Plant Physiol. (1981) 68, 261-264
0032-0889/81/68/0261/04/$00.50/0

Cycloheximide Resistance in Carrot Culture: A Differentiated Function[1]

Received for publication September 24, 1980 and in revised form February 18, 1981

ZINMAY R. SUNG[2]
Department of Genetics and Department of Plant Pathology, University of California, Berkeley, California 94720
GABOR B. LAZAR AND DENES DUDITS
Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, H6701 Szeged, Hungary

ABSTRACT

Cultured carrot cells grow as unorganized callus tissue in medium containing auxin. Upon removal of the auxin from the medium, they grow in an organized manner and differentiate into embryos. In the normal cell line, W001C, the callus growth can be inhibited by cycloheximide, but the embryonic growth cannot. A variant cell line, WCH105, whose callus growth is resistant to cycloheximide, was isolated. The mechanism of cycloheximide resistance in embryos of both lines and in WCH105 callus was found to be cycloheximide inactivation. In addition to auxin, bromodeoxyuridine can also promote callus growth in carrot culture. Callus cultures maintained by bromodeoxyuridine behave the same as do those maintained by auxin. WCH105 callus is resistant, whereas W001C callus is sensitive to cycloheximide inhibition. Except for the onset of embryogenesis, cycloheximide inactivation is expressed throughout the embryo developmental stages up to the plantlets. These results suggest that cycloheximide inactivation is a function expressed in the differentiated, but not in the undifferentiated, tissues.

Our present knowledge about the mechanisms regulating gene expression during differentiation is limited, largely because of the lack of experimental systems equipped with appropriate mutants. Plant cells grown in culture often divide as unorganized cells, but many of them can differentiate back to whole plants. The reversible process of obtaining plants from cultured cells and converting them back to tissue culture provides a convenient system in which to study developmentally regulated gene functions. It also allows selection of mutants expressing differentiated functions in unorganized culture cells.

Carrot cultures regenerate *via* embryogenesis (7). Embryo development undergoes characteristic morphogenetic stages, *i.e.* globular, heart, and torpedo embryos. The process is similar to zygotic embryogeny (1). The somatic embryos develop directly into plantlets in culture without a period of dormancy. This work describes CH^r[3] as a function that is expressed in somatic embryos and plantlets but not in callus of normal carrots. A variant resistant to CH as callus, WCH105, has been isolated (9). We have shown by somatic hybridization that CH^r in WCH105 is a recessive trait (3). In this paper, we report the characterization of the mechanism of CH^r, expressed both in the WCH105 callus and in the somatic embryos.

MATERIALS AND METHODS

Plant material and culture conditions have already been described (8). Briefly, a diploid tissue culture of wild carrot, W001C (10), was maintained in Murashige and Skoog medium supplemented with 0.1 mg/l 2,4-D (callus medium). To regenerate plants from the culture or to initiate embryogenesis, the cells were transferred to the same medium devoid of 2,4-D (embryogenic medium), according to our published procedure (10). For convenience, cultures grown in liquid or on semisolid callus medium are referred to as 'callus culture,' and cultures grown in liquid or semisolid embryogenic medium are called 'embryo culture.' Growth of liquid suspension cultures was measured by the sidearm-turbidity method. The number of cells at any point of the growth stage was estimated from the turbidity expressed in arbitrary units in a Klett-Summerson colorimeter. For example, Klett 40 corresponds to about 8×10^5 cells/ml of the culture grown in liquid callus medium (8).

In the course of a mutagenic study on carrot culture, a CH^r cell line, WCH105, was isolated from the W001C line (9). Since 1975, it has been maintained as callus at 25 C by alternating transfers on semisolid medium with and without the supplementation of 10 μg/ml CH.

