Bot. Bull. Academia Sinica **17**: 192-202 (1976)

CYTOCHEMICAL STUDIES ON THE CHANGES OF NUCLEAR HISTONES AND RNA SYNTHESIS ACTIVITY OF ADVENTITIOUS ROOT INITIALS IN MUNGBEAN HYPOCOTYL CUTTINGS[1]

WEI-CHIN CHANG and SHYUE-JAN CHAN

Institute of Botany, Academia Sinica, Nankang, Taipei Taiwan, Republic of China

(Accepted for publication April 12, 1976)

Abstract

Changes of nulear histones and RNA synthesis activity in rooting regions of mungbean (*Vigna radicata* L.) hypocotyl cuttings have been studied by light microscopic cytochemical methods. There was no marked difference in histone composition of nuclei of all tissues at the rooting regions when cuttings were made, however, after 24-48 hr, the phloem parenchyma of potential rooting zones might have more lysine-rich histones as shown by eosin Y-fast green and ammonical silver reactions. There was more RNA synthesis activity in these potential rooting zones before any defined root primordium occurred. The phloem parenchyma of non-potential rooting zones and the adjacent cortical tissues, however, still showed same responses in cytochemical reactions as in 0 hr, and were quite inactive in RNA synthesis.

Introduction

Eukaryotic nuclei contain considerable amounts of histones associated with nucleic acids. Apart from their role as structural proteins (Alfert and Geschwind, 1953), histones have been considered to be candidates for the regulation of gene expression (Georgiev *et al.*, 1966; Huang and Bonner, 1962; Stedman and Stedman, 1950). Changes in the nuclear histone pattern in a variety of animal developmental systems have been reported (Alfert, 1960; Block and Brack, 1964; Bloch, 1966b; Bloch and Hew, 190; Claypool and Bloch, 1967). In plant morphogenetic systems, histone changes based on histochemical staining reactions have been observed during floral induction (Gifford, 1964; Gifford and Nitsch, 1970; Gifford and Tepper, 1961, 1962; Knox and Evans, 1966), bud

(1) This investigation was supported by a grant (1974-1975) from Biology Research Center, Academia Sinica, Republic of China. Paper No. 183 of the Scientific Journal Series, Institute of Botany, Academia Sinica.

activation (Chang, 1970; Dwivedi and Naylor, 1968), leaf initiation (Evans, 1970), pollen nuclear differentiation (Sauter, 1969), root differentiation (Kusanagi Yanagi, 1970) and in the growth of suspension cultured cells (Arima and Kusanagi, 1975).

In histochemical studies on root initiation of mungbean cuttings a buildup of RNA and protein in the loci of root initiation appeared before formation of organized root primordia (Chang, unpublished), and puromycin inhibited this early metabolic activity (Chang, unpublished). Using antimetabolites of RNA synthesis, Jalouzot (1979) demonstrated that an early RNA synthesis was essential for the root initiation process. Thus. remeristemation of usually non-dividing cells of the potential rooting loci in the early stage of root initiation might involve gene activation. In this paper we report on the cytochemical specificity in nuclear histone and RNA synthesis activities of root initials during early stages of adventitious root formation in mungbean hypocotyl cuttings.

Material and Methods

Preparation of plant material

Seeds of mungbean (*Vigna radicata* L.) were surface sterilized in 10% commercial Chlorox for 15 min., rinsed, and soaked in distilled water for 24 hr. Imbibed seeds were planted in peat moss and grown in a growth chamber at 26 C with a 12-hr photoperiod for 8 days. Cuttings were made by removing cotyledons and cutting off the root system 2 cm below the cotyledonary node. Thus, the cuttings consisted of 2 cm of hypocotyl, about 3 cm epicotyl, a pair of primary leaves and small terminal meristem. The basal end of each cutting was submerged in a 50-ml beaker containing 6 ml distilled water with 1 ppm $CuSO_4$ as an antiseptic. The basal 5 mm segments of the cuttings were excised at various time intervals, fixed in 10% neutral buffered formalin for 24 hr under partial vacuum, dehydrated in an ETOH-TBA series, embedded in Tissuemat and sectioned transversely at 10 μ.

