EXPRESSION OF CYCLOHEXIMIDE RESISTANCE IN CARROT SOMATIC HYBRIDS AND THEIR SEGREGANTS

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ABSTRACT

Cycloheximide resistance (CHr) was shown to be a function expressed in differentiated plant tissues, but not in unorganized callus tissues. A variant, WCH105, expressing CHr in the callus, as well as in regenerated plantlets, was isolated from a cell line derived from a wild carrot plant. The plantlets regenerated from WCH105 are green, but do not produce normal, dissected leaves. Protoplasts of WCH105 were fused with that of a cycloheximide-sensitive (CHs) cell line derived from an albino, domesticated carrot. Hybrid selection was based on (1) irreversible growth inhibition of WCH105 protoplasts by iodoacetamide, and (2) restoration of green plants producing dissected leaves. Analysis of the CHr trait as an unselected marker in the callus cells of the somatic hybrids indicated that it behaved as a recessive. The combined recessive and resistant phenotype of this trait allowed the recovery of CHr segregants from CHs hybrids at a frequency of 10⁻⁴, 1000 times higher than the spontaneous frequency of CHr. The recovery of CHr somatic segregants confirmed the recessiveness of the CHr trait.

Since Carlson's reports on the isolation of mutants and production of somatic hybrids from tobacco tissue cultures (Carlson, Smith and Dearing 1972; Carlson 1970), there has been interest in the development of somatic cell genetic systems in plants. Increasing numbers of biochemically selectable markers have been reported in the past ten years (Maliga 1978); protoplast fusion and plant regeneration have become useful tools for generating interspecific and intergeneric hybrid plants (Vasil, Ahuja and Vasil 1979). These techniques allow the combining of genetic material from different somatic cells. In addition to permitting genetic exchange, an efficient genetic system should provide opportunities for gene mapping, e.g., by somatic recombination and chromosome elimination. Somatic segregation by means of chromosome elimination has been exploited extensively for mapping gene functions on specific chromosomes in animal tissue cultures (Ruddle 1973). Although chromosome elimination has been observed in plant somatic hybrids (Binding and Nehls 1978; Maliga et al. 1978; Wetter and Kao 1980), definitive proof for somatic segregation requires recessive markers that are selectable in culture.
Cycloheximide resistance (CH') is a function related to the differentiated state of tissues. CH' is expressed only in embryos and plantlets regenerated from callus cultures of carrots, not in the callus per se (Sung, Lazar and Dudits 1981). Normally, growth of callus cultures is inhibited by 10 μg/ml CH. A variant (WCH105) resistant to CH was isolated, Sung 1976b); it expressed CH' both in the undifferentiated callus and in the plantlets. The mechanism of CH', in both plantlets and WCH105 callus was found to be CH-inactivation (CH') (Sung, Lazar and Dudits 1981). This work reports the characterization of the CH' variant by somatic hybridization. Use of somatic hybridization is discussed as a means of studying the mechanisms regulating the expression of differentiated functions.

**MATERIALS AND METHODS**

The isolation and genetic characterization of the albino mutant (A,) from a biennial diploid domesticated carrot (Daucus carota, cv. Nantaise Slendero) has been described (Dudits et al. 1977; Dudits et al. 1977a, b). The albinism is the result of a nuclear mutation and reverts at a frequency less than 5 x 10^-9. The CH' cell line (WCH105) was isolated from a cell line, W001C, initiated from a diploid wild carrot, Daucus carota L. (Sung 1979). Spontaneous appearance of CH' colonies occurred at a frequency of 5 x 10^-8 (Sung 1976b). The characterization of CH' in the plantlets and callus of WCH105 and in the callus of W001C was provided by Sung, Lazar and Dudits 1981. Cultures were maintained in MS medium (Murashige and Skoog 1962) supplemented with 0.1 or 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Growth was monitored as dry weight increase or by the sidearm-turbidity method (Sung 1976a). To initiate embryogenesis and regenerate plants from the culture, cells were transferred to the MS medium free of 2,4-D (regeneration medium), according to procedures described previously (Sung, Smith and Horowitz 1979). WCH105 forms green plantlets, but they do not develop mature dissected leaves, nor do they flower. Hence, genetic characterization was attempted by means of somatic hybridization.