The CH^i tests were carried out in liquid medium. Callus media, supplemented with 10 μg/ml CH, were preincubated with suspension callus cells at Klett 40, 8×10^5 cells/ml until the culture resumed growth following the lag period. Embryogenic media, supplemented with CH, were incubated with 4-day-old embryo cultures consisting mostly of globular embryos at a density of approximately 800 globular embryos/ml or 12-day-old embryo cultures consisting mostly of torpedo embryos at about 500 torpedo embryos/ml. Plantlets of 0.5 to 1.0 cm were incubated at 200 plantlets per 20 ml of CH-containing embryogenic media. Following several days' incubation with the above cultures, the media were separated from the cells by filtration through sterile filters of 0.45-μm pore size. 2-4-D was added to the embryogenic media at a concentration of 0.1 mg/l. These media were subsequently reinoculated with CH^s, W001C cells at a density of 8×10^5 cells/ml. The ability of W001C to grow in the media was used as an assay for CH^i. Alternatively, CH was estimated by agar-gel diffusion assay with *Saccharomyces cerevisiae* strain GRT-18, according to the method of Maliga *et al.* (4). Media (0.1 ml) from liquid cultures, water-soluble extracts of agar media, or washed callus homogenates were applied in a well punctured in Yeast

[1] Supported in part by United States Department of Agriculture Competitive Grant No. AG5901-0410-9-0370-0 (to Z. R. S.).

[2] To whom requests for reprints should be addressed.

[3] Abbreviations: CH^r, cycloheximide resistance; CH, cycloheximide; CH^i, cycloheximide inactivation; BUdR, 5-bromo-2'-deoxyuridine; NAA, naphthaleneacetic acid; CH^s, cycloheximide sensitivity.

Extract Peptone Dextrose agar medium inoculated with yeast. After a 20-h incubation at 30 C, a zone of growth inhibition formed around the well if the media contained active CH. A zone of inhibition with a diameter of 1.8 cm is equivalent to 1 μg CH.

In analysis of the correlation between the CH sensitivity and callus tissue, cell lines were subcultured every 3 days for 3 weeks in media supplemented with various callus-maintaining agents other than 2,4-D such as IAA, at 1 mg/l; NAA, at 0.5 mg/ml; and BUdR, at 6 μM (2). The growth rate of these cultures was the same as that of callus culture maintained in 2,4-D. At the end of 3 weeks, 10 μg/ml CH was added to the cultures of 4×10^6 cells/ml for 8 h; the media were subsequently assayed for growth inhibition of W001C cells.

RESULTS

The CH Sensitivity of Carrot Calli and Plantlets. The sensitivity towards CH was compared in the 3-week-old embryo culture and the callus culture. Figures 1A and 1B show that, while somatic plantlets of W001C were resistant up to 100 μg/ml, the growth of callus cells was completely inhibited by 5 μg/ml CH. On the other hand, WCH105 callus was resistant to CH up to 15 μg/ml; plantlets were also resistant to CH to the same extent as was W001C. Figure 2A shows the growth of W001C and WCH105 cells in liquid culture. While 10 μg/ml CH inhibited the growth of W001C, WCH105 grew in it at the same rate as it did in the CH-free medium after a 2-day growth lag (Fig. 2A).

The CH^r trait of WCH105 callus was stable in the absence of selective pressure, CH, for at least 16 months (over 100 cell generations). Calli initiated from regenerated plantlets were found to be resistant to CH (3). Since the embryos of WCH105 line are incapable of developing into mature, flowering plants, genetic analysis of the CH^r trait was carried out by somatic hybridization. Although CH^r was not expressed in the hybrids, it was reexpressed in some segregants following somatic segregation (3).

Mechanism Responsible for CH^r in Callus Culture of WCH105.

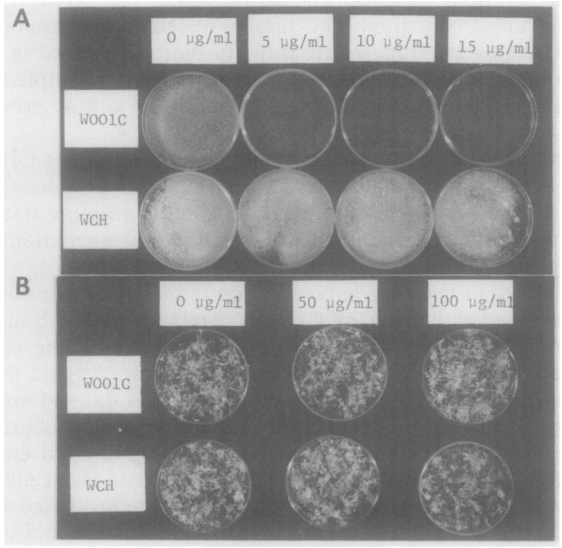

FIG. 1. Callus growth and plantlet development in cell lines of wild carrot grown in the presence of increasing concentrations of CH. A, Liquid suspension culture of W001C and WCH105 (WCH) were plated at a density of approximately 5×10^6 cells/plate, supplemented with various concentrations of CH. Pictures were taken after a 4-week incubation of the cultures at 27 C. B, Three-week-old embryo cultures, consisting mostly of torpedo embryos and some plantlets of about 0.5 cm in length, were plated at a density of approximately 10^4 embryos/plate on regeneration media supplemented with CH. Pictures were taken 2 weeks after the inoculation.