Cytochemistry and microspectrophotometry

Pyronin Y staining (Tepper and Gifford, 1962) was employed for histological observation and for demonstrating RNA accumulation. For cytochemical specificity in nuclear histones, the following two established staining procedures were used: (1) ammonical silver reaction (Black and Ansley, 1964): deparaffinized sections were immersed in 10% acetate-neutralized formalin for 1 hr at room temperature. After a thorough rinse in distilled water, sections were stained for 10 min in an ammonical silver solution, which was

freshly prepared by adding dropwise 10% silver nitrate to 4 ml of concentrated ammonium hydroxide until a persistent turbidity was obtained. The sections then were thoroughly rinse in distilled water, immersed in 3% formalin for a few minutes, dehydrated through a graded ethanol series, cleared in xylene, and mounted. (2) Eosin Y-fast green procedure after Bloch (1966a): deparaffinized sections were hydrolyzed for 15 min in 5% trichloroacetic acid (TCA) at 90 C to remove nucleic acids. The slides were rinsed three times in distilled water and then stained in 0.1% fast green FCF in 0.07 M tris-HCl buffer at pH 8.3 at 4 C. They were subsequently stained in mixture of 0.05% fast green FCF and 0.5% eosin Y in the same buffer at 4 C. The sections were then differentiated in the same cool buffer and 70% ethanol before being mounted.

To check the validity of the above differential staining, the stepwise of histone prior to staining was done according to the procedure of Littau *et al.*, (1965). The acetylation technique (Deitch, 1966) was also employed to block lysine amino groups before conducting histone stainings.

The absorption spectra of nuclei stained by the eosin Y-fast green procedure and the relative amount of RNA of cells treated by the pyronin Y procedure were scanned and measured with a microspectrophotometer. In the case of the eosin Y-fast green procedure, the E_{535} and E_{640} of a plug (12.5 μ^2) through each nucleus were measured for calculating E_{535}/E_{640} value.

^{3}H-uridine histoautoradiography

After hypocotyl cuttings were made the base ends of hypocotyl cuttings were dipped in 5 μc/ml ^{3}H-uridine (S. A. 10 c/mmole, The Radiochemical Center, Amersham. UK) at various stages for 2 hr. The basal 5 mm segments of the cuttings were excised and fixed, dehydrated and embedded in Tissuemat for preparation of 10 μ sections. Deparaffinized and hydrated sections were coated with Ilford L4 emulsion and autoradiographed for 21 days. Autoradiogram were developed and stained with pyronin Y (Tepper and Gifford, 1962).

Results

Histology of the adventitious root formation and pyronin Y-RNA cytophotometry

Adventitious roots were visible at the basal ends of hypocotyl cuttings 4 days after the cuttings were planted in water. These adventitious roots occurred in four rows (Fig. 1A) and the roots were associated with 4 pairs of vascular traces of primary leaves and cotyledons (Fig. 1A). Outward from and between the two bundles of vessels associated with leaf vascular traces were five to ten large phloem parenchymal cells (Fig. 1B). These cells were

