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ISOLATION OF VARIANT CARROT CELL LINES WITH ALTERED PIGMENTATION

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; cultured cells; carotenoid, mutagen; variant cells.

Abstract—Cultured carrot cells were treated with a known mutagenic compound, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and plated on a nutrient agar medium. Four variant cell lines whose pigmentation properties differed from stock calluses have been isolated. The contents of major carotenoid components, β-carotene and lycopene, of these cells were determined and compared with those of parent strains.

INTRODUCTION

IT IS known that the production of secondary metabolites by cultured plant cells is controlled by environmental factors such as growth regulators.[1–3] The synthetic activity may also be dependent on the state of differentiation of the cells and their genetic constitution. The present studies were undertaken to develop a technique for the isolation of "variant" cells with altered synthetic activity of plant products. In this paper, we report the effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) on the ability to synthesize carotenoids in cultured carrot cells. Mutagenesis of MNNG has been examined in a variety of microbial systems such as *Escherichia coli*[4] and yeast.[5] Heimer and Filner[6] have found growth inhibition of tobacco cell by L-threonine and obtained a variant cell line resistant to the amino acid by treating the parent cells with MNNG.

RESULTS

Growth of carrot cells on agar plate

The formation of colonies on nutrient agar plates was markedly influenced by the conditions of inoculation and composition of the medium. When the carrot cells were plated on the standard agar medium, the minimum inoculum density resulting in the formation of visible colonies within 30 days of incubation was about 2×10^4 cells per plate. At this critical density, the plating efficiency was less than 10%. Conditioning of the medium by the addition of culture filtrate did not reduce the critical initial density but was effective in increasing the plating efficiency. Table 1 shows the increase in cell number in each colony developing on the conditioned agar plate. Approximately 80% of the cells or cell units have started to divide by 7 days after plating.

[1] SUGANO, N., MIYA S., and NISHI, A. (1971) *Plant Cell Physiol.* **12,** 525.
[2] TABATA, M., YAMAMOTO, H., HIRAOKA, N., MARUMOTO, Y. and KONOSHIMA, M. (1971) *Phytochemistry* **10,** 723.
[3] FURUYA, T., KOJIMA, H. and SYONO, K. (1971) *Phytochemistry* **10,** 1529.
[4] ADELBERG, E. A., MANDEL, M. and CHEN, G. C. C. (1965) *Biochem. Biophys. Res. Commun.* **18,** 788.
[5] HARTWELL, L. H. (1967) *J. Bacteriol.* **93,** 1662.
[6] HEIMER, Y. M. and FILNER, P. (1970) *Biochim. Biophys. Acta* **215,** 152.

TABLE 1. INCREASE IN CELL NUMBER IN INDIVIDUAL COLONIES DEVELOPING ON CONDITIONED AGAR PLATES. (FREQUENCY AS PERCENTAGE OF TOTAL NUMBER OF COLONIES). INITIAL CELL DENSITY WAS 2×10^4 CELLS PER PLATE

		Cells per colony				
		1–5	6–10	11–20	21–30	>30
Day	0	61	23	10	5	1
	2	36	25	15	10	13
	5	29	19	11	10	32
	7	10	11	10	14	55

Isolation of clones with altered pigmentation activity

When the carrot cells exposed to 2·8 mM MNNG for 1 hr were plated at a cell density of 2×10^5 per plate, several hundreds of colonies were formed on a plate after incubation for 30 days at 28°. No colony at all was formed when the cells were treated for more than 2 hr. The origin of these colonies, however, cannot be traced to single cells since the cell suspension used for the inoculum contained cells with septa and small cell aggregates. For this reason, cells from a colony were repeatedly plated on the agar medium. Finally, 4 clones were established which differed from each other and from the parent strains with respect to pigmentation.

TABLE 2. CAROTENOID CONTENTS OF VARIOUS STRAINS OF CULTURED CARROT CELLS

Strain	Culture condition*	Carotenoid content (μg/g dry wt) Total†	Av.	β-Carotene	Av.	Lycopene	Av.
GD-1	S	139–165	149	78–122	103		
GD-3	S	961–1184	1069	150–179	166	631–792	706
GD-1NO	C	182–203	193	39–132	91	46–134	86
	S	208–214	210	87–90	88	40–45	42
GD-1NB	C	342–583	501	74–161	117	183–283	235
	S	501–559	530	236–258	247	177–210	194
GD-3NR	C	1666–2016	1782	160–352	220	995–1454	1218
	S	2336–3215	2831	113–305	241	1970–3126	2344
GD-3NY	C	350–396	372	105–178	139	126–295	208
	S	423–491	454	130–154	138	154–335	279
Root‡			647		121		447

* S, suspension culture. C, callus culture.
† Total carotenoid was calculated from the value of $E^{1\%}_{1\,cm}$ of lycopene.
‡ Data from Sugano and Hayashi.[7]

Carotenoid content in various carrot cell strains

The major components of carotenoid pigment accumulated in the root of red carrot, Kintoki, are β-carotene and lycopene.[7] Various callus strains, whose pigmentation properties were different from the original plant tissue, had been derived from an initial explant. GD-1 cell line contains β-carotene and a small amount of xanthophyll but lycopene was absent from the cells, or only present in a minute amount. On the other hand, GD-3 cell line was particularly rich in lycopene; the β-carotene content in this strain was comparable to that of the original tissue. In Table 2, the contents of the major carotenoids in cell clones