Protoplasts were isolated from both cultured cells and leaf material according to published procedures (Dudits et al. 1976). To reduce the survival of parental cells, protoplasts prepared from WCH105 cell cultures were inactivated with 30 mM iodoacetamide for 30 min at 0° (Nehls 1978). Leaf protoplasts of WCH105 were prepared from aseptically cultivated plantlets. Since leaf protoplasts of carrot plants could not divide in culture (Dudits et al. 1979a), this property can be used to counterselect unfused WCH105 protoplasts and its homokaryons.

In fusion experiments, protoplasts were mixed in a 1:1 ratio at 2 x 10^6 cells/ml and treated with polyethylene glycol (PEG) Mw: 1540 according to published methods (Kao and Michayluk 1974). Parental cultures and mixed protoplast cultures without PEG treatment were established in each experiment to serve as controls.

Protoplasts were cultured in plastic petri dishes in MS/VI medium prepared from MS medium with the following supplements: (mg/l) yeast extract, 200; L-glycine, 10; L-glutamine, 100; L-tryptophan, 10; L-cysteine, 10; L-methionine, 5; choline, 10; coconut milk, 100 ml; conditioned cell culture medium, 100 ml, obtained from a 10-day-old A, culture grown at the logarithmic phase. The osmotic stabilizer was glucose (0.38 M). Naphthalene acetic acid (0.18 mg/l), 2,4-D (0.55 mg/l) and zeatin (0.11 mg/l) were used as plant hormones.

Two weeks after fusion, cultures were gradually diluted with cell culture medium at two-day intervals. Each culture was divided after one month. One part was further cultivated under hormone-free conditions to induce embryogenesis. The regenerated small embryos were transferred into large Erlenmeyer flasks and were subcultured every month. Aseptically cultured plants were grown in a photoperiod of 16/8 hr, with approximately 1500 lux illumination. Plant-
lets thus produced were potted and grown in a greenhouse. The other parts of the PEG-treated cultures were plated on agar plates containing 10 μg/ml CH for direct selection of CH⁺ colonies.

Cycloheximide-inactivation tests were performed by agar-gel diffusion bioassay based on the growth of Saccharomyces cerevisiae (MALIGA et al. 1976). Callus or plantlets were incubated in liquid medium containing 10 μg/ml CH (SUNG, LAZAR and DUDITS 1981). Media from liquid cultures (0.1 ml), water extracts of agar media, and washed callus homogenates were applied in a well punctured in a YEPD medium inoculated with yeast. After 20 hr incubation at 30°, a zone of growth inhibition was formed around the well if the media contained active CH. The amount of CH was estimated by the size of the inhibition zone. A zone of inhibition with a diameter of 1.8 cm is equivalent to 1 μg CH.

Chromosome counting of the cultured cells and the root tips was performed according to published procedures (DUDITS et al. 1976). Thirty metaphases were counted for each sample.

RESULTS

Somatic hybridization through protoplast fusion: Fusion between protoplasts from callus suspension or primary leaves of WCH105 and from callus of the albino carrot (Aₜ) was achieved by PEG treatment. The nuclear chlorophyll-deficient mutation in Aₜ and the failure to differentiate normal leaves in WCH105 were characters shown to be highly stable and presumed to be recessive. A subsequent selection scheme for somatic hybrids was devised based on the restoration of these two recessive traits. Colonies were isolated that produced green plantlets capable of differentiating normal, dissected leaves (Figure 1).

Predominantly nondividing leaf protoplasts or iodoacetamide-treated cell culture protoplasts of the WCH105 line were used in order to reduce the survival of WCH105. Three hybrids were obtained by fusion between leaf protoplasts of WCH105 with Aₜ protoplasts, and 57 hybrids were obtained by fusion between iodoacetamide-treated WCH105 protoplasts with Aₜ protoplasts. Hybrids obtained from the two kinds of fusion experiments behaved similarly, but the experiments employing iodoacetamide-treated WCH105 yielded 20 times more fusion products than did the ones employing leaf protoplasts. For simplicity, only the experiments employing iodoacetamide-treated WCH105 are reported in Table 1.

In a total of four experiments, approximately 4 × 10⁶ albino protoplasts and 4 × 10⁶ WCH105 protoplasts were treated with PEG to effect fusion. Although 285 green plantlets were recovered from the fused protoplasts, only 57 of them developed normally (Figure 1).