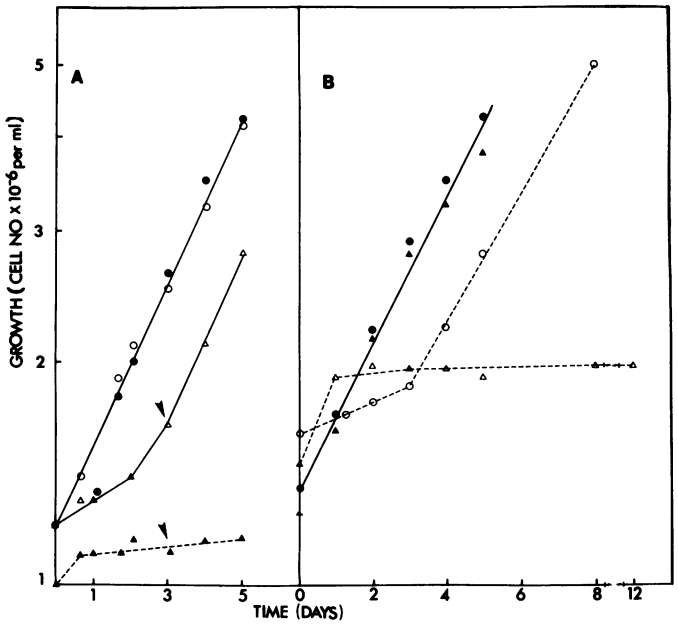

FIG. 2. A, Effect of cycloheximide on the growth of callus cultures. In the CH-free media, both W001C (●) and WCH105 (○) cultures showed a mass doubling time of 3 days. In the CH-containing media, the growth of W001C (▲) culture was completely inhibited, but WCH105 (△) exhibited the same growth rate as it did in control medium after a 2-day time lag. Arrows indicate the time when the culture media were collected from the two cultures grown in CH; each medium was incubated again with WCH105 and W001C cells, as shown in Figure 2B. B, The growth-inhibitory effect of the CH-containing media after incubation with W001C and WCH105 cells. Callus cultures of W001C and WCH105 were grown in callus media with and without 10 μg/ml CH. The media collected from the W001C and WCH105 cultures, as shown in Figure 2A, were divided into two portions; one portion was incubated with fresh WCH105 and one was incubated with fresh W001C cells. Growth of W001C cells in CH medium collected from the W001C culture (△) and from the WCH105 culture (▲); growth of WCH105 cells in CH-medium collected from the W001C culture (○) and from the WCH105 culture (●).

In the course of characterizing the CH^r trait, we found that WCH105 cells were permeable to the drug, and their protein synthesis could be inhibited by CH, as evidenced by the same rate of [^{3}H]leucine incorporation (over a period of 15 h) into WCH105 as into W001C cells in the presence of CH. Subsequently, we found that WCH105 cells acquired an ability to inactivate CH. After 3 days incubation with W001C or the WCH105 callus, the culture media were separated from the cells and reinoculated with either WCH105 or W001C cells. The arrows in Figure 2A point to the time when media were harvested; Figure 2B shows the growth of new cells in these harvested media. CH-containing media previously inoculated with WCH105 cells did not inhibit the growth of W001C and did not cause the characteristic lag in the growth of WCH105. In contrast, media previously cultured with W001C cells were still potent in inhibiting the growth of W001C and in causing the growth lag in WCH105. The control experiments employing the W001C cells indicated that simple decomposition of the drug or adsorption and subsequent removal of CH by the cells were not responsible for the loss of CH potency in media cultured with the WCH105 cells.

The time lag in the growth of WCH105 in CH-containing media appears to be the period required to inactivate CH rather than the period needed to express the inactivation function, because this period is cell density-dependent. Figure 3A shows the growth of WCH105 callus cultures in callus media containing 10 μg/ml CH at various cell densities. As the initial cell density increases, the

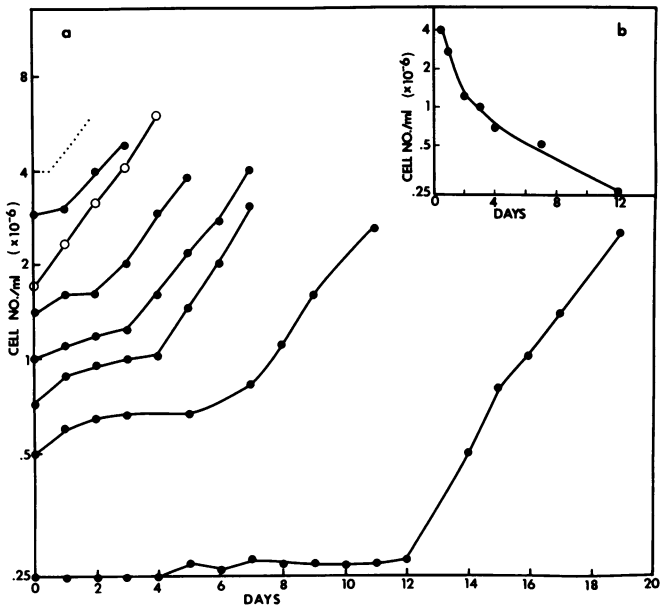

FIG. 3. The effect of cell density on the lag period of WCH105 grown in CH-containing callus medium. A, Liquid callus culture of WCH105 grown at the logarithmic phase were washed and suspended in callus medium containing 10 μg/ml CH at different cell densities. The growth of the culture was followed daily in a Klett-Summerson colorimeter. (○), Control, callus medium without CH; (●), callus medium containing 10 μg/ml CH. Inset B, Lag period in each growth curve plotted against cell density.

lag period shortens. Figure 3B shows the lag period plotted against the initial cell density. The dotted line in Figure 3A is a growth curve of WCH105 callus inferred from the experiment described below. Because growth cannot be measured accurately in the Klett-Summerson colorimeter at cell densities higher than 3×10^6 cells/ml, CH was incubated with WCH105 cells at 4×10^6 cells/ml for various numbers of h; media were separated and reinoculated with W001C at 8×10^5 cells/ml to test for CH^i. After a 15-h incubation, the medium could no longer inhibit the growth of W001C. At this point, one would expect that WCH105 would resume growth in the callus medium originally supplemented with 10 μg/ml CH. Furthermore, the CH-inactivating ability is constitutively expressed in the WCH105 callus and is probably not an inducible function. We found that WCH105 cells grown in the absence of CH for 6 months were capable of inactivating CH in 5 h when cultured at a density of 2×10^7 cells/ml. Inasmuch as it is difficult to imagine how the cells might start to express the trait in the absence of protein synthesis, this result suggests that the trait is expressed constitutively.

Temporal Expression of the CH^i Function in Carrot Culture. Subsequent to the discovery of WCH105 callus cells' ability to inactivate CH, we found that the plantlets of W001C and WCH105 were also capable of inactivating CH. Thus, the expression of the CH^i throughout the developmental period from the callus to the plantlets was investigated. Table I shows that CH inhibited growth of carrot culture at all developmental stages. The varying degree of inhibition was caused by the lag period required to inactivate CH. But some cultures resumed growth, *e. g.* torpedo; plantlets of W001C and callus; and globular, torpedo, and plantlets of WCH105 culture; the CH in these media was found to have lost the ability to inhibit growth. In other cultures, *e. g.* globular embryos of W001C, growth never resumed; however, CH in the medium was inactivated, probably because cells were irreversibly inhibited before CH was completely inhibited. CH not only inhibited growth and elongation in plantlets but also stopped

Table I. *Developmental Changes in the Expression of CH^r and CH^i in Carrot Culture*

Growth of callus and globular, torpedo embryos was measured by turbidity as increase in Klett units. Growth of plantlets was measured by fresh weight. The inoculum size of these cultures was described in "Materials and Methods." The lag period for the embryo cultures grown in CH-containing media was about 3 days. After 6 days' incubation, media were collected for the CH^i assay. The lag period for the plantlet culture was not determined. Fresh weight of the plantlets was determined after the culture was incubated for 1 week in CH-containing media. In cultures incapable of inactivating CH, media were assayed after 10 days' incubation.

