cell division and growth in the presence of auxins.
Shoots and roots can be formed by cytokinins from cultured tobacco
cells[20-22]. During organogenesis from these cultured cells, many
metabolic changes involving, for example, changes in RNA and protein
syntheses have been observed[23]. Cytokinins and auxins are pre-
sumed to play an important role in organogenesis and transcriptive
regulation by cytokinins and/or auxins seems to exist.

This chapter describes the partial purification of DNA-depen-
dent RNA polymerases from cultured tobacco cells at different
stages of shoot formation and presents a comparison of these RPases.

MATERIALS AND METHODS

Chemicals.

Uridine-5-^{3}H-5'-triphosphate (^{3}H-UTP, sp. act. 26.9 Ci/mM) was obtained from New England Nuclear and ethanol as solvent was removed by bubling with N_2. Highly polymerized calf thymus DNA was purchased from Warthington Biochemical Corp.; whole histones, from Sigma Chemical Co.; α-amanitin, from C. H. Boehringer Sohn; rifamycin SV, from Calbiochem, and four kinds of nucleoside triphosphates, from Sigma Chemical Co. All other chemicals were of reagent grade.

Cell culture.

Three kinds of cultured cells (2,4-D cells, kinetin cells and redifferentiated shoots) originating from the pith tissue of the tobacco plant, Nicotiana tabacum var. Bright Yellow, were used. 2,4-D cells[24] were transferred to a liquid medium containing 10^{-5} M kinetin and cultured at 27°C in the dark. Three days after this transfer the kinetin cells were harvested (3DK5 cells). Redifferentiated green shoots (RS cells) were induced with 10^{-5} M kinetin using cells cultured with 10^{-5} M indole-3-butyric acid(IBA), then subcultured on an agar medium containing 10^{-5} M kinetin at 25°C in the light. RS cells were harvested 15-20 days after transfer to the same medium. The basic composition was that of the Linsmaier and Skoog medium[25]. These three day-old 2,4-D cells (3DD6 cells), 3DK5 and RS cells were stored at -20°C for use as the enzyme source.

Enzyme assay.

RPase activity was determined as described previously[1,11] with some modifications. The standard reaction mixture contained in 0.15 ml: 50 mM Tris-HCl, pH 7.8; 2 mM DTT; 1-2 mM $MnCl_2$; 0.32 mM of ATP, GTP and CTP; 0.05 mM of UTP containing 1-2 uCi of ^{3}H-UTP; 12 ug of native calf thymus DNA (highly polymerized) or pea DNA; and 50 ul of enzyme solution. Incubated lasted for 10-15 min at 37°C RPase activity was represented by the ^{3}H-UMP incorporated into the trichloroacetic acid (TCA) insoluble materials. Radioactivity was determined with a Beckman LS-100 liquid scintillation spectrometer.

Purification of RNA polymerases.

RPase preparation was essentially according to the methods . described by Sasaki et al[11]. Sixty grams of frozen tobacco cells and 20 g of Polyclar AT were ground in a chilled mortar for 10 min in a final volume of 120 ml containing 0.15 M Tris-HCl (pH 7.8); 0.1 M EDTA; 5 mM DTT; 0.01 M $(NH_4)_2SO_4$ and 25 %(v/v) glycerol. The mixture was sonicated for 2 min. The homogenate was filtered through gauze and centrifuged at 100,000 g for 60 min. The supernatant was dialysed for 4 hr against 3 liters of 50 mM Tris-HCl (pH 7.8); 0.5 mM $MgCl_2$; 1 mM DTT; 0.01 M $(NH_4)_2SO_4$ and 25 % glycerol (0.01 M ammonium sulfate standard buffer; 0.01 M ASS buffer). After the enzyme solution was loaded on a DEAE-Sephadex column (1.5 x 11 cm) equilibrated with 0.01 M ASS buffer.

The column was washed with 150 ml of 0.01 M ASS buffer and eluted with 150 ml of 0.1 M ASS buffer, then with 100 ml of 0.1-0.4 M linear gradient ASS buffer. RPase II fractions after DEAE-Sephadex chromatography were collected and dialysed for several hours against 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM DTT and 50 % glycerol. Purified RPase IIs were stored in liquid nitrogen.

Preparation of pea DNA.

About 50 g of etiolated Alaska pea seedlings which had been grown for 5 days, was homogenized in a Waring Blendor with 50 mM Tris-HCl, pH 7.8, containing 0.25 M sucrose, 10 mM mercaptoethanol and 1 mM $MgCl_2$ (Buffer I). After passage through 6 layers of gauze, the filtrates were centrifuged at 6,000 g for 30 min. The resulting pellet was resuspended in Buffer I and collected by centrifugation at 10,000 g for 15 min. This pellet was washed with 1/10 x SSC buffer, then suspended in a small amount of 1/10 x SSC buffer containing 2 M NaCl and stirred gently for a few hours. The viscous solution was centrifuged at 10,000 g for 15 min. Three volumes of cold ethanol was added to the supernatant obtained. The resulting white fibrous precipitate (crude chromatin) was collected with tweezers, then dissolved in 50 mM Tris-HCl, pH 7.8, containing 1 % SDS after which the solution was stirred gently for several hours. An equal volume of phenol saturated with buffer was added to the solution and the whole was stirred for 30 min at room temperature. After low speed centrifugation the

water phase was collected. The phenol treatment was repeated 2 more times, then three volumes of cold ethanol was added to the collected water phase solution. A white fibrous precipitate (crude DNA) was collected and dissolved in a small amount of SSC buffer. After adding the RNase (100 ug/ml) the solution was incubated at 37°C for 10 min. DNA was purified three times using the phenol treatment. This purified DNA was precipitated with cold ethanol, then washed with acetone and dried.

RESULTS

Cell culture.

When tobacco cells cultured with 10^{-6} M 2,4-D (2,4-D cells) were transferred to a medium containing 10^{-5} M kinetin (kinetin cells) shoot formation was observed. In the early stage of shoot formation the growth rate of the kinetin cells decreased 3 days after transfer compared to that for the 2,4-D cells, as shown in Fig. 5 in CHAPTER I. The degree of organogenesis proceeded in the order of 2,4-D cells (3DD6 cells) through kinetin cells (3DK5 cells) to RS cells (redifferentiated shoots).

Isolation and purification of RNA polymerases.

Fig. 1 shows that cultured tobacco cells (3DD6) had two peaks of RPases. α-Amanitin inhibited RPase activity of peak II completely but did not inhibit peak I (Table I). From this result cultured tobacco cells had two different types of RPases, RPase I and RPase II, which were eluted with 0.1 M and 0.25-0.30 M

Table I. Effects of Rifamycin and α-Amanitin on RNA Polymerases from 3DD6 Cells.

Drugs	Concn. ug/ml	^{3}H-UMP Incorporated, CPM	
		Peak I	Peak II
Control	-	44 (100)	637 (100)
Rifamycin	63.0	28 (64)	627 (98)
α-Amanitin	0.65	-	59 (9)
	1.30	40 (91)	21 (3)

1) RNA polymerases after DEAE-Sephadex column chromatography were used.

2) Incubation lasted for 10 min with 1 uCi of ^{3}H-UTP and 2 mM MnCl$_2$.

(NH$_4$)$_2$SO$_4$ in DEAE-Sephadex column chromatography respectively, as well as other eukaryotic cells.