[7] SUGANO, N. and HAYASHI, K. (1967) *Bot. Mag. Tokyo* **80**, 440.

isolated after treating with MNNG were compared with those of the parent strains. GD-1NO and NB derived from GD-1 strain contained appreciable amount of lycopene which was not detected in the parent cells. GD-1NB cells contained an unidentified brown pigment other than carotenoid. GD-3NR cells are intensely red and contain more lycopene than the parent strain, GD-3. The accumulation of this carotenoid was enhanced when the cells were grown in suspension culture. On the other hand, a yellow callus, GD-3NY derived from GD-3, had a lower level of carotenoid, in which a decrease in lycopene content was particularly marked.

Cell growth and carotenoid accumulation

When the cell growth was measured by increases in protein content and dry weight, the growth patterns of variant cells resembled those of the mother strains. In suspension culture, they grew exponentially for 7 days with a doubling time of about 2 days. Accumulation of the carotenoid was observed only in the logarithmic growth phase. During this growing period, the amount of carotenoid increased in parallel with the protein content.

DISCUSSION

The parent callus strains used in this experiment had been cultured *in vitro* for 10 yr. Among various cell lines isolated from an initial callus, GD-1 and GD-3 have stable properties of carotenoid synthesis. The frequency of spontaneous variants was very low. Treatment with MNNG significantly increases the occurrence of variant cells. After the treatment, many differently pigmented colonies were found on a plate. Four clones selected for the present study have retained their pigmentation characteristics for 1 yr. The stability of these cell clones suggests that they differ from one another and from parent strains in their inherent properties. Kao and Puck[8] have reported that MNNG was effective in chromatid breaks and single gene mutagenesis in mammalian cultured cells. In the present studies, however, whether the observed differences among these clones arose from the change in their genetic constitution was not critically examined. Another possibility is that the mutagenic carcinogen altered the stable states of differentiation of the carrot cells. Specialization of the cells in cultured tissue could arise through a process similar to cell differentiation in intact plant. Tabata *et al.*[9] reported that stem callus of tobacco contained only a small amount of nicotine while the intact plant regenerated from the callus produced a normal amount of nicotine. From these findings, they considered that the cultured cells of tobacco possess the genetic potentiality to form nicotine, but that its quantitative expression is dependent on the events peculiar to the organization of the plant.

EXPERIMENTAL

Organism. Parent strains were GD-1 and GD-3 which had been obtained from a root of red carrot, cv. Kintoki.[1] These strains have been maintained by subculturing every 2 weeks on the synthetic medium of Murashige and Skoog[10] supplemented with 3% sucrose and 1 ppm 2,4-dichlorophenoxy acetic acid. Suspension cultures were maintained in 500 ml flasks containing 100 ml of the liquid medium. The flasks were agitated on a reciprocal shaker at 160 oscillations per min with a horizontal excursion of 6 cm at 28°. Callus tissue was grown on the surface of the same medium solidified with 1% agar.

Variant cell clones were isolated by plating the cells on nutrient agar medium prepared in 9 cm Petri dishes. The dishes contained 15 ml standard medium and 5 ml culture filtrate in which the cells had been cultivated for 10 days. Cells in suspension culture were harvested by centrifugation and suspended in a medium containing

[8] KAO, F. and PUCK, T. T. (1971) *J. Cell Physiol.* **78,** 139.
[9] TABATA, M., YAMAMOTO, H. and HIRAOKA, N. (1968) *Japan. J. Genet.* **43,** 319.
[10] MURASHIGE, T. and SKOOG, F. (1962) *Physiol. Plant.* **15,** 473.

2 mM MNNG. At the end of the treatment, cells were washed and resuspended in a fresh medium at a cell density of *ca* 2×10^5/ml. 1 ml aliquot was poured into the Petri dish containing the conditioned solid medium. The dish was sealed with cellophane tape to minimize evaporation and incubated at 28° for 30 days. Discrete colonies were removed, diluted with medium and streaked again onto the agar medium.

Determination of carotenoids. Washed cells were immersed in acetone and ground in a mortar with quartz sand. Further fractionation of carotenoids was performed essentially according to the methods described by Goodwin.[11] The pigments dissolved in petrol. (30–70°) were chromatographed on an alumina column and eluted with petrol. with increasing amounts of acetone.[7] The amount of each carotenoid eluted from the column was calculated from the value of its $E^{1\%}_{1\,cm}$.[11,12]

Measurement of cell growth. In a suspension culture growth was estimated by measuring dry wt and the protein content determined by the method of Lowry *et al.*[13] Cell number in each colony was counted under a microscope. At least 500 cells or colonies in randomly selected areas of plate were counted.

[11] GOODWIN, T. W. (1955) In: *Modern Methods of Plant Analysis* (PAECH, K. and TRACEY, M. V., ed.), Vol. III, p. 272, Springer, Berlin.
[12] DAVIES, B. H. (1965) In: *Chemistry and Biochemistry of Plant Pigments* (GOODWIN, T. W., ed.), p. 489, Academic Press, New York.
[13] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.