These 57 plants exhibited hybrid morphology: they were biennial, like the domesticated carrot—none bolted in 6 months as does the wild carrot—but they developed dissected, mature green leaves and white roots with a morphology intermediate between the domesticated and the wild carrot. Sexual hybrids between different varieties of carrots often exhibit a morphology intermediate between the parents (McCOLLUM 1977). No differences were observed between plants selected after fusion with callus or with leaf protoplasts of WCH105.

Callus cultures were initiated from 30 of the 57 plantlets, and their sensitivity to CH was tested. All 30 callus cultures were found to be CH⁺. The growth rate of cell suspension cultures initiated from two of the hybrid plants (no. 25–12
**Figure 1.**—Morphology of plants regenerated from different carrot cell lines. Upper left: Plantlets of WCH105 (WCH) incapable of normal development and of producing mature, dissected leaves. The cotyledons of the plants are light green. They are cultured on MS medium free of growth regulators. Lower left: Albino plants from the carrot. Upper right: Plants from hybrid no. 25-12, exhibiting normal plant development and mature, dissected leaves; thus, they can be grown in soil. Lower right: Albino somatic segregants isolated from the hybrid no. 25-12. Cultured cells from these plants are sensitive to cycloheximide. The plants appear stronger than the albino parent.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Number of A₁ and iodoacetamide-treated WCH105 protoplasts and resulting hybrids</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCH105* + PEG</td>
<td>A₁ × WCH105 + PEG</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>4 × 10⁶</td>
</tr>
<tr>
<td>Green plantlets</td>
<td>0</td>
</tr>
<tr>
<td>Green plants with the hybrid phenotype and normal development</td>
<td>0</td>
</tr>
<tr>
<td>Green plants with normal development and sensitive to 10 μg/ml of CH in callus</td>
<td>0</td>
</tr>
</tbody>
</table>

* All the WCH105 protoplasts have been previously treated with iodoacetamide according to procedure described in Materials and Methods.
† Calli were initiated from 30 of the 57 hybrids. They were tested for CH sensitivity in culture according to procedures described in Materials and Methods.
‡ The origin of these plantlets were not clear; they were probably WCH105, whose survival, after iodoacetamide treatment, was aided by the presence of A₁ protoplast.
and no 26–3) was tested at 5 and 10 μg/ml CH, along with cell cultures initiated from WCH105 and A1 plantlets regenerated from the protoplasts. Both nos. 25–12 and 26–3 were as sensitive as the parental A1 culture. Only cell cultures initiated from WCH105 plantlets proved to be resistant (Figure 2). An additional 28 hybrid cell lines selected after fusion with protoplasts derived from cell culture and three hybrids selected after fusion with leaf protoplasts of WCH105 were tested on agar plates; they did not form colonies on agar plates containing 5 or 10 μg/ml CH. The chromosome number counted in cell cultures of the WCH105 line was found to be \(2n = 18\), while that of A1 showed variation between \(2n = 18\) and 46; however, 50% of the cells possessed \(2n = 18\) chromosomes. Chromosome numbers of the hybrids were counted in the root tips of the

![Graphs showing growth of liquid cultures](image)

**Figure 2.** Cycloheximide sensitivity test of carrot cultures reinitiated from plants regenerated from various cell lines, WCH105 (WCH), A1, and somatic hybrids no. 25–12 and no. 26–3. Growth of liquid cultures in the absence of CH (●), in the presence of 5μg/ml (○) and 10 μg/ml CH (△).
selected green plantlets, where the tetraploid nature of the hybrids remained stable. For example, the all 30 metaphases counted in hybrid no. 25-12 contained 36 chromosomes (Table 2).

In direct selection for CH⁺ hybrids, the colonies grown from PEG-treated protoplast cultures were plated on an agar medium supplemented with 10 μg/ml CH. The colonies that grew on a CH-containing medium were isolated, and plants were regenerated from them. Plantlets that developed from these cultures all exhibited the WCH105 phenotype, with its abnormal development. The total elimination of the hybrid category and the albino parent in this experiment agreed with the results of indirect selection, in that CH⁺ of the WCH105 line was not expressed in callus cells of somatic hybrids.