RPase I and II were found in 3DK5 and RS cells as well as in 3DD6 cells. However, as the RPase Is showed low activities and were very labile, only the RPase IIs were partially purified and characterized. Fig. 2 shows the elution profile of each RPase II from the DEAE-Sephadex column chromatographies. Each RPase activity eluted at 0.28 M (NH$_4$)$_2$SO$_4$ was identified by its inhibition with α-amanitin as RPase II (Table II). Each RPase I was eluted at less than 0.1 M (NH$_4$)$_2$SO$_4$ and was not inhibited by α-amanitin. RPase IIs in 50 % glycerol-50 mM Tris-HCl-1 mM DTT buffer were stable in liquid nitrogen for more than 4 months. No appreciable RNase activity was detected in any RPase II fraction. RS cells
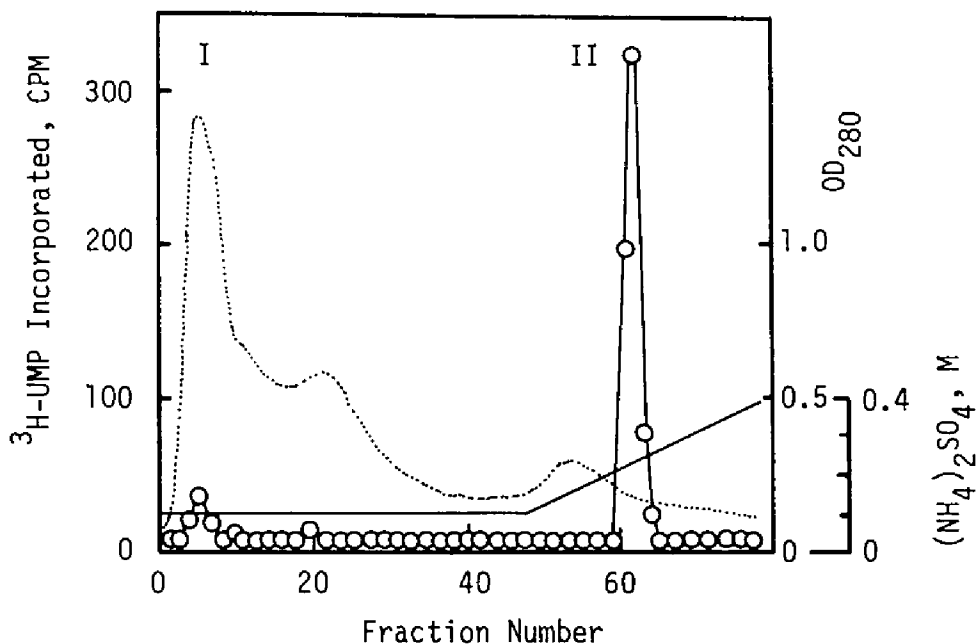
Fig. 1. DEAE-Sephadex Column Chromatography of RNA Polymerases
from 3DD6 Cells
(····) shows OD_{280} and (-O-), radioactivity of ^{3}H-UMP incorporated.

had other RPase activity (peak III) eluted with 0.22 M $(NH_4)_2SO_4$.
This seems to be an RPase originating from chloroplasts because
rifamycin inhibited this activity but α-amanitin did not.

Table III show changes in the total RPase II activities after
DEAE-Sephadex column chromatography. Total activity decreased
after to the kinetin-medium (3DK5 cells), then the activity of the
RS cells increased to the level of the 3DD6 cells.

Effects of templates on RNA polymerase activity.

The RPase IIs isolated from cultured tobacco cells required DNA as a template (Fig. 3). Each RPase II transcribed denatured calf thymus DNA as the template better than it did native DNA.

The effects of the DNA species on the RPase IIs are shown in Fig. 4. RPase II from 3DD6 cells transcribed calf thymus DNA at a constant rate at a concentration ranging from 3.75 to 22.75 ug. However, enzyme activity gradually increased for pea DNA as the amount of added DNA increased. At a concentration of more than 6.50 ug DNA, the RPase II activity of the 3DD6 cells for pea DNA was greater than that for calf thymus DNA, while the opposite was true at a low concentration of DNA.

In contrast, the RPase II from RS cells showed slight increases for both calf thymus and pea DNA as the amounts of DNA increased. No inversion of the enzyme activity for calf thymus and pea DNA

Fig. 2. DEAE-Sephadex Column Chromatography of RNA Polymerase II from 3DD6 (A), 3DK5 (B) and RS cells (C).
The crude extract after dialysis was loaded on a 1.5 x 11 cm DEAE-Sephadex column equilibrated with 0.01 M ASS buffer (50 mM Tris-HCl-25% glycerol containing 0.5 mM $MgCl_2$, 1 mM DTT and 0.01 M $(NH_4)_2SO_4$; 0.01 M ammonium sulfate standard buffer). After loading, the column was washed with 150 ml of 0.01 M ASS buffer and eluted with 150 ml of 0.1 M ASS buffer, then with 100 ml of 0.1-0.4 M linear gradient ASS buffer. Part of linear gradient elution (RPase II) is shown in the figure.
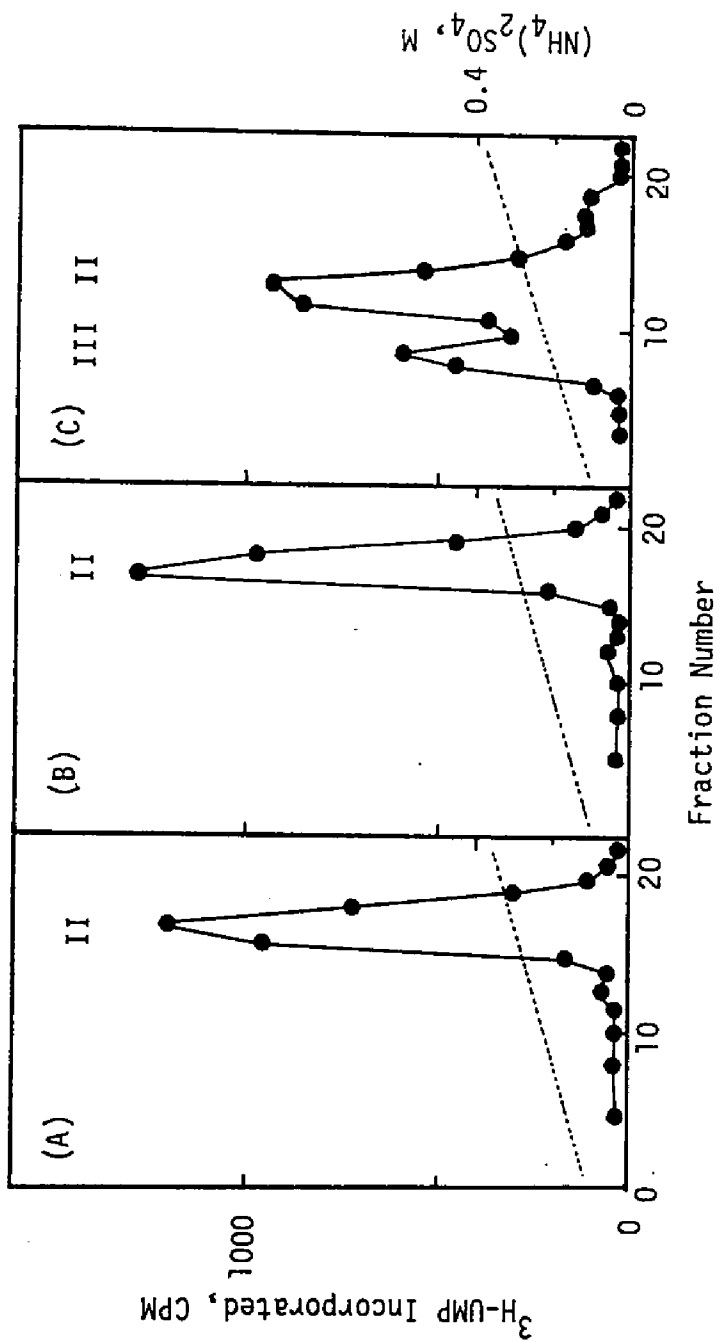
Table II. Effects of Rifamycin and α-Amanitin on RNA Polymerase IIs from 3DD6, 3DK5 and RS Cells.

