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Chlorsulfuron resistance in Daucus carota cell lines and plants: involvement of gene amplification

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Abstract *Daucus carota* L. cell lines stably resistant to the herbicide chlorsulfuron (CS) have been isolated according to a stepwise selection. Studies carried out during different selection steps show that the specific activity of the target enzyme acetohydroxyacid synthase (AHAS) increases along with CS resistance. Southern hybridization analysis performed with a *Brassica napus* AHAS probe in a CS highly-resistant cell line reveals the presence of a greatly amplified *Eco*RI fragment of genomic DNA. This indicates that AHAS overproduction induced by stepwise selection is due to gene amplification. Regenerants from some resistant cell lines maintained the CS-resistant trait at the whole plant level.

Key words *Daucus carota* L. · Cell selection · Chlorsulfuron resistance · Acetohydroxyacid synthase · Gene amplification

Introduction

In higher plants the site of action of a large number of new herbicides has been investigated and the target molecule identified (Ray 1989). Sulfonylurea herbicides act by inhibiting the enzyme acetohydroxyacid synthase (AHAS, EC 4.1.3.18) which catalyses the first step in the biosynthesis of leucine, isoleucine and valine (Chaleff and Mauvais 1984; Ray 1984).

Cell lines and plants resistant to sulfonylurea herbicides have been isolated in several species: in these cases resistance has been related to a dominant, semidominant, or recessive mutation in the nuclear gene encoding AHAS, which becomes less sensitive to the herbicide (Chaleff and Ray 1984; Haughn and Somerville 1986; Chaleff and Bascomb 1987; Jordan and McHughen 1987; Sebastian and Chaleff 1987; Saxena and King 1988; Swanson et al. 1988; Saxena et al. 1990; Harms and DiMaio 1991; Saunders et al. 1992; Caretto et al. 1993).

In different plant cell lines selected for resistance to the herbicide glyphosate, amplification of the gene for the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) was found coupled to an increased level of EPSPS (Shah et al. 1986; Hauptmann et al. 1988; Goldsbrought et al. 1990; Shyr et al. 1992). Phosphinotricin ressistance in alfalfa cells is also associated with an increase in the copy number of the gene encoding for the target enzyme glutamine synthetase (Donn et al. 1984).

It has been reported recently that in addition to mutations in the AHAS gene, sulfonylurea resistance in tobacco can be achieved by gene amplification (Harms et al. 1992). This is the first case where two different herbicide-resistance mechanisms selected by cell selection have been found operative.

To further investigate the nature of plant resistance to this class of molecules, we have carried out a stepwise cell selection on *Daucus carota* L. cells based on chlorsulfuron (CS, commercial name "Glean", DuPont). The choice of carrot cells was motivated by the high reproducibility of this cell system. We have isolated several resistant cell lines from which resistant plants were regenerated. The biochemical and molecular characterization of a highly-resistant cell line indicates an overproduction of the AHAS enzyme depending on amplified AHAS sequences.

Materials and methods

Selection of resistant cell lines

Callus cultures (SC0), initiated from aseptically-grown *D. carota* L. var. Scarlet Nantes seedlings, were maintained on agarized (1% w/v) Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 0.5 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0.25 mg/l 6-benzylaminopurine (6-BAP). The plates were placed at 23 °C under cool fluorescent light (3000 lux) with a 12 h photoperiod.

SCO was selected stepwise for CS resistance by transferring callus, at 1–2-month intervals, onto B5 medium supplemented with the

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above mentioned hormones and gradually increasing CS concentration. CS, 2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl] benzene-sulfonamide, was kindly provided by DuPont (Wilmington, Del.); the stock solution (10 μM) was filter sterilized. The CS concentration used for the first step was 10 nM.

Cell growth (I_{50}) was determined by transferring approximately 50 mg of callus tissue on a filter paper disk (Whatman No. 1) placed on the surface of a Petri dish containing agarized medium supplemented with increasing CS concentrations; the increase in fresh weight was determined after 3 weeks.