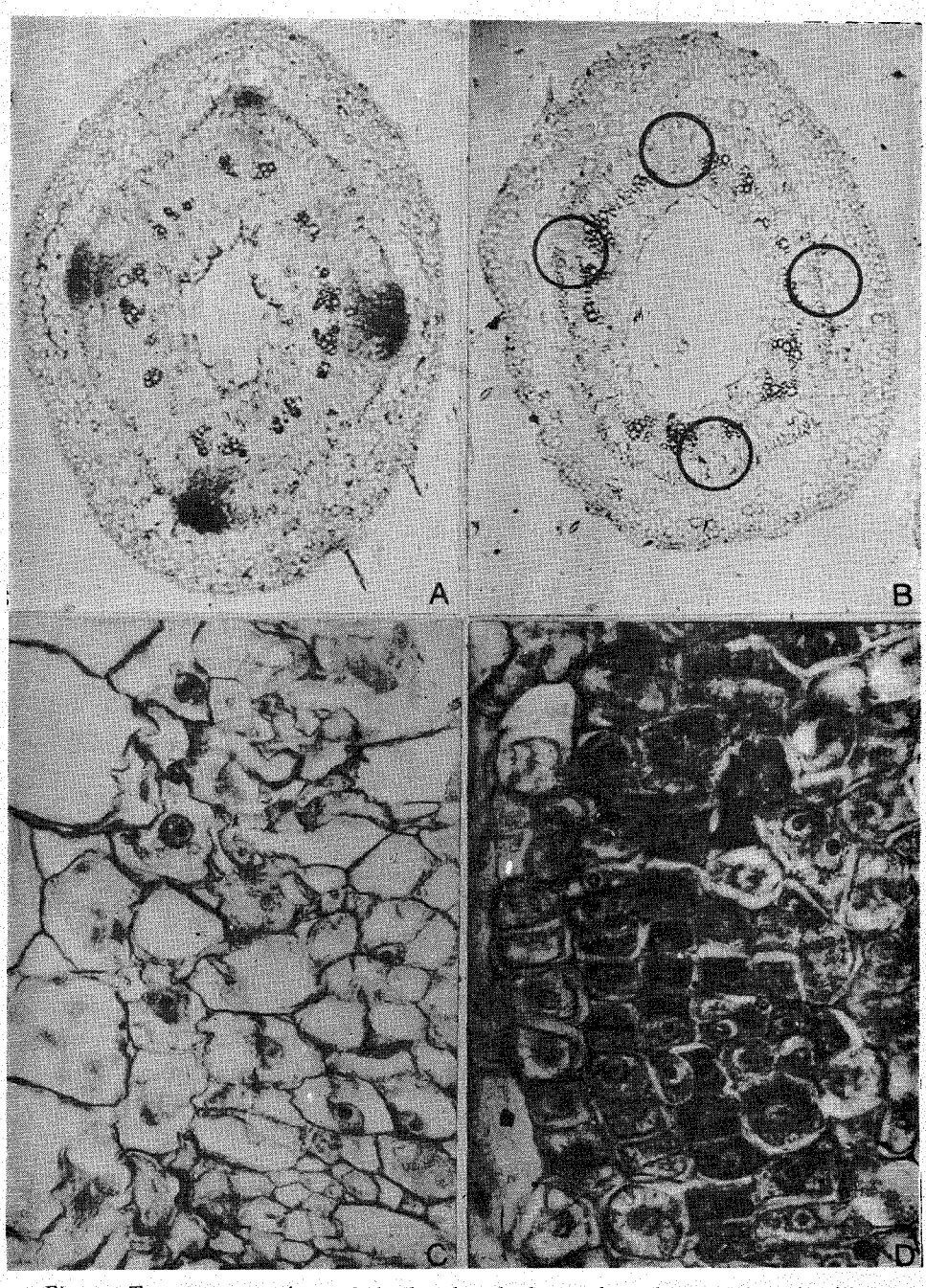

Fig. 1. Transverse sections of the basal end of mungbean hypocotyl cutting illustrating the early development of of adventitious root primordia: (A) 72-hr cutting showing the four primordia, X128, (B) 0-hr cutting showing potential rooting zones within the marked cycle, X128, (C) a higher magnification of the area circled in B in 0-hr cutting, X1028, (D) root initial in 48-hr cutting, X1028. Sections were stained with pyronin Y; thus the dense color areas represent greater RNA accumulation.

usually between rows of smaller phloem cells, including the sieve tubes. These phloem parenchyma cells were the tissue from which a root initial might form (Fig. 1B, C). Twenty-four to 48 hr after the cuttings were made, cells of the potential rooting zones increased considerably in size and had enlarged nuclei, nucleoli and dense cytoplasm (Fig. 1D). This might be considered as a part of the dedifferentiation or remeristematization phase of adventitious root initiation.