Drug	Concn. ug/ml	^{3}H-UMP Incorporated, CPM		
		3DD6	3DK5	RS
Control	–	1826 (100)	836 (100)	428 (100)
Rifamycin	63.0	1913 (105)	834 (100)	310 (73)
α-Amanitin	1.30	31 (2)	11 (1)	51 (12)

1) Figures in parentheses show the relative activities(%).

2) Incubation lasted for 15 min at 37°C with 1.5 uCi of ^{3}H-UTP and 1 mM MnCl$_2$.

Table III. Changes in Total RNA Polymerase II Activities during Shoot Formation.

Cells	Total Activity[1)
3DD6	396×10^3 (100)
3DK5	193×10^3 (49)
RS	351×10^3 (89)

1) Total activity is represented as total ^{3}H-UMP incorporated (CPM) per 60 g of original material, after DEAE-Sephadex column chromatography.

2) Incubation lasted for 15 min with 2 uCi of ^{3}H-UTP and 1 mM MnCl$_2$.
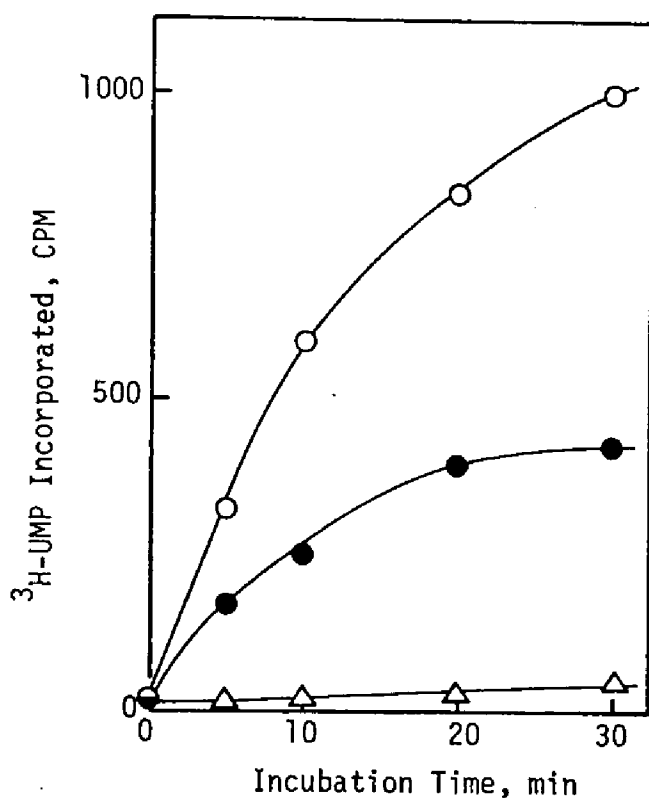
Fig. 3. DNA Dependency of the RNA Polymerase II from 3DK5 Cells.
Incubation lasted for 15 min with 1.5 uCi of ^{3}H-UTP and 1 mM
MnCl$_2$. (-O-) shows the activity in the presence of denatured
calf thymus DNA; (-●-), in the presence of native calf thymus DNA;
(-△-), in the absence of DNA.

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was observed at the concentrations tested.

The RPase IIs from 3DD6 and RS cells showed different template
specificity which seems to reflect the difference in regulative
functions in the RPase IIs from these cells.

Additions of histones (whole histones from calf thymus)
decreased the template activity of pea DNA. At the ratio of
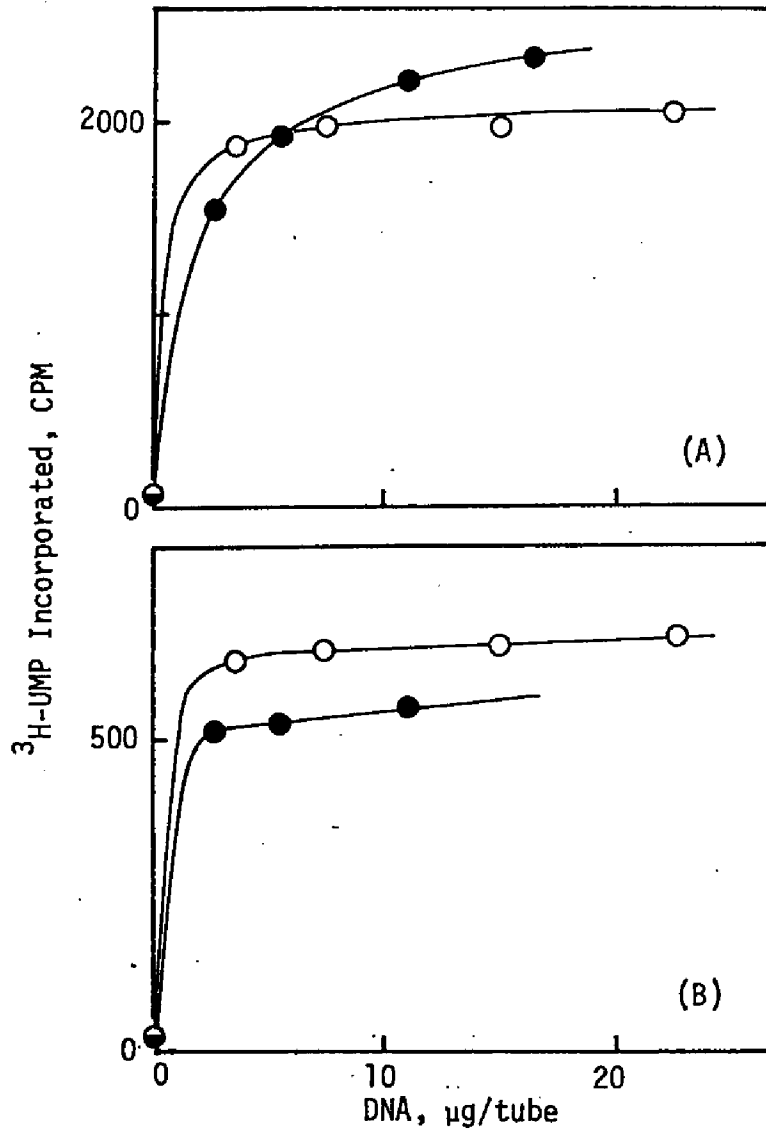
Fig. 4. Effects of Pea and Calf Thymus DNA on RNA Polymerase IIs from 3DD6 (A) and RS Cells (B).
(-●-) shows activity for native pea DNA as template; (-O-), for native calf thymus DNA.

Table IV. Effects of 2,4-D and Kinetin on RNA Polymerase IIs
in vitro.

Additions	Concn. (M)	^{3}H-UMP Incorporated, CPM	
		3DD6 Cells	RS Cells
Control	-	1023 (100)	418 (100)
2,4-D	6.3×10^{-6}	1079 (105)	417 (100)
Kinetin	6.3×10^{-6}	1121 (110)	403 (96)

1)   Figures in parentheses show relative activities (%).
2)   Incubation lasted for 15 min at 37°C with 1.5 uCi of ^{3}H-UTP
and 1 mM $MnCl_2$.

1:1.5 (DNA: histone), 50 % of the activity was found and there was

no activity when the ratio was 1: 2.5.   However, no significant

difference between the RPase IIs from 3DD6 and RS cells was found.

Effects of auxins and cytokinins on enzyme activities in vitro.

As shown in Table IV, 6.3×10^{-6} M 2,4-D or kinetin had no

effect on RPase II activities in vitro.   Other plant regulators,

IBA, NAA and benzyladenine, also had no effects on RPase II activi-

ties.   This indicates that auxins and cytokinins do not directly

take part in transcriptive regulation.   GA also had no effects on

enzyme activity[20].