Plant regeneration and resistance test

Calli, actively growing at various CS concentrations, were induced to regenerate plants by inoculating them into herbicide-free liquid B5 medium supplemented with 2, 4-D. After 3 weeks, cells were washed, transferred in B5 liquid medium without hormones and, after a further 2 weeks, plated on the same agarized medium.

A resistance test in the putatively-resistant and control plants was carried out by foliar application of CS. Each plant was sprayed daily for 1 month with an aqueous solution containing 0.5 μM CS and 0.2% Tween 20 (v/v). The level of resistance was estimated by evaluating the typical toxic effects on plant growth (extreme growth inhibition, chlorosis, necrosis). Plants were classified in three groups characterized by: lack of resistance (−), an intermediate degree of resistance (+) and a high degree of resistance (++).

AHAS activity assay

AHAS activity was determined in extracts of callus cultures 10 days after the last transfer. Extraction and assay were performed according to Chaleff and Mauvais (1984) and Ray (1984) with minor modifications. Calli were frozen in liquid nitrogen and ground in a mortar in 2 ml/g fresh weight of 0.1 M K_2HPO_4, 1.0 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 10 μM flavine-adenine dinucleotide (FAD), 10% v/v glycerol, pH 7.5. The homogenate was centrifuged (27 000 g, 15 min, 4 °C) and an equal volume of a saturated solution of ammonium sulfate was added to the supernatant fraction. The resultant precipitate was redissolved in 10 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM $MgCl_2$ and 1 mM sodium pyruvate. AHAS activity was assayed in a total volume of 500 μl containing 100 μl of extract in 20 mM phosphate buffer pH 7.0, 20 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate, 0.5 mM $MgCl_2$, 10 μM FAD. CS was added at various concentrations. The mixture was incubated for 90 min at 30 °C. The reaction was terminated by adding 50 μl of 6 N H_2SO_4. A further incubation at 55 °C for 10 min was carried out. To evaluate the acetoin content, to each tube were added 100 μl of 50% NaOH, 150 μl 0.5% creatine and 150 μl 5% α-naphtol (solubilized in 2 M NaOH). Tubes were incubated for 15 min at 55 °C and the optical density was measured at 525 nM.

Protein concentrations were tested by the Biorad protein assay.

DNA isolation and Southern blotting

DNA was extracted according to R. M. Hauptmann (personal communication) as described by Menancio et al. (1990). Ten micrograms of CsCl-purified DNA was digested with *Eco*RI, separated on an 0.8% agarose gel and transferred to a nylon membrane as described by Maniatis et al. (1982). The DNA was crosslinked to the nylon membrane with the GS Genelinker (Biorad). Prehybridization was performed at 42 °C for at least 3 h in 30% formamide, 6 × SSC, 5 × Denhardt's (1 × Denhardts is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumine), 1% SDS, 100 μg/ml denatured salmon sperm DNA and 2.5% dextran sulfate. An AHAS probe was made by radiolabeling a 2 000-bp fragment of a *Brassica napus* AHAS cDNA clone pBI-284, kindly provided by W. L. Crosby, National Research Council, Canada. The 2 000-bp AHAS fragment is in an *Eco*RI site of pTZ-18R (Bekkaoui et al. 1991). The specific activity of the probe was approximately 1×10^8 cpm per μg DNA. Hybridization was performed in 30% formamide, 6 × SSC, 1 × Denhardts, 1% SDS, 100 μg/ml salmon sperm DNA and 5% dextran sulfate, with a probe concentration of 1.5×10^6 cpm/ml at 42 °C for 20 h. The membrane was washed in 2 × SSPE (1 × SSPE is 1 mM Na_2EDTA, 8 mM NaOH, 10 mM NaH_2PO_4, 180 mM NaCl) and 0.5% SDS at room temperature for 15 min and twice at 50 °C for 30 min and then subjected to autoradiography.

Copy-number reconstructions were done assuming that the total carrot genome comprises 0.9×10^9 bp (Arumuganathan and Earle 1991).