Cell division occurred about 24–48 hr after the cuttings were made, and tissues with definite root primordia developed within 48–72 hr. In phloem parenchyma of 0-hr cuttings, the light pink coloration of the cytoplasm as showed by pyroniophilia indicated a low concentration of RNA throughout the tissue (Table 1). An increasing pyroniophilia was observed in potential rooting zones of 24–48 hr cuttings, while the phloem parenchyma of nonpotential rooting zones still stained a light pink color (Table 1). This pattern of increasing pyroniophilia persisted throughout the potential rooting zones and was seen in young primordia sampled on later days. No RNA buildup was found in adjacent cortical and phloem ray parenchyma.

Cytochemical variations of nuclear histone dye binding

By the ammonical silver reaction most nuclei in the cortex and phloem parenchyma of both potential rooting zones and non-potential rooting zones at 0 hr stained brown-black, while all the phloem ray parenchyma of potential rooting zones, root initials formed in 24 hr samples and root primordia formed at later hours stained yellow brown (Table 2). According to Black and Ansley (1966), the brown-black staining might be due to the quanido ε-amino groups groups of lysine.

Table 1. *RNA concentrations (pyronin Y-RNA cytophotometry) in root initials, primordia and adjacent tissues in the basal ends of hypocotyl cuttings of mungbean*

Tissue	OD_{540} per plug (12.5 μ^2) through cytoplasm				
	0	24	48	72	96 hr
Phloem parenchyma of potential rooting zone	0.31	0.45	—	—	—
Root primordia	—	—	0.95	0.84	0.89
Phloem parenchyma of non-potential rooting zong	0.34	0.36	0.30	0.41	0.37
Cortical tissue	0.25	0.21	0.29	0.23	0.27

Bloch's eosin Y-fast green method produced green-stained nuclei in zero

Table 2. *Cytochemical tests for nuclear histones of adventitious root initials and adjacent tissues* (*Vigna radicata* L.)

Cytochemical tests	Phloem parenchyma in non-potential rooting zones		Phloem parenchyma in potential rooting zones		Cortex		Root initials	
	0 hr	48 hr	0 hr	48 hr	0 hr	48 hr	0 hr	48 hr
Eosin Y-fast green staining	green	green	green	pink purple	green	green	—	pink purple
Ammoniacl silver	brown black	brown black	brown black	yellow brown	brown black	brown black	—	yellow brown
Eosin Y-fast green staining after acetylation	green	green	green	faint pink	green	green	—	faint pink
Eosin Y-fast green staining after 70% EtOH-0.18 N HCl extraction	no color	no color	light green	pink	no color	no color	—	pink
Eosin Y-fast green staining after 30% EtOH-0.18 HCl extraction	no color	no color	no color	no color	no color	no color	—	no color

hr samples. In the 24–48 hr samples, a few nuclei in the potential rooting zone stained eosinophilic with deep pink nucleoli. The nuclei of meristematic cells of developing root primordia stained pink-purple, while the cortex of mother root tissue stained green (Table 2). According to Bloch (1966a) when the dye were allowed to compete with one another, the eosin Y and fast green bond preferentially to lysine-rich and arginine-rich histones respectively, Thus most of the cells in our samples contained both lysin-rich and arginine-rich histones. The intensity of the eosinophilic staining increased in the nuclei of the differentiated cells of the potential rooting zones, suggesting that more lysine-rich histones were available for eosin Y binding in these cells than in the adjacent nondivided cells.

After acetylation and staining with the eosin Y-fast green procedure, the phloem parenchyma cells of potential rooting zones, root initials and primordia were exclusively affected and almost completely lost their dye binding ability. In the stepwise extraction of nuclear histones the following results were obtained: when the ethanol concentration was adjusted to 70%, the nuclei of cortical tissue and phloem parenchyma in non-potential rooting zones became unstainable with fast green in eosin Y-fast green and ammonical silver reactions. But the root initials and primordia still stained clearly. When the ethanol concentration was adjusted to 30%, root initials and primordia began