Effects of Mn^{2+}, Mg^{2+} and $(NH_4)_2SO_4$ on enzyme activities.

The divalent cation requirements of the RPase IIs shown in

Fig. 5 indicate that each RPase II prefered Mn^{2+} to Mg^{2+}.   The

optimum concentration was 1 mM of Mn^{2+} and 10 mM of Mg^{2+} for each

RPase II. The ratio of maximum activity at 10 mM of Mg^{2+} to that at 1 mM of Mn^{2+} decreased from 74 to 43 % as organogenesis proceeded (from 3DD6 to RS cells). A high concentration of Mg^{2+} repressed the activity of the RPase II from the 3DD6 cells, while the RPase II from the RS cells maintained 74 % of its activity at 40 mM of Mg^{2+}. Repression at a high concentration of Mg^{2+} was enhanced in the presence of $(NH_4)_2SO_4$ (about 0.08 M) as shown in Fig. 6. The addition of $(NH_4)_2SO_4$ to the reaction mixture caused changes in the enzyme activities (Fig. 7). In the presence of 1 mM Mn^{2+}, maximum activities occurred at 0.05 M of $(NH_4)_2SO_4$ for the RPase IIs from the 3DD6 and 3DK5 cells, and when there was no addition of $(NH_4)_2SO_4$ there was maximum activity for the RPase II from the RS cells. In contrast, in the presence of 10 mM Mg^{2+}, $(NH_4)_2SO_4$ repressed the transcribing activities of each RPase II and no activity was found at 0.1 M of $(NH_4)_2SO_4$. However, the degree of repression differed for each RPase II at the concentrations tested (Fig. 7).

Fig. 5. Divalent Cation Requirements of the RNA Polymerase IIs from 3DD6 (A), 3DK5 (B) and RS cells (C).
Incubation lasted for 10 min at 37°C with 1.5 uCi of ^{3}H-UTP and the given concentration of divalent cation. (-O-), activity in the presence of $MgCl_2$; (-●-), in the presence of $MnCl_2$.
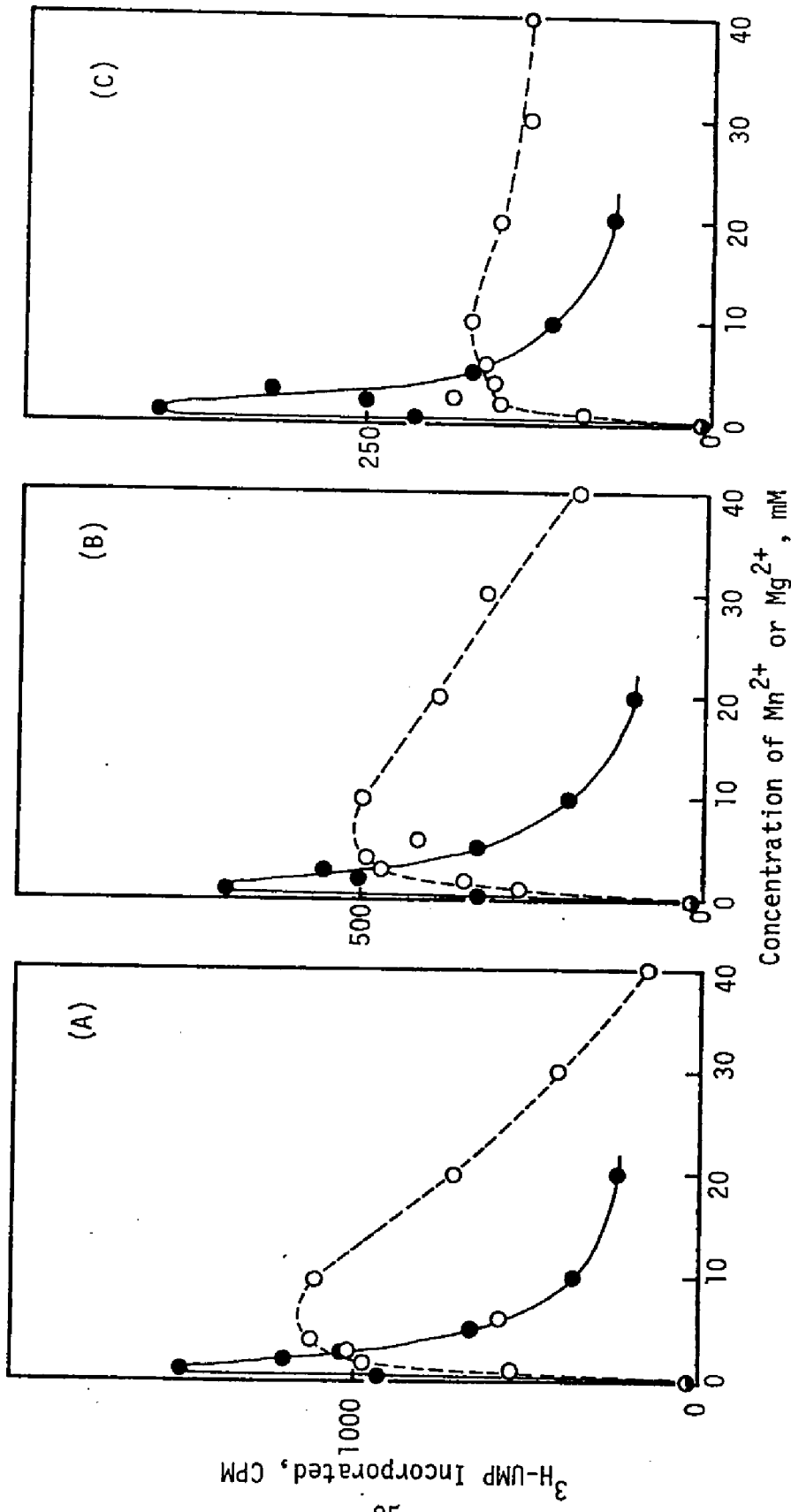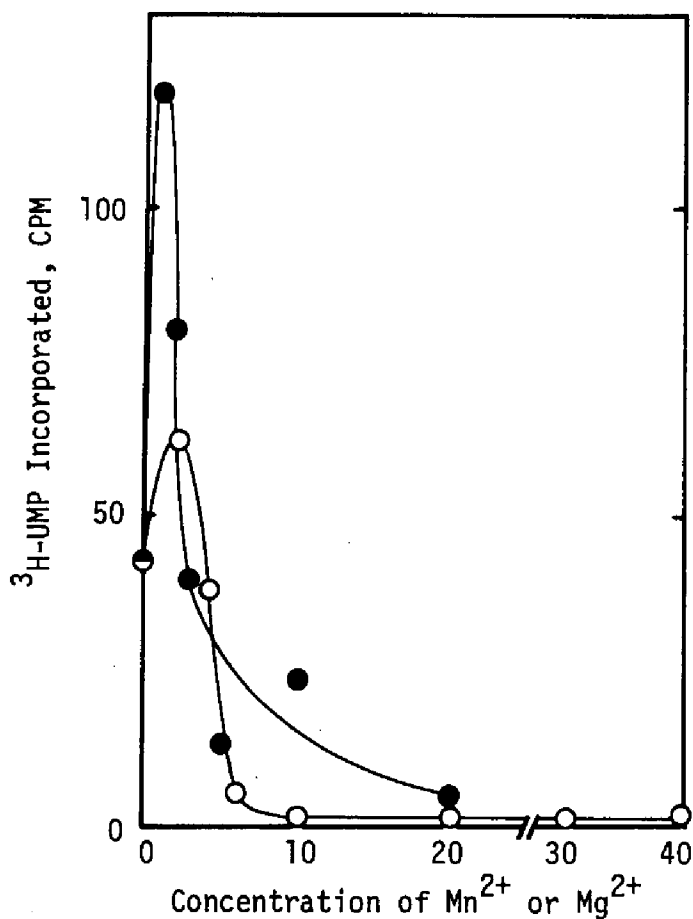
Fig. 6. Effects of Mn^{2+} and Mg^{2+} on RNA Polymerase II Activity from 3DD6 Cells.