The EP2 cDNA clone (Sterk et al. 1991) was kindly provided by Sacco De Vries.

Results

Cell selection and resistance stability

A stepwise chlorsulfuron selection was carried out using a wild-type *D. carota* L. callus cell line, designated SC0, starting with a CS concentration of 10 nM. This concentration inhibited wild-type cell growth by 50%. No growth was observed in the presence of 50 nM CS (Fig. 1). By transferring cells at a few month intervals onto increasing concentrations of the herbicide, cells stably growing on 1 μM CS were obtained after eight steps (a culture designated SC1000). One-step selection did not induce the appearance of highly-resistant cell lines.

To verify the stability of CS resistance, SC1000, and some of the intermediate selection cultures, designated SC10, SC50 and SC100 (respectively growing on 10 nM, 50 nM and 100 nM CS), were maintained on herbicide-free medium for a few months and subsequently transferred onto medium containing increasing concentrations of the herbicide. The I_{50} was calculated by means of the increases in fresh weight for each cell line and results are shown in Table 1. The CS concentration required to obtain 50% of growth inhibition was about 4-fold in SC10 and progressively increased up to 80-fold in SC1000 with respect to the control.

Fig. 1 Growth of wild-type carrot cells in the presence of various concentrations of chlorsulfuron (values are given as a percentage of the control)

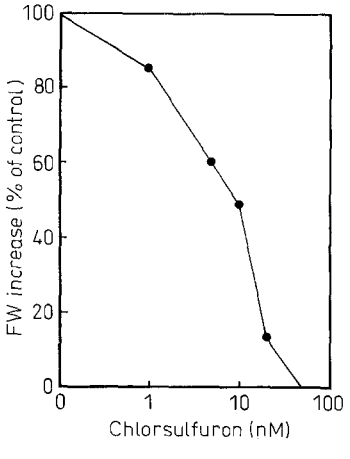

Table 1 Sensitivity of different carrot cell lines of chlorsulfuron

Cell line	I_{50} (nM)[a]
SC0	10
SC10	38
SC50	170
SC100	300
SC1000	800

[a] I_{50}: concentration required to inhibit cell growth by 50%

Table 2 AHAS activities expressed as nmoles acetoin/mg protein per h in different carrot cell lines. The SD did not exceed ±7%

Cell line	nmol acetoin (mg protein)$^{-1}$(h)$^{-1}$
Control	248
SC10	362
SC50	610
SC100	923
SC1000	1380

Characterization of AHAS activity

To clarify the mechanism of the observed high CS resistance in SC1000, the activity of the target enzyme AHAS was investigated. AHAS was extracted from SC1000, SC10, SC50, SC100 and from a control line (SC0). The enzyme activity was measured in the cellular extracts in the presence of increasing concentrations of CS ranging from 10 nM to 10 µM. Inhibition profiles showed that AHAS from SC50, SC100 and SC1000 was less sensitive to CS than AHAS from SC0. AHAS from SC10 did not show any significant difference. The AHAS activity of SC1000 was not inhibited by 10 nM CS, a concentration which only slightly inhibited the AHAS activity of SC50 and SC100, but which induced an average inhibition of 38% in the control line. At the highest CS concentration tested (10 µM), AHAS from SC50, SC100 and SC1000 was inhibited by approximately 30% in comparison with a 70% inhibition in SC0 (Fig. 2).

Table 2 compares the values of AHAS activity, expressed as nmoles acetoin/mg protein/h, among the analyzed lines. AHAS activity progressively increased along with resistence from 1.5-, 2.5-, 3.7- up to 6-fold in SC10, SC50, SC100 and SC1000 respectively, indicating a higher level of expression of AHAS in the resistant lines. The increase of AHAS activity in SC50, SC100 and SC1000 crude extracts accounts for the observed resistance to CS inhibition of the enzyme from these lines. Experiments of AHAS sensitivity to CS, where the crude extract from SC1000 was diluted to make the enzymatic activity comparable to the one of SC0 control, showed that the AHAS inhibition profile in SC1000 was not different from that of the control: this suggests that on a equimolar basis AHAS from SC1000 behaved as sensitive as that of the control (data not shown).