Spontaneous somatic segregation: One year after the hybrids had been cultured under nonselective conditions, they were plated on a medium containing 10 μg/ml CH to test their sensitivity to CH. Six out of 18 hybrid lines tested formed colonies at a frequency of 1.3 × 10⁻⁴ to 1.5 × 10⁻⁵. Analyses of hybrid line no 25-12 and three of the resistant cell lines isolated from no 25-12 are summarized in Table 2. The data show a correlation between the increase in variability in chromosome number and the ability to grow in CH and to inactivate CH in line 25-12 before and after subculture. Partial CH⁺ in line no. 25-12 after subculture suggests a mixed culture of CH⁺ and CH⁻ cells. Indeed,

**TABLE 2**

*Karyotypic and cycloheximide resistance analysis of the parental lines, hybrid no. 25-12 and three CH⁺ segregants*

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Growth in liquid medium containing 10 μg/ml CH* (percentage of control)</th>
<th>Colony-forming ability on agar plates containing 10 μg/ml CH** (percentage of control)</th>
<th>Cycloheximide inactivation ability</th>
<th>Chromosome numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>18-46</td>
</tr>
<tr>
<td>WCH105</td>
<td>95</td>
<td>92</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>Hybrid line no. 25-12 at the first test</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>36</td>
</tr>
<tr>
<td>Hybrid line no. 25-12 after subculture for one year</td>
<td>18</td>
<td>0.002</td>
<td>±</td>
<td>32-72</td>
</tr>
<tr>
<td>25-12-11</td>
<td>89</td>
<td>18</td>
<td>+</td>
<td>20-70</td>
</tr>
<tr>
<td>25-12-12</td>
<td>82</td>
<td>29</td>
<td>+</td>
<td>32-58</td>
</tr>
<tr>
<td>25-12-30</td>
<td>99</td>
<td>9</td>
<td>+</td>
<td>30-100</td>
</tr>
</tbody>
</table>

* The growth of liquid cultures were measured by dry-weight increase after 10 days cultivation with and without CH.
** Colony-forming ability was estimated on the basis of number of colonies per plate. Numbers represent averages of triplicate samples from one experiment.
† CH inactivation was performed by Saccharomyces agar-gel diffusion test after 20 hr incubation of cells with 10 μg/ml CH.
"+" means CH is inactivated. There is no zone of yeast inhibition; less than 0.1 μg CH is present.
"-" means CH is not inactivated; 1.6-1.8 cm of zone of inhibition is present.
"±" means results were variable, but the inhibition zone never exceeded 1 cm.
Cytochrome c353 colonies can be isolated from 25-12. The properties of three segregants, 25-12<sup>+</sup>-11, 25-12<sup>+</sup>-12 and 25-12<sup>+</sup>-30, are shown in Table 2. They resist and inactivate CH at a much higher degree than does 25-12. They showed nearly the same growth rate in liquid CH-containing cultures as did the WCH105 cells. Their poorer colony-forming ability on agar plates, however, indicated that these cultures might be heterogenic and possess both resistant and sensitive cells. The varying chromosome numbers observed in these cell lines suggested genetic instability in somatic hybrids grown in culture.

Somatic segregation for the albino trait was also observed in the hybrids. Plantlets were regenerated from a somatic hybrid cell line. Three of 3.5 × 10<sup>4</sup> plantlets were albinos (Figure 1). All cultures initiated from these albinos were sensitive to 10 µg/ml CH. In root tips of albino segregants, 32 to 34 chromosomes were counted. CH<sup>+</sup> and albino phenotypes from the hybrids appeared at a frequency at least 1000 times higher than the spontaneous mutation frequency of CH<sup>+</sup> and albino traits. This observation indicates that these colonies were somatic segregants rather than new spontaneous mutants.

**DISCUSSION**

Our results showed that the phenotype of hybrids derived from the CH<sup>+</sup> and CH<sup>-</sup> parents was CH<sup>-</sup>. There are several explanations for the disappearance of CH<sup>+</sup> phenotype in the somatic hybrids: (1) CH<sup>+</sup> is an epigenetic trait that is unstable in the presence of another genome; (2) CH<sup>+</sup> is a stable dominant trait, but the chromosome carrying the trait is lost subsequent to hybridization; (3) CH<sup>+</sup> is a recessive nuclear mutation, thus not expressed in the presence of a dominant allele; or (4) CH<sup>+</sup> is a recessive trait coded by organellar DNA. CH<sup>+</sup> in WCH105 callus was shown to be a stable trait (Sung, Lazar and Dudits 1981). The doubled-chromosome nature of the initial hybrids suggests that the chromosome complements of both parents were present after hybridization. The subsequent recovery of CH<sup>+</sup> colonies from the hybrids indicates that CH<sup>+</sup> is a stable, recessive trait that was present in the hybrids since their inception. Our results do not indicate whether the CH<sup>+</sup> trait is coded by the organellar genome or by the nuclear genome. The CH<sup>+</sup> trait segregates, however, in a pattern similar to that of the albino marker, which has been proven by sexual crosses to be a recessive, nuclear mutation.