Indicated concentration of Mn^{2+} or Mg^{2+} was added instead of 2 mM of $MnCl_2$. About 0.08 M $(NH_4)_2SO_4$ and 0.17 mM Mg^{2+}, originating from enzyme solution after DEAE-Sephadex chromatography, were included in all reaction mixture. Incubation lasted for 10 min at 37°C with 1 uCi of ^{3}H-UTP. (-●-) and (-O-) indicate the Mn^{2+} and Mg^{2+} effects, respectively.
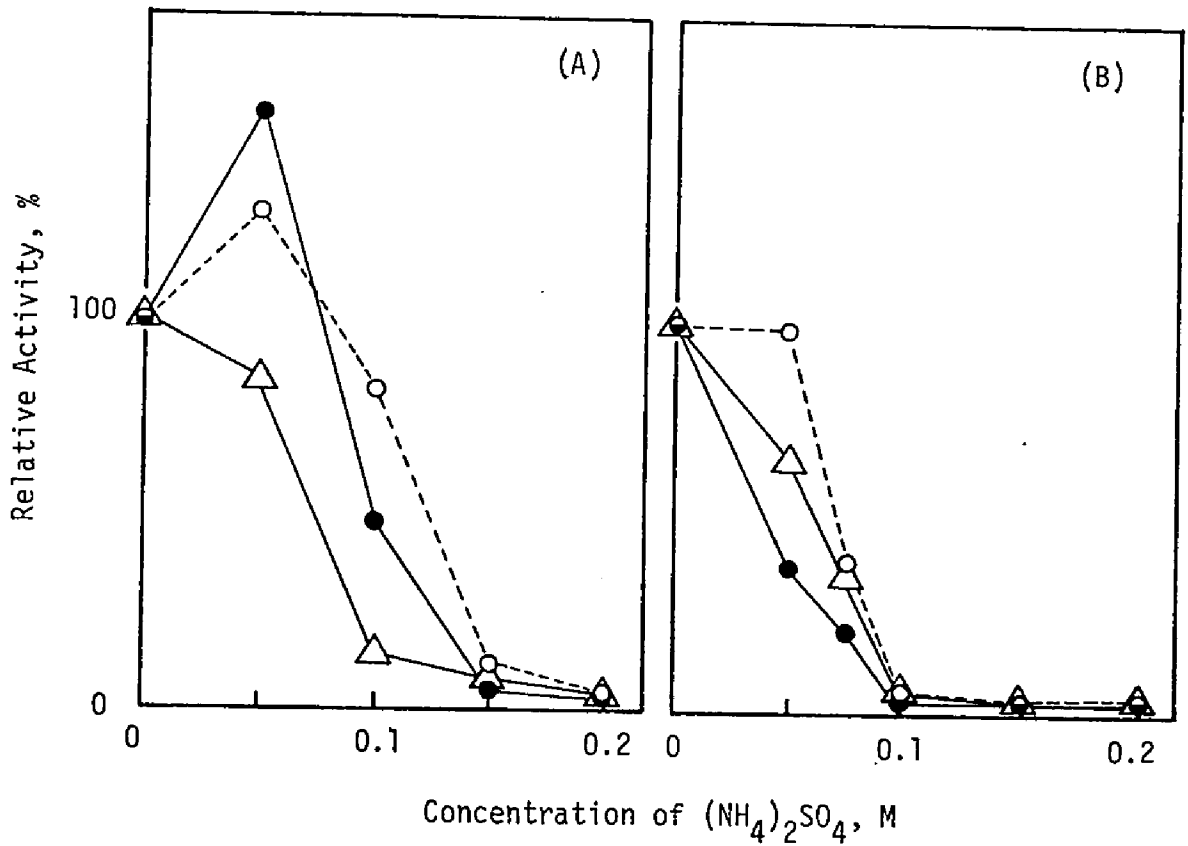
Fig. 7. Effect of $(NH_4)_2SO_4$ on RNA Polymerase II Activity. Incubation lasted for 15 min with 2 uCi of ^{3}H-UTP and given concentration of $(NH_4)_2SO_4$ in the presence of 1 mM $MnCl_2$ (A) and 10 mM $MgCl_2$ (B). (-●-) shows RPase II activity from 3DD6 cells; (-O-), from 3DK5 cells; (-△-), from RS cells.

DISCUSSION

Changes in protein and RNA syntheses have been found during shoot formation from cultured tobacco cells by cytokinins[23] and during callus formation from Jerusalem artichoke tubers[26]. To clarify what this transcriptive regulation is, RPases from the different stages of shoot formation were purified and characterized. The RPases of cultured tobacco cells(3DD6 and 3DK5 cells) and re-differentiated shoots (RS cells) were shown to have multiple forms as in many other plants. The total RPase II activity of the 3DD6 cells was higher than that of the 3DK5 and RS cells. Changes in the levels of RPase II activities have been observed during the embryogenesis of $\underline{Xenopus}$[27] and the germination of wheat[14]. One possibility is that increased or decreased $\underline{de\ novo}$ synthesis of RPase II occurs during shoot formation.

The RPase IIs purified from 3DD6, 3DK5 and RS cells possessed differing properties for template specificity (Fig. 4), their divalent cation requirements (Fig. 5) and their behaviors toward ionic strength (Fig. 7). The difference in transcribing activity for pea and calf thymus DNAs (Fig. 4) indicates that the RPase IIs from 3DD6 and RS cells differ in their regulatory functions. These RPase IIs from the 3DD6 and 3DK5 cells were repressed at a high concentration of Mg^{2+}, especially in the presence of $(NH_4)_2SO_4$, while less repression was found in the RPase II from RS cells. RPase II activity decreased rapidly as the concentration of

$(NH_4)_2SO_4$ increased.   In preliminary experiments the RNAs synthesized by RPase IIs from 3DD6 and RS cells with pea DNA appeared to be different in molecular size.   Changes in divalent cations and the ionic strength of the reaction mixture are known to produce a qualitative difference in the RNA synthesized by various animal nuclear preparations[28].   These findings might imply the presence of polymerases with different ionic requirements or they could be the results of the effects of divalent cations and ionic strength on the template, or on other regulatory components of the systems.

The detailed relationships between auxins and cytokinins and RPase II activity are not clear.   But auxins or cytokinins had no direct effect on RPase II activity in vitro (Table IV) nor did GA[20].   Non-histone proteins[29] and some types of activating factors[18,19,30,31] involved with auxins or kinetin, have been reported to be regulatory factors for plant RPases.

Two molecular species for RPase II have been reported.   Thus, an RPase II with a low molecular weight may be derived from one with a high molecular weight by proteolysis[8,13,30].

Because of the different properties of the RPase IIs from the three kinds of cells, modifications in the structure of the enzyme are presumed.   However, it is not yet clear what kinds of modifications exist in cultured cells.

SUMMARY

During tobacco (Nicotiana tabacum var. Bright Yellow) cell culture, cytokinins can induce shoot formation. As the syntheses of specific proteins progressed during shoot formation from cultured cells, the RNA polymerases (RPases) were extracted and partially purified from 3 day-old cells cultured with 2,4-D (control) or kinetin, and from redifferentiated shoots. RPases from cultured tobacco cells were composed of at least 2 kinds, RPase I and II. RPase I was low activity and very labile. Total RPase activities changed during shoot formation. Furthermore, the isolated RPase IIs from redifferentiating shoot cells showed properties which differed in template specificity, divalent cation requirements and behavior to ionic strength when compared with the control. In contrast, no direct effects of auxins and cytokinins on RPase II activities were found in vitro. It is presumed that changes in de novo RPase II synthesis and some modifications of RPase II occur during shoot formation.