Gene amplification

Genomic DNA isolated from SC1000 was analyzed by Southern blotting. A *B. napus* AHAS cDNA was used to hybridize *Eco*RI-digested DNA from SC1000 and SC0. Two fragments of 8.9 kb and 4.9 kb hybridized in the control cell line with a weak signal, as expected where a single-copy gene is hybridized by a heterologous probe. The 8.9-kb band was also present in SC1000 but at a greater intensity, indicating that this DNA sequence was amplified in the CS-resistant cells. Compared to the control the 4.9-kb fragment was also much stronger in SC1000, thus confirming the presence of a greatly amplified AHAS sequence. From copy-number reconstruction experiments it was calculated that in SC1000 the 4.9-kb amplified sequence contains at least ten copies of AHAS gene (Fig. 3a). The two *Eco*RI fragments amplified in CS-resistant cells might represent two separate

Fig. 2 Responses to chlorsulfuron of acetohydroxyacid synthase activities in crude extracts of control and chlorsulfuron-resistant carrot cell lines. Response activity is expressed as a percent of values of uninhibited assays

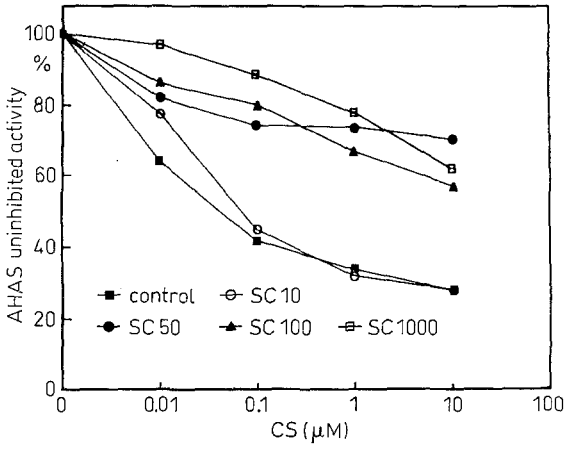

Fig. 3a, b Southern hybridization of *Eco*RI-digested genomic DNA from SC0 (*lane 1*) and SC1000 (*lane 2*). **a** with a *B. napus* AHAS probe, on the right a copy number reconstruction experiment is reported; **b** with an EP2 cDNA probe

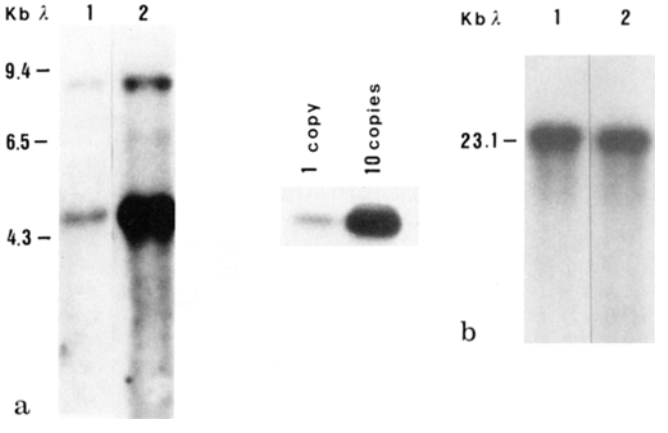

Table 3 Effects of foliar application of chlorsulfuron to SC0 control and putatively-resistant SC50 and SC100 carrot plants. The values represent the number of plants characterized by: lack of resistance (−); an intermediate degree of resistance (+); a high degree of resistance (+ +)

Regenerant	Number of tested plants	(−)	(+)	(+ +)
SC0	53	53	0	0
SC50	58	47	10	1
SC100	51	27	20	4

AHAS genes. This was supported by the fact that they are amplified to a different extent.