Tissue	Growth in CH		CH^i[a]	
	W001C	WCH105	W001C	WCH105
	% of control			
Callus	0	67	–[b]	+[c]
Embryo initiation	0	0	–	–
Globular	0	63	+	+
Torpedo	70	100	+	+
Plantlet	20	45	+	+

[a] The media were separated from the cell by filtration. They were incubated with W001C callus cells at a density of about 4×10^5 cells/ml. Their growth was followed by an increase in Klett units. These results were confirmed by the yeast bioassays (3).

[b] –, CH was not inactivated; Klett units did not double after 6 days' (2 generation time) incubation.

[c] +, CH was inactivated; W001C cells grew in the media.

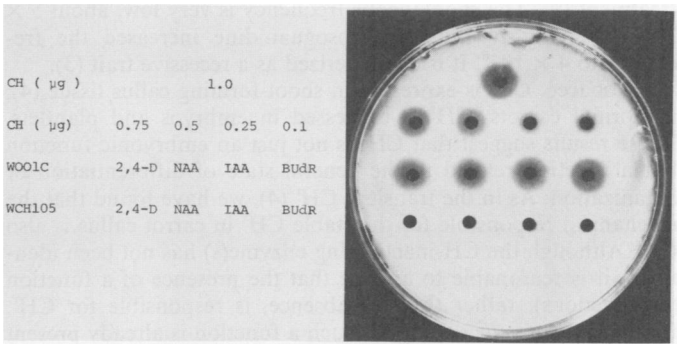

FIG. 4. CH^i of callus cultures maintained at different dedifferentiation agents. Top and second row (CH standards), 0.1 ml CH solutions at 10, 7.5, 0.5, 0.25, and 0.1 μg/ml were applied in the agar hole 0.7 cm in diameter. Third row, 10 μg/ml CH were added to W001C cultures grown in 2,4-D, IAA, NAA, and BUdR. After an 8-h incubation, 0.1 ml media was applied in each well. Fourth row, CH^i by WCH105 cells grown in various dedifferentiating agents.

greening, initially. Following CH^i, plantlets resumed growth and turned green again. The only time that tissue is incapable of inactivating CH is at the stage when embryogenesis is initiated. When transferred from callus medium directly into embryogenic medium containing CH, neither WCH105 nor W001C could inactivate CH.

CH^i as a Differentiated Function. Since the CH^i function in W001C is expressed in the embryos and plantlets of W001C, but not in the callus, it may be either a function correlated with the state of differentiation or one that is controlled by 2,4-D. We have found that callus cultures of W001C maintained by NAA or IAA were also sensitive to CH (Fig. 4).

BUdR was found to interfere with embryo differentiation in carrot culture, thus maintaining callus growth in the absence of exogenous auxin (2). After WCH105 and W001C cultures had

been grown in 2,4-D medium containing 6 μM BUdR for 3 weeks, CH was added at 10 μg/ml for 8 h. As with callus cultures maintained by 2,4-D, NAA, or IAA, BUdR-maintained W001C culture could not inactivate CH; the WCH105 culture did (Fig. 4). This result supports the hypothesis that the expression of CH^r or CH^i is a part of the differentiation process. Any disturbance that disrupts differentiation will also interfere with its expression.

DISCUSSION

When carrot cells were subjected to CH on 2,4-D-containing medium during mutant selection, green plantlets or semiorganized tissues were often observed to arise on selective medium. In tobacco, a higher level of CH^r has been detected in shoot-forming tobacco tissues, as compared with the calli (4). When carrot cells are plated on CH-containing callus medium, most of the colonies grown on the selective medium look tightly clumped and semiorganized. These colonies are likely to have arisen from cells that underwent differentiation and escape killing by CH rather than from cells mutated to resist CH as undifferentiated tissue. Consequently, we picked friable callus colonies only for further characterization. Because of the developmentally variable expression of the CH^i trait, it is not surprising to find transient, unstable CH^r cell lines as well (4). The unstable cell lines appear more frequently than do the stable CH^r cells; 5×10^{-6} (4) *versus* 5×10^{-8} (9), for spontaneous frequency.