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CHAPTER VI

CELL-CELL INTERACTION IN CULTURED TOBACCO CELLS.

-THE INDUCTION OF PHENYLALANINE AMMONIA LYASE IN

CULTURED TOBACCO CELLS[1].-

INTRODUCTION

Phenylalanine ammonia lyase(PAL) plays an important role in plants. Many phenolics, including a large number of secondary metabolites, are developed from the t-cinnamic acid produced from L-phenylalanine by a PAL catalyzed reaction. Since Koukol et al[2] discovered this enzyme, a number of reports on the interrelationship of PAL with the synthesis of phenolic compounds have been made. However, PAL responds to a wide variety of stimuli in a short time; especially to illumination[3,4]. In cultured cells, Rubery et al[5], Thorpe et al[6], Davies[7], Gregor et al[8] and Innerarity et al[9] reported evidence of PAL or a large increase in PAL activity due to different regulations. This chapter describes PAL induction after the transfer of cells to a fresh culture medium and cell-cell interaction is discussed.

MATERIALS AND METHODS

Cell cultures.

Cultured tobacco cells(Nicotiana tabacum var. Bright Yellow), Strain T5S, derived from tobacco callus Strain T5 and cultured in a liquid medium containing 10^{-6} M 2,4-D were used. The basic

composition of the medium was the same as that of Linsmaier and Skoog[11]. All cells were cultured in suspension under reciprocal shaking (80 r/min) at 25°C in the dark.

The inoculate of cells was about 10 g in 20 ml of liquid medium in a 100 ml Erlenmeyer flask. Growth was measured by the turbidity at 610 nm. Protein content was also measured during growth. Protein was extracted with 0.05 M Tris-HCl buffer, pH 7.8, then precipitated by 5 % trichloroacetic acid. The precipitate was collected by centrifugation and dissolved in 0.2 N NaOH. Protein content was determined by the method described by Lowry et al[12].

Extraction of PAL.

The enzyme was extracted from acetone powder. To do this, about 10 g of cultured cells was ground in a mortar with acetone previously chilled to -20°C. The homogenate was filtered on a glass filter funnel and its residue was washed with cold acetone, then dried in vacuo. The acetone powder was homogenized with 75 mM borate buffer, pH 8.8, and Polyclar AT. The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was used as the enzyme solution.

Enzyme assay.

The spectrophotometric method[13] was used for the enzyme assay. The reaction mixture contained 112 u moles of borate at pH 8.8; 30 umoles of L-phenylalanine; 1.5 ml of enzyme solution (0.4-0.7 mg of protein) in a volume of 3 ml. The reaction at 37°C
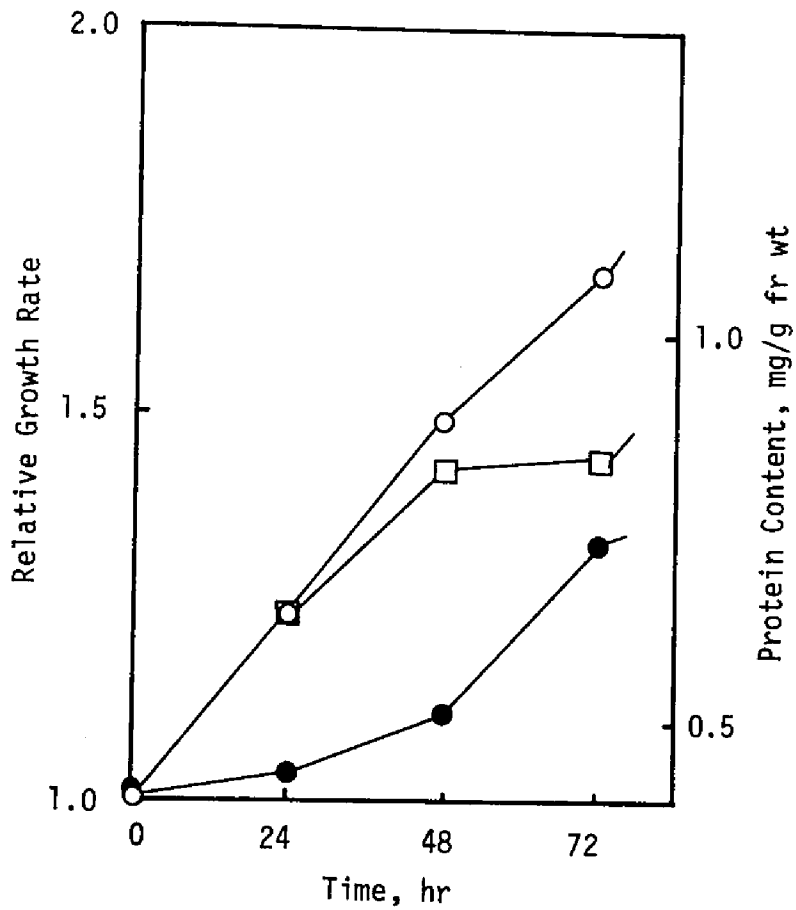
Fig. 1.   Growth Curves and Changes in Protein Content.
(-O-) shows the growth of 2,4-D cells and (-□-), that of kinetin
cells.   (-●-) shows the protein content of 2,4-D cells.

lasted for 30 or 60 min. The amount of t-cinnamic acid produced
was calculated from the increase in absorbance at 290 nm. One
unit(U) of activity represents the amount of enzyme which produces
1 nmole of cinnamic acid in 60 min at 37°C in the above reaction
mixture. The amount of protein in the enzyme solution was deter-
mined by the method of Lowry et al[12].

RESULTS AND DISCUSSION

Strain T5S cultured tobacco cells transferred to a medium
containing 10^{-6} M 2,4-D (2,4-D cells) grew rapidly without a lag
period, as shown in Fig. 1, and continued to grow for 10 days
without producing tracheid-like cells. The growth of cells trans-
ferred to a medium containing 10^{-5} M kinetin (kinetin cells) became
slow at 72 hr after transfer, though up to 48 hr had been almost
the same as that for 2,4-D cells.

Fig. 2 shows changes in PAL activity during culture. After
transfering cells to a fresh medium, the PAL activity increased and
reached a maximum at 24 hr, then the PAL activity decreased rapidly.
The magnitude of maximum activity varied with the age of the cells,
but the pattern of the activity change was the same. During the
increase in growth the protein contents per fresh weight only
slightly increased (Fig. 1). Therefore, increased PAL activity
appears to be caused by the induction or activation of the PAL
enzyme. Until 10 days after the first 48 hr PAL activity was at
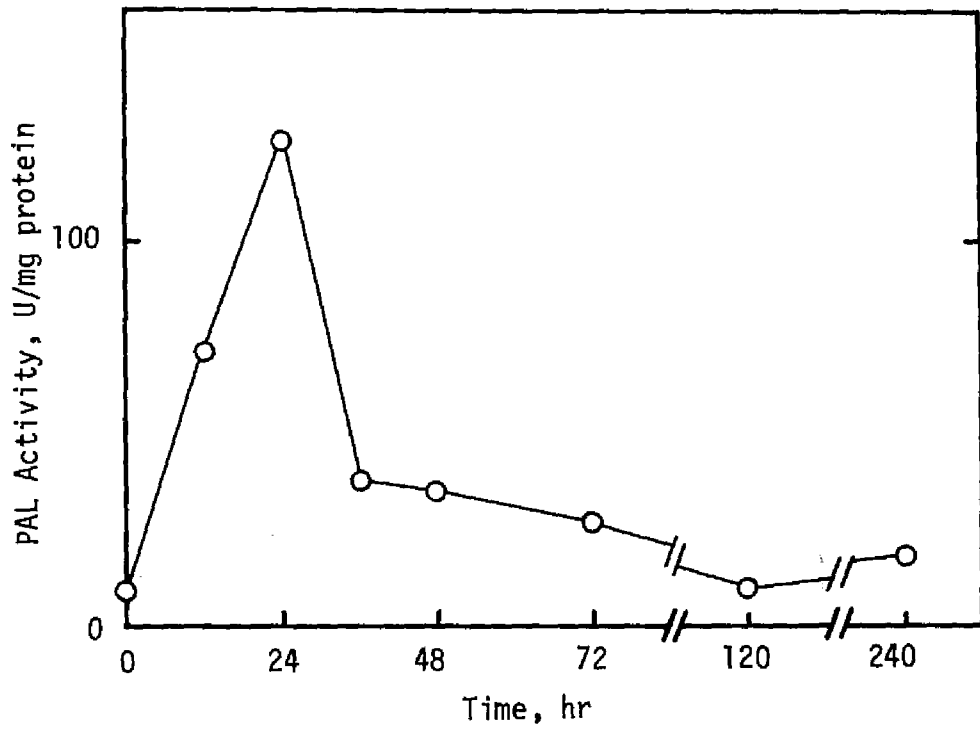a low level. Kinetin cells also showed similar changes in PAL
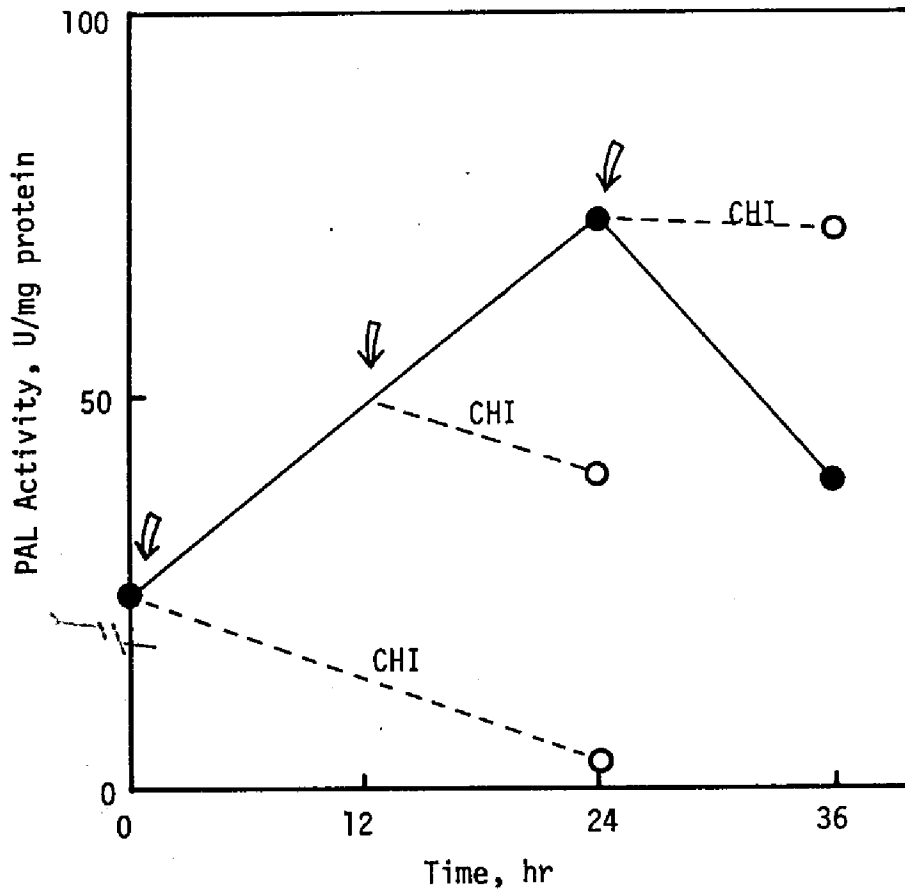
Fig. 2. Changes in PAL Activity in 2,4-D Cells.

Fig. 3. Effect of Cycloheximide (CHI) on the PAL Activity Induced by Cell Transfer. Arrows show the time when CHI (5×10^{-5} M) was added to the medium.

activity during the early period, up to 72 hr.   These inductions

of PAL activity seems to be due to transfering the cells to fresh

medium because different growth regulators produced little signifi-

cant differences.   A similar induction was reported in citrus

fruit callus by Thorpe et al[6].   In citrus callus the PAL activity

reached a maximum at 48 hr after cell transfer to a fresh medium.

The decrease in PAL activity after the maximum was different, which

probably reflects a different utilization of the t-cinnamic acid

produced.

   The effects of cycloheximide (CHI) in PAL induction and PAL

reduction due to cell transfer are shown in Fig. 3.   A CHI ($5x10^{-5}$M)

addition at 0 or 12 hr inhibited the increase in PAL activity after

that time.   An addition of CHI at 24 hr inhibited the reduction

of PAL activity and kept it constant.   The results of these

inhibitor experiments are similar to those for the light induction

of PAL[3,14,15].

   In many cases, PAL activity was induced by illumination.

However, in the cultured cell system, light which had been used

for the cell transfer was not very effective (Fig. 4).   Cells

treated without transfer and with an illumination for 30 min, which

corresponds to the time required for transfer precedures in the

light(a and d in Fig. 4), showed very low activity as compared to

transferred cells and cultured cells(b in Fig. 4).   Even with

illumination for 12 hr (e in Fig. 4) PAL activity was induced only

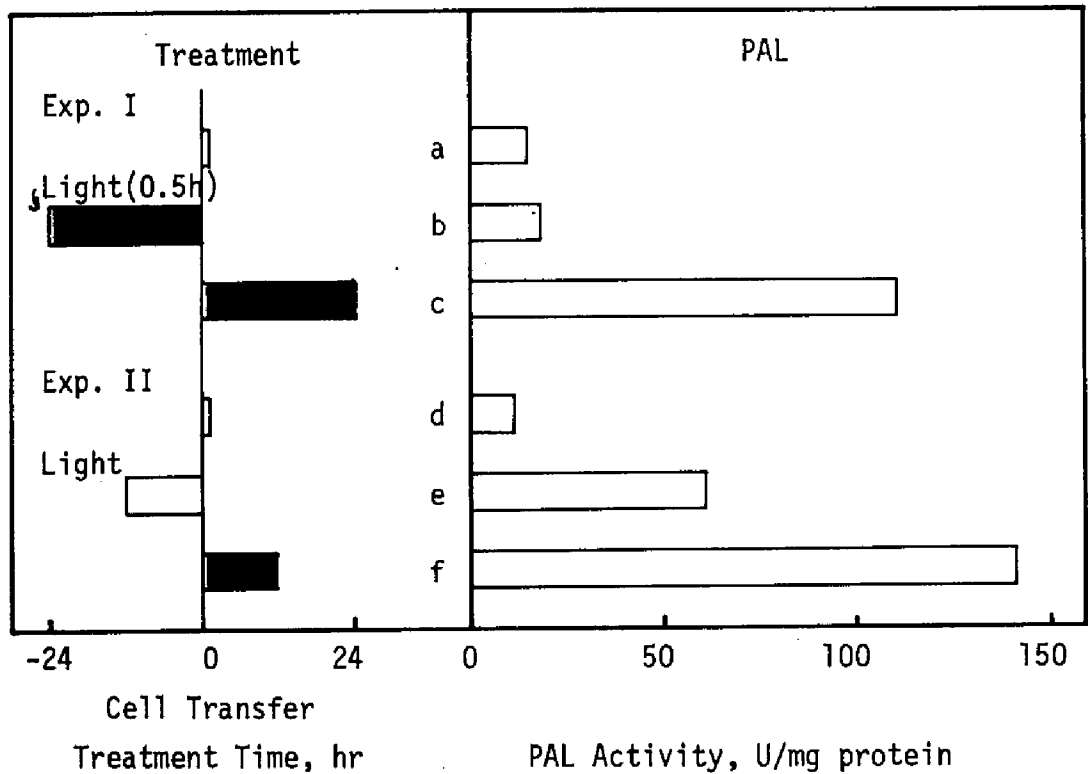half as much as with transferred and 12 hr cultured cells kept in