EP2, a single-copy gene in the carrot genome, has been used as a control. The same filter membrane was hybridized with a cDNA clone containing the EP2 coding sequence (Sterk et al. 1991): no differences in the signal intensity were noticed between the *Eco*RI-digested 23-kb fragments in SC0 and SC1000 (Fig. 3 b).

Plant regeneration

To show that morphogenetic ability was not lost after the prolonged cell selection in the presence of CS, plant regeneration was carried out in the resistant cell lines. Several plants have been easily regenerated from SC50 and SC100 through few transfers in herbicide-free liquid medium. After transfer to soil, about 50 regenerated plants of both SC50 and SC100 were tested by foliar spray application of chlorsulfuron. One SC50 and four SC100 plants were found highly resistant showing a growth similar to untreated controls. Ten SC50 and 20 SC100 plants had an intermediate degree of resistance. As expected the SC0 controls were extremely sensitive to CS action and did not survive the treatment (Table 3).

Discussion

The results presented in this study show the first isolation, by means of stepwise selection, of a carrot cell line, SC1000, which is 80-fold more resistant to CS than the wild-type. By analyzing cell growth at different steps of selection it is evident that in our cell system resistance to CS was reached gradually. Our data prove that a stepwise selection is needed to obtain high resistance. One-step selection was ineffective for this purpose. Supported by Shyr et al. (1993), we hypothesize that wild-type cell populations contain no cells able to grow at very high concentrations of CS. Probably, the small number of moderately-resistant cells in this original population can be increased by a gradual selective pressure. Further selections with an increasing CS concentration lead to the isolation of highly-resistant cell lines.

It is interesting to note that in our carrot cell system CS resistance is a trait stably acquired and maintained through cell division. The herbicide concentration required to inhibit 50% of the growth of SC10 cells (the first-step selected line) was four times higher than that used for the wild-type cells, even when the cells were grown for an extended period in the absence of selective pressure.

During the stepwise selection a correlation has been observed between CS resistance and AHAS specific activity. This increased 6-fold in SC1000 with respect to the wild-type cells.

The decreased sensitivity to CS of AHAS from the selected lines can, in principle, be explained by the presence of an altered form of enzyme. However, this does not seem to be the case, since AHAS from SC1000 proved to be as sensitive as AHAS from the control. It is noteworthy in this respect that the AHAS inhibition profile from SC10 did not show any difference with respect to that of the wild-type, and that the enzyme specific activity was only 1.5-fold higher than the one recorded for SC0. The progressive increase of AHAS specific activity accounts for the decreased sensitivity of the enzyme to the herbicide. These data suggest that CS resistance is due to an overproduction of AHAS enzyme in the selected lines.

The overproduction of the target enzyme in cell lines selected for resistance to glyphosate and phosphinotricin correlated with the amplification of the gene encoding the corresponding enzyme. In these cases a stepwise selection was used (Donn et al. 1984; Shah et al. 1986; Hauptmann et al. 1988; Goldsbrough et al. 1990; Shyr et al. 1992). Hybridization of the genomic DNA from our cell lines with a *B. napus* AHAS cDNA results in two bands, both in the wild-type and in the SC1000. Both bands seem amplified in the selected line. The different degree of amplification of the two bands suggests the presence of two different DNA sequences homologous to the AHAS probe. These observations imply that in carrot at least two genes encode AHAS. The relative intensity of the 4.9-kb amplified band in SC1000 indicates that this cell line contains approximately ten copies of one of the two AHAS genes.

Several cell cultures of species other than *D. carota* L. have been selected for resistance to sulfonylurea herbicides. In most of these cases resistance was associated with a mutation of the gene encoding the target enzyme AHAS. Only recently, Harms et al. (1992) reported a case of AHAS gene amplification in a tobacco cell line selected sequentially on two different sulfonylurea herbicides, with one of the two used at increasing concentrations. Since gene amplification is one of the typical events occurring during "in-vitro" cell multiplication, we hypothesize that the "stepwise" schemes succeeded in preferentially selecting herbicide-resistant cells by inducing gene amplification.

The carrot cell lines produced in this study were proven to be highly regