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# Cycloheximide Resistance in *Daucus carota* Cell Cultures

P.M. Gresshoff
Genetics Department Research School of Biological Sciences Australian National University Canberra City (Australia)

**Summary.** Cycloheximide resistant wild carrot cultures were derived by selection for growth on the otherwise inhibitory concentrations of 10 and 25 μg/ml cycloheximide. The resistance phenotype was maintained by culture in the presence of the drug but was lost in its absence. Only cycloheximide-grown cultures were capable of embryogenesis on cycloheximide media. Callus regenerated from such plantlets grown in the presence of cycloheximide still retained the resistance phenotype. Co-culture and media transfer experiments indicated that the resistant cell cultures detoxify the drug.

**Key words:** Cycloheximide resistance – Instability – Carrot culture

The value of genetically marked cell lines in somatic tissue culture system is exemplified by their use as complementing markers in the selection of protoplast hybrids (Cocking 1975; Glimelius et al. 1978). Such cell lines could have further application in the study of chromosome elimination in interspecific hybrids (Kao 1977), gene transfers (Gresshoff 1975), organism/organelle uptake and symbiotic studies (Giles 1978; Fowke et al. 1979).

A broad spectrum of auxotrophic requirements, which are expressed and thus selectable in in vitro culture, would be optimal. Such mutants have been of value in the analysis of the biochemical genetics of bacteria and lower eukaryotes. With plant systems little progress has been made along this line (Widholm 1978) and alternative methods may have to be employed to allow auxotrophic mutant isolations with plants (Gresshoff 1978b).

The isolation of antimetabolite or antibiotic resistant, or tolerant, cell cultures has been more successful. This research area was reviewed by Maliga (1976a,b) and Widholm (1978). In only a few instances was the tissue culture phenotype checked for maintenance through redifferentiation, or analysed genetically (for example; Maliga et al. 1973; Carlson 1973; Gengenbach et al. 1977; Dix 1977; Bourgin 1978).

Successful isolation of a cycloheximide resistant mutant has been accomplished in such other biological systems as *Saccharomyces cerevisiae* (Wilkie and Lee 1965), *Physarum* (Haugli and Dove 1972), *Dictylostelium discoideum* (Katz and Sussman 1972) *Chlamydomonas reinhardi* (Gresshoff, unpublished data) and Chinese hamster cells (Pöche et al. 1975).

All these mutants retained their resistance to cycloheximide through culture in the absence of the drug.

The isolation of cycloheximide resistance in plants has been limited to a report by Maliga et al. (1976), who isolated cycloheximide resistance in haploid *Nicotiana tabacum* cell lines at a frequency of $5 \times 10^{-6}$. Plant regeneration was successful from the resistant material. However, the overall phenotype for resistance was found to be transient and was lost in the absence of the drug. This communication reports a similar observation made in the wild carrot (*Daucus carota*) line WC2-91 (Baldwin and Gresshoff 1978; Fowke et al. 1979). This line retains a high embryogenic potential after years of successive subculture and is well adapted to suspension culture growth.

Preliminary results showed that 10 μg/ml cycloheximide completely inhibited callus formation from suspension cultured material (including aggregates up to 100 cells). Furthermore, it was found that embryo-formation following 2,4-D withdrawal was severely inhibited at 5 μg/ml cycloheximide, and totally suppressed at 10 μg/ml.

Washed suspension cultured cells (3 days after subculture-grown in MS medium (Murashige and Skoog 1962) were plated onto selective and control media in 3 ml of soft water-agar (0.6%) at a density of about 10,000 colony-forming-centres per plate. The yield of 'resistant' colonies is tabulated on Table 1. Spontaneous mutation frequencies were not determined, but seemed to match roughly those reported by Maliga et al. (1976). It was

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**Table 1.** Isolation of cycloheximide resistant colonies from carrot suspension cultures

| Cycloheximide concentration[a] (μg/ml) | Number of plates[b] screened | Number of colonies growing[c] |
|---|---|---|
| 0 | 1 | ~ $10^4$ (confluent) |
| 10 | 72 | 8 |
| 25 | 66 | 3 |
| 50 | 30 | 0 |

[a] All culture conditions as described by Baldwin and Gresshoff (1978)

[b] At approximately $10^4$ colony forming centres per plate

[c] After 4 week culture at 27°C, (dark) in Nescofilm selaed petri dishes. A colony was scored as growing if it was larger than 2 mm in diameter. Such growth rate represents about 20-30% of control growth rates. Some smaller colonies were seen on 50μg/ml, but these were not tested further

possible to isolate 11 colonies with apparent cycloheximide resistance at either 10 or 25 μg/ml. Spontaneous resistance was not seen at the 50 or 100 μg/ml level.

All colonies were tested for their range of tolerance/resistance to the drug, their stability of the 'resistance' to subculture and embryoid formation in the absence/presence of the drug.

Of the eleven original callus colonies (CY-1 to CY-11), three lost their resistance within the first subculture on 10 μg/ml cycloheximide. Of the remaining eight isolates, six lost the ability to grow on 10 μg/ml cycloheximide after the third to sixth transfer in the presence of the drug. The decrease of growth capability was gradual as successive subcultures gave progressively decreased growth yield. In contrast, cultures on control media grew without any noticeable alteration in growth rate.

Two isolates have retained their cycloheximide resistance over lengthy subculture periods in the presence of the drug (18 months). These are CY-4 and CY-7 (one selected on 10 μg/ml, the other on 25 μg/ml cycloheximide).

These, when exposed to further selection, adapted, and were able to grow on cycloheximide at concentrations as high as 150 μg/ml. The extent of resistance was progressive, which means that cell material growing marginally at a certain cycloheximide concentration grew efficiently at that and marginally at a higher concentration in its next subculture. Renewed subculture gave excellent growth on the higher level, indicating the adaptive nature of the resistance.

Culture in the absence of the drug quickly (within one subculture period) removed the resistance phenotype, which could be regained by selection.

Suspension culture experiments of highly resistant cell material (CY-7, adapted to grow at 150 μg/ml) at 10 μg/ml cycloheximide, followed by harvesting the media and reinoculation with susceptible cell material (WC2-91) resulted in near normal growth rates of the susceptible cells. This provided evidence that the 'resistance' of CY-7 was based on degradation of the drug. Control experiments with sensitive cells (at equal cell densities) in equivalent drug media showed that adsorption and subsequent removal of the drug by cell harvesting was not a probable mechanism.

Similarly, when highly resistant callus material was placed adjacent to sensitive callus on 10 μg/ml cycloheximide, the latter grew better than isolated controls.

These two findings indicate that, similar to the observations of Maliga et al. (1976) in tobacco, detoxification was the most likely mechanism of resistance. Rapid detoxification of cycloheximide was previously reported in insects (Campbell and Birt 1975).

Since CY-4 and CY-7 cultures maintained and increased their resistance phenotype during culture in the presence but not absence of the drug, it was attempted to study the stability of the phenotype through embryogenic development.

Suspension cultures of WC2-91, CY-4 or CY-7 were precultured on MS medium containing 0.5 μM kinetin, which enhanced embryoid formation, most likely by increasing the degree of clumping of the tissue culture. Embryoids were induced by plating about 100 embryo-forming units onto agar plates containing MS medium (hormone free) supplemented with $2 \times 10^{-8}$M NAA (naphthalene acetic acid) and 0.5 μM kinetin. All embryogenic cultures were incubated at 22°C with continuous light. Cycloheximide was added in a range from 0 to 50 μg/ml to the embryo-inducing medium. The results were consistent for all variables and both resistant cultures tested. Only cells which were pre-grown (and thus adapted) on cycloheximide continued to grow and to produce high yields of embryos and plantlets. These had an appearance similar to that obtained in control experiments of wild type cells grown without cycloheximide.

Callus cultures re-initiated from 'cycloheximide-resistant' embryos and plantlets continued to show resistance in callus culture. Genetic analysis was not carried out.

Cell cultures derived from other plant species varied significantly in their inherent tolerance to cycloheximide under identical growth conditions. The following threshold toxicities were found: *N. tabacum* NT-1: 1 μg/ml; *Trifolium repens* (white clover) CLO-1: 100 μg/ml; WC2-91: 5-10 μg/ml, *Datura inoxia* crown gall tumour line (Baldwin and Gresshoff 1978): 5-10 μg/ml; *Glycine max* (soybean) SB-1: 200 μg/ml (higher levels were not tested). Although the number of plant species tested here is limited, it raises the question of legumes possessing an inherent resistance to cycloheximide as compared to non-legumes. This differential, if based on dominant (detoxification)

character rather than an uptake barrier, can be of value for genetic marking of cell cultures. Its application in other biological systems is nicely illustrated by the selection of parasexual diploids in *Dictyostelium* between cobalt resistance and cycloheximide hypersensitivity (Williams 1978).

The existence of unstable 'mutants' in both tobacco and carrot, coupled with the occurrence of inherent insensitivity as seen in the clover and soybean cultures, display a promise that further selection using diploid cultures will provide stable induced cycloheximide resistances in cultured plant cells.

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Dr. P.M. Gresshoff
Genetics Department
Research School of Biological Sciences
Australian National University
P.O. Box 475, Canberra City
A.C.T. 2601 (Australia)