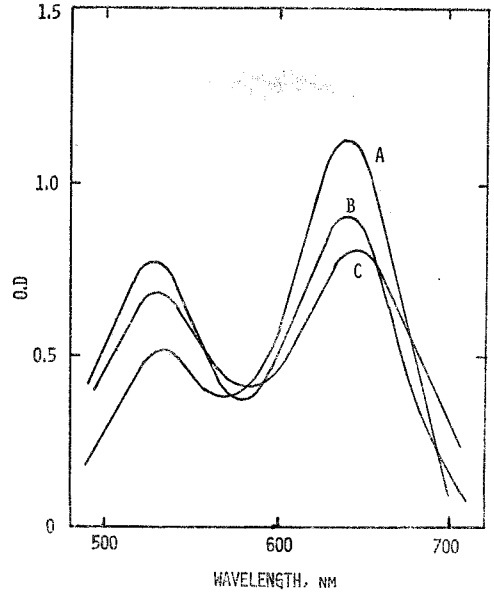

Fig. 2. Absorption spectra of nuclear histones of adventitious root initial (A), adjacent cortical cells (B) and phloem in non-potential rooting zones (C) stained by the eosin Y-fast green procedure.

losing their dye-binding ability. According to John *et al.* (1960), in the stepwise extraction process, arginine-rich histones were extracted when the ethanol concentration was 80%, and lysine-rich histones were extracted when the ethanol concentration went down to 30% (John *et al.*, 1960; Littau *et al.*, 1965; Yanagi and Kusangi, (1970). Thus the information obtained by cytochemical tests suggested that the nuclei of root initials in the potential rooting zones and the nuclei of root primordia contained mainly lysine-rich histone available for dye binding, while the nuclei of cortical tissue and phloem parenchyma in the non-potential rooting zones contained mainly arginine-rich histones.

Cytophotometry of the eosin Y-fast green reaction

The absorption spectra of nuclei of root initals, primordia and adjacent cortical cells showed that the nuclei of root initials and primordia were more eosinophilic than that of non-divided phloem parenchyma and the cortical tissue (Fig. 2). The ratio of the mean E_{525}/E_{640} and the standard error (Table 3) demonstrated that great differences in the cytochemical nature of nuclear histone occurred in the nuclei of different kinds of tissue during adventitious root initiation.

Table 3. *Mean ratios of extinction at 535 and 640 nm and their standard errors for nuclei of different kinds of tissue during early stages of adventitious root formation*

Tissue	mean E_{535}/E_{640}±standard error		
	0 hr	24 hr	48 hr
Phloem parenchyma of potential rooting zone	0.42±0.09	0.97±0.10	—
Formed root initials and primordia	—	—	0.99±0.14
Phloem parenchyma of non-potential rooting zone	0.51±0.13	0.53±0.16	0.49±0.08
Cortical tissues	0.49±0.09	0.51±0.17	0.53±0.14

Table 4. *Incorporation of ^3H-uridine into nuclei of different tissues in the rooting portion of cuttings at early stages of adventitious root initiation*

Tissue	Average grains/cell		
	0 hr	24 hr	48 hr
Phloem parenchyma of potential rooting zones	41.2	213.5	—
Root initials	—	—	276.8
Phloem parenchyma of non-potential rooting zones	49.6	60.3	62.1
Cortical cells	29.1	35.2	37.7

^{3}H-uridine histoautoradiography

RNA synthesis activities of the nuclei of different kinds of tissues were demonstrated by ^{3}H-uridine histoautoradiography. The considerable amount of ^{3}H-uridine was incorporated into the phloem parenchyma of potential rooting zones at 24 hr after cuttings were made, and in the root initials (Table 4). Only slightly incorporation was found in the cells of adjacent cortical tissues and phloem parenchyma at non-potential rooting zones.

Discussion

During the early period of adventitious root initiation of mungbean hypocotyl cuttings, stainable histones were identified only in nuclei. There was no evidence of cytoplasmic histone staining as recorded by Gifford (1964) in *Xanthium* and Knox and Evans (1966) in *Lolium*. Results of the cytochemical studies indicated that there were remarkable histone changes in the nuclei of the phloem parenchyma in the potential rooting zones and the forming root initials. The lack of staining observed following acetylation suggested that the most of the histones present in the phloem parenchyma of potential rooting zones of 24 hr samples, the initials and the primordia were lysine-rich (Table 2). Results of stepwise extraction prior staining by the eosin Y-fast green method supported this finding.