WCH105 differs from the reported transient CH^r cell lines in the following ways. The resistance is highly stable, even in the absence of the selective pressure, CH. Calli initiated from plantlets regenerated under CH-free conditions maintain their CH^r. The frequency of CH-resistant colonies can be increased by mutagenic treatment (9). The spontaneous frequency is very low, about 5×10^{-8}; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine increased the frequency to 4×10^{-6}. It is characterized as a recessive trait (3).

In tobacco, CH^r is expressed in shoot-forming callus tissue (4); in normal carrots, CH^r is expressed in embryos and plantlets. These results suggest that CH^r is not just an embryonic function but a function related to the general state of differentiation or organization. As in the transient CH^r (4), we have found that the mechanism responsible for the stable CH^r in carrot callus is also CH^i. Although the CH-inactivating enzyme(s) has not been identified, it is reasonable to assume that the presence of a function (or functions), rather than its absence, is responsible for CH^r. Inasmuch as a gene coding for such a function is already present in W001C, WCH105 differs from W001C in mechanisms regulating the expression of the same differentiated function or a similar one, *e. g.* an isozyme.

The observation that the onset of embryogenesis in WCH105 is susceptible to inhibition by CH implies either that the selected CH^i is not expressed at that moment or that it is insufficient to inactivate the drug before protein synthesis is inhibited, thus resulting in growth inhibition. Sengupta and Raghaven (6) found that macromolecular synthesis was elevated in the first 2 to 4 h after the transfer of callus culture to embryogenic culture. We found that two specific polypeptides were greatly enhanced in the 4 h after culture transfer to embryogenic medium (11). At this initial stage of embryogenesis, cells may be more sensitive to CH due to their higher rate of macromolecular synthesis (5). However, if CH^i was not expressed at the onset of the embryogenesis, there are at least two possibilities to explain the CH sensitivity at this stage. (1) The selected CH^i in WCH105 callus is the same CH^i expressed in the embryos and plantlets, but it was temporarily turned off during the onset of embryogenesis. (2) Two different gene products are responsible for the CH^i in the callus and embryos, *e. g.* isozymes. Although both callus and embryo CH^i functions are expressed in WCH105, there is a delay after the callus CH^i is turned off and before the embryo CH^i is turned on. Resolution of the two possibilities will have to await identification and characterization of the protein(s) conferring resistance.

LITERATURE CITED

1. Borthwick MA 1931 Development of macrogametophyte and embryo of *Daucus carota.* Bot Gaz 92: 23–44
2. Dudits D, G Lazar, G Bazszar 1979 Reversible inhibition of somatic embryo differentiation by bromodeoxyuridine in cultured cells of *Daucus carota* L. Cell Differ 8: 135–144
3. Lazar GB, D Dudits, ZR Sung 1981 Expression of cycloheximide resistance in carrot somatic hybrids and their segregants. Genetics. In press
4. Maliga P, G Lazar, Z Svab, F Nagy 1976 Transient cycloheximide resistance in a tobacco cell line. Mol Gen Genet 163: 145–151
5. Sengupta C, V Raghavan 1980 Somatic embryogenesis in carrot cell suspension. I. Pattern of protein and nucleic acid synthesis. J Exp Bot 31: 247–258
6. Sengupta C, V Raghavan 1980 Somatic embryogenesis in carrot cell suspension. II. Synthesis of ribosomal RNA and poly $(A)^+$ RNA. J Exp Bot 31: 259–268
7. Steward FC, MO Mapes, AE Kent, RD Holsten 1964 Growth and development of cultured plant cells. Science 143: 20–27
8. Sung ZR 1976 Turbidimetric determination of plant cell culture growth and its applications. Plant Physiol 57: 460–462
9. Sung ZR 1976 Mutagenesis of cultured plant cells. Genetics 84: 51–57
10. Sung ZR, J Horowitz, R Smith 1979 Quantitative studies in normal and 5-methyltryptophan resistant cell lines of wild carrot: the effects of growth regulators. Planta 147: 236–240
11. Sung ZR, R Okimoto 1981 Embryonic proteins in somatic embryos of carrot. Proc Natl Acad Sci USA. In press