Fig. 4. Effect of Illumination on PAL Activity.
(a-f) show the different treatments in various experiments with cell culture: a) 30 min of illumination without cell transfer; b) 30 min of illumination and a continuous 23.5 hr in the dark; c) cell transfer and 24 hr in the dark; d) 30 min of illumination without cell transfer; e) 12 hr of illuminaiton; f) cell transfer and 12 hr in the dark.   In treatments, ⬜ and ⬛ indicate cell culture in light and dark conditions, respectively.   Time 0 is the point of cell transfer.

the dark(f in Fig. 4). The PAL induction in Fig. 2 was independent
of the illumination used for transfering cells.

Table I shows the effect of the culture media on PAL activity
when cells were transferred to a habituated medium or to a freshly
prepared one. If PAL induction in the cultured cells is due to
changes in pH, the mineral and sugar contents, 2,4-D concentration
or to the reduction of accumulated phenolics in a medium, then
activity should be better induced when cells are transferred to a
freshly prepared medium as opposed to a habituated one. The
results show that a habituated medium was better and that changes
in the medium's composition were not very significant.

Table II shows the effect of cell density on PAL activity.
Cell transferred under low density(small amounts of the inoculate)
have higher PAL activity than do cells transferred under high
density.

Cells in the late log or early stationary period were used
as the original material for these experiments. These cells are
very dense. When cells are transferred to a fresh medium, a part
of them is suspended in the medium; this causes changes in cell
density (high to low), immediately after transfer which seems to
be the first stimulus for PAL induction. As the magnitude of PAL
activity after transfer is determined by cell density, this suggests
that cell-cell interaction plays a very important role in cultured
tobacco cells and it is also presumed that cell-cell interaction
with metabolic changes is an important factor to induce shoot

Table I. Effects of Culture Media on PAL Activity.

Medium	PAL Activity[*] U/mg protein
Freshly prepared medium	211
Habituated medium[**]	291

*) Twenty four hr culture after transfer. The
Average of 2 replications.

**) Ten day cultured old medium was collected and
autoclaved.

Table II. Effects of Cell Density on PAL Activity.

	Grams of Inoculate[*]	PAL Activity[**] U/mg protein
Exp. I	7.8	159
	12.1	97
Exp. II	10.2	191
	16.6	125

*) Each inoculate was transferred to 20 ml of medium in a 100 ml
Erlenmyer flask.

**) Twenty four hr culture after transfer.

formation.

SUMMARY

The induction of phenylalanine ammonia lyase (PAL) activity in a suspension of cultured tobacco cells(2,4-D cells) was observed when the cells were transferred to fresh medium.   It was concluded that this induction of PAL activity was caused by changes in cell density in the medium and was independent of illumination during cell transfer and of changes in the composition of the medium. Hence, it is considered that cell-cell interaction is important in cultured cells.

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CONCLUSION

Recent developments in plant tissue and cell culture have provided a basis for an experimental system for differentiation. To date, results indicate that cultured plant cells produce de-differentiation of differentiated tissue and cells and, in addition, that some organs or the whole plant can be regenerated from de-differentiated cultured cells under appropriate condition (re-differentiation). The purpose of this study was to obtain information on the biochemical mechanism of shoot redifferentiation in cultured tobacco cells and on some properties of these cultured cells.

When 2,4-D cells or IBA cells were cultured on a medium with 5×10^{-5} M of kinetin, zeatin or ($\pm$)-dihydrozeatin with little or no auxin, shoot formation was observed after 6 to 8 weeks of culture. The natural cytokinins, zeatin and ($\pm$)-dihydrozeatin showed higher activity for shoot formation than did the synthetic cytokinin, kinetin. Cytokinin was required continuously throughout the culture for shoot formation.

Growth rates for the zeatin and kinetin cells were reduced after several days of culture with cytokinin, while the dry material percentage increased.

Morphological changes, cell organization and tracheid-like cell production were simultaneously observed about 1-2 weeks after inoculation to the zeatin medium. RNase activity also changed in

correspondence to a change in the growth. The initiation of shoot redifferentiation may have already begun morphologically and physiologically one week after transfer to the cytokinin medium.

($\pm$)-Dihydrozeatin and optically active cytokinins were tested for their ability to induce shoot redifferentiation in cultured tobacco cells. ($\pm$)-Dihydrozeatin was active in inducing shoot formation. Levorotatory compounds were active in inducing shoot formation, while the corresponding dextrorotatory compounds were inactive at all the concentrations tested. This suggests that the N^6 position of the cytokinins binds to a stereo-specific receptor site to bring about shoot formation.

Kinetin-8-^{14}C was incorporated into cultured cells and subsequently metabolized. About 20 % of its radioactivity was located in the 80 % EtOH insoluble materials and 80 %, in the 80 % EtOH soluble materials. About 50 % of the EtOH insoluble radioactivity was found in RNA and 3-30 % in protein. The EtOH soluble materials were separated into 5 main fractions. It is presumed that cytokinins are metabolized after their incorporation into cells and that free cytokinin or some metabolite acts as a trigger for shoot redifferentiation with some types of proteins.

RNA, buffer-soluble proteins and DNA increased in this order in zeatin cells during the early stages of shoot formation. A different incorporation pattern of ^{14}C-leucine into the protein fraction was observed in zeatin cells when compared to for 2,4-D cells, using DEAE cellulose column chromatography.

The results of disc electrophoresis of the proteins in the 2,4-D and zeatin cells indicated that qualitative differences occurred during shoot formation. These qualitative changes in protein synthesis were also verified by immunological studies which indicated that cytokinins caused the induction of certain characteristic proteins when leaves began to form from cultured cells in vitro. During shoot formation some proteins which are synthesized for cell proliferation were repressed. These changes in protein synthesis suggests the existence of transcriptive regulation during organogenesis.

One participant in transcription, the RNA polymerases(RPases), was investigated. RPases were found in multiple forms in cultured tobacco cells. RPase I showed very low activity and was very labile while RPase II was relatively stable. RPase II was influenced by the degree of redifferentiation. The levels of RPase II activities varied during shoot formation. Template specificity, the requirement for divalent cations and the behavior toward the ionic environment changed according to the degree of redifferentiation. The RPase II in dedifferentiated cultured cells is believed to differ from other plant RPase IIs. Thus, the existence of transcriptional regulation by RPase during shoot formation is indicated. In addition, the fact that the RPase in cultured cells had different properties involves a problem: the difficulty of preserving a cell-specific phenotype when cell culture is used for practical purposes.

When dedifferentiated cultured tobacco cells were cultured with relatively high concentrations of cytokinins, shoot redifferentiation occurred. Cytokinin is incorporated into the cell and free cytokinin or some metabolite acts as a trigger for shoot redifferentiation. At this time the cytokinin (or its metabolite) takes part in transcriptional regulation through the RPases probably with a cytokinin-reactive protein. Subsequently, organ-specific or tissue-specific RNA and protein are synthesized. Simultaneously, such morphological changes as cell organization and the production of tracheid-like cells take place. These changes have begun 1 to 2 weeks after the transfer to the cytokinin medium. These changes also enhance the process of the development of organ formation and produce an organ specific shape.

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