Interestingly the eosin Y-fast green method stained some meristematic nuclei eosinophilic (pink), while adjacent cell nuclei in 24 hr or 48 hr samples were unaffected. It was reported by Bloch (1966) that the eosin Y and fast green bound preferentially to lysine and arginine-rich histones respectively, when the dyes were permitted to compete with one another. Thus, most of the cells in the rooting region contained both lysine-rich and arginine-rich histones. The increased intensity of the eosinophilic staining in nuclei of the meristemic cells of the potential rooting zones indicated that more lysine-rich histones were available for eosin Y binding in these than in the adjacent cortical cells. This might suggest that dedifferentiation or the remeristematization stage of early adventitious root initiation involved histone differentiation, with more lysine-rich histones available for dye binding at this time. Moreover, there was marked correlation among RNA synthesis activity, amount of pyronin Y binding and eosinophilic of the nuclei of each tissue (Table 1, 2, 3, 4).

The eosinophilic staining of lysine-rich protein in the rooting region of hypocotyl cutting was also of interest because of the proposed role of f_1-histones (lysine-rich histones) in gene regulation (Georgiev *et al.*, 1966). Removal of lysine-rich histone (f_1-histones) resulted in a significant increase

in template activity (Georgiv, 1969; Georgiev *et al.*, 1966). Furthermore, lysine-rich histones crosslinked with chromatin fibrils might be the most probable cause of dense, inactive chromatin (Stedman and Stedman and Stedman, 1950). A pronounced eosinophilic nature might mean an increase in the absolute amount of lysine-rich histones in the nuclei, or that more lysine-rich histones were free from DNA binding.

It is worthy recalling here that the nuclei of meristematic cells of garlic roots were highly eosinophilic and exhibited a lack of response to fast green whereas the nuclei of well differentiated or elongated cells had a high affinity for fast green as reported by Kusagi and Yanagi (1970).

As to the role of histones in adventitious root initiation, it is interesting to note that exogenous histones applied at 100 mg/ml inhibited the rooting of *Cicer arietinum* cuttings (Jalouzot, 1970).

Literature Cited

ALFERT, M. 1969. Chemical differentiation of nuclear proteins during spermatogenesis in the salmon. J. Biophys. Biochem. Cytol. **2**: 109-114.

ALFERT, M. and J. J. GESCHWIND. 1953. A selective staining method for the basic proteins of cell nuclei. Proc. Natl. Acad. Sci. (U. S. A) **39**: 991-999.

ARIMA, T. and A. KUSANAGI. 1975. Chromatin structure, histone variation, and RNA synthetic activity in *Alcineae* cultured cells. Protoplasm **83**: 297-310.

BLACK, M. M. and H. R. ANSLEY. 1964. Histone staining with ammonical silver. Science **143**: 693-695.

BLOCH, D. P. 1966a. Cytochemistry of histones. Protoplasmatologia V/3/d: 1-56.

BLOCH, D. P. 1966b. Histone differentiation and nuclear activities. Chromosoma (Berl.) **19**: 317-337.

BLOCH, D. P. and B. D. BRACK. 1964. Evidence for cytoplasmic synthesis of nucleic histone during spermatogenesis in the grasshopper *Chortophaga viridifasciate* (de Geer). J. Cell Biol. **22**: 327-340.

BLOCH, D. P. and H. Y. C. HEW. 1960. Schedule of spermatogenesis in the pulmonate snail *Helix aspersa*, with special reference to histone transition. J. Biophys. Biochem. Cytol. **7**: 515-532.

CHANG, W. C. 1970. Histochemical studies on bud activation of the Alaska pea (*Pisum sativum* L.). Ph. D. dissertation, Univ. Calif., Riverside, 129p.

CLYPOOL, J. C. and D. P. BLOCH. 1967. Synthesis of ribonucleic acid and histone change during spermatogenesis in the grasshopper *Chortophaga viridifasciata*, Nature **215**: 966-967.