Both somatic segregation and chromosome instability have been observed in mammalian somatic hybrids. They also are associated with an increase in ploidy level (Harris and Whitmore 1977). The mechanism of somatic segregation in carrot hybrids is not known; however, its association with chromosome instability suggests that chromosome loss or chromosome rearrangement may be responsible for its occurrence (Chasin and Urlaub 1975), although it does not exclude other possibilities (Harris 1979), such as chromosome inactivation or gene dosage effects. Increased chromosome variability and chromosome elimination have been shown to occur simultaneously in callus cells derived from somatic hybrids (Wetter and Kao 1980; Maliga et al. 1978). Somatic segregation of morpho-
logical traits and isozyme patterns observed in these cells could result from chromosome elimination as a consequence of chromosome instability. The CH' and albino traits permit selection and quantification of these events, thus providing further evidence that the phenomenon of somatic segregation occurs in hybrids formed by protoplast fusion and maintained in culture. Potentially, somatic segregation of two traits can be employed to study their linkage relationships. While we have not found simultaneous recovery of both recessive traits, it does not mean that the responsible genetic factors are linked. Since the segregation frequency of one trait is 10⁻⁴, recovery of two unlinked recessive traits as a result of simultaneous loss of the four corresponding dominant alleles (tetraploid) would appear at a frequency of 10⁻⁸. To isolate one albino plantlet from the CH' segregant line with a 99% probability of success requires screening 46,049 plantlets regenerated from CH' segregants.

Genetic imbalance was suggested as the mechanism responsible for chromosome instability and elimination in somatic hybrids constructed between remotely related species (BINDING and NEHLS 1978). If genetic imbalance exists in hybrids of wild and domesticated carrots, it occurs only in culture, not in the plants. Chromosome variability was not observed in root tips of the selected hybrid plantlets, but only in the suspension cultures initiated from the hybrid plants. Some chromosome variability has been observed in one of the parents, the albino, domesticated carrot culture (Table 2). Chromosome instability may also be a trait of the albino cell line grown in culture.

Plants regenerated from somatic hybrids often exhibit developmental abnormalities. Both the somatic hybrids and their segregants from the WCH105 × A₁ parents were capable of normal vegetative development, but they produced abnormal flowers that did not set seeds. Hence, somatic hybridization was an alternative means by which the trait could be characterized genetically.

Although frequently observed in animal tissue cultures (HARRIS 1971), the isolation of a recessive marker from diploid plant cells remains a puzzling phenomenon. The mechanism remains to be resolved.

Somatic hybridization has been employed to study the mechanisms regulating the expression of differentiated functions. For example, the expression of tyrosine aminotransferase was suggested to be negatively controlled by the steroid hormones, because fusion between tyrosine aminotransferase-inducible and uninducible cells produces somatic hybrids that are unresponsive to the hormone (THOMPSON and GELEHRTER 1971). CH' in carrots results from CH-inactivation (CH'), which is shown to be a differentiated function (SUNG, LAZAR and DUDITS 1981). Since the A₁ parent already possesses a CH¹ function, WCH105 differs from A₁ in the regulation of the CH¹. Like the mammalian cell hybrids that cannot express the specialized gene functions, the callus cells of somatic hybrids constructed between WCH105 and A₁ cannot inactivate CH. The recessive nature of these differentiated functions suggests that diffusable substances may be involved in the regulation of their expression. It seems more reasonable to assume that the presence, rather than the absence, of a product(s) (e.g., an enzyme) is responsible for CH¹. Under this assumption, it is possible to deduce the
mechanism of the diffusable substance that regulates the expression of CH'. If CH' (or CH⁺) is positively regulated, the synthesis of the diffusable substance is a recessive trait. On the other hand, if CH' is negatively regulated, the synthesis of the diffusable substance should be a dominant trait.

LITERATURE CITED


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