DEITCH, A. D. 1966. Cytophotometry of nucleic acids. *In:* Introduction to Quantitative Cytochemistry (G. L. Wied, ed.). pp. 327-353. Academic Press, New York.

DWIVEDI, R. A. and J. M. NAYLOR. 1968. Influence of apical dominance on the nuclear proteins in cells of the lateral bud meristem in *Tradescantia palidosa*. Can. J. Bot. **46**: 289-298.

EVANS, L. S. 1970. Histochemical studies of leaf initiation in *Triticum aestivum* L. Ph. D. dissertation, Univ. Calif., Riverside, 90p.

GEORGIEV, G. P. 1969. Histone and the control of gene action. Ann. Rev. Genet. **3**: 155-180.

GEORGIEV, G. P., L. V. ANANIEVA and J. V. KOZLOR. 1966. Stepwise removal of protein from a deoxyribonuleoprotein complex and de-repression of the genome. J. Mol. Biol.

22: 265-271.

GIFFORD, E. M. 1964. Developmental studies of vegetative and floral meristems. Brookhaven Symp. Biol. **22**: 365-371.

GIFFORD, E. M. and C. NITSCH. 1970. Cytohistological and hormonal studies of floral initation in *Plumbago indica*. *In:* Cellular and Molecular Aspects of Floral Induction (G. Bernier, ed.). pp. 304-315. Longman, London.

GIFFORD, E. M. and H. B. TEPPER. 1961. Ontogeny of the inflorescence in *Chenopodium album*. Amer. J. Bot. **48**: 657-667.

GIFFORD, E. M. and H. B. TEPPER. 1962. Histochemical and autoradiographic studies of floral induction in in *Chenopodium album*. Amer. J. Bot. **49**: 706-714.

HUANG, R. C. and J. BONNER. 1962. Histone, a suppressor of chromosomal RNA synthesis. Pro. Natl. Acad. Sci. (U. S. A.) **48**: 1216-1222.

JALOUZCT, R. 1970. Etude du metabolisme des acide nucleiques et des proteines au cours de racines adventives. Planta (Berl.) **97**: 16-27.

JOHNS, E. W., D. M. P. PHILLIPS, P. SIMSON and H. A. V. BUTLER. 1960. Improved fraction of arginine-rich histones from calf thymus. Biochem. J. **77**: 631-636.

KNOX, R. B. and L. T. EVANS, 1966. Inflorescence initiation in *Lolium temulentum* L. VIII. histochemical changes at the shoot apex during induction. Aust. J. Biol. Sci. **19**: 233-245.

KUSANGI, A. and T. YANAGI. 1970. Cytochemical determination of changes in nuclear histone content in differentiating root cells of barley and garlic. Protoplasma **69**: 279-282.

LITTAU, U. C., C. L. BURDICK, U. G. ALLFREY and A. E. MIRSKY. 1965. The role of histones in the mainteance of chromatin structure. Proc. Nat. Acad. Sci. (U. S. A.). **54**: 1204-1214.

SAUTER, J. J. 1969. Cytochemische Unters chung der Histone in Zellen mit unterschiedlicher RNA-und Protein-synthese. Z. Pflanzenphysiol. **60**: 434-449.

STEDMAN, E. and E. and E. STEDMAN. 1950. Cell specificity of histones. Nature **166**: Detection of ribonucleic acid with pyronin. Stain Technol. **37**: 52-53.

YANAGI, T. and A. KUSANAGI, 1970. Cytochemical and biochemical determinations of changes in nuclear histone content during differention of barley root cells. Development, Growth and Differentiation (DGD) **12**: 2-12.

綠豆下胚軸扦插不定根發生初期 Histone 和 RNA 的細胞化學變化

張 唯 勤　陳 學 貞

中央研究院植物研究所

用 Eosin Y-fast green 法和 Ammonical Silver 法顯示綠豆 (*Vigna radicata* L.) 下胚軸插條發根部位的靱皮部薄皮細胞形成根原體之初期有較多的 lysine-rich histones 出現，及較高的 ^{3}H-uridine-RNA 合成活性。非根原體發生部位的組織却少有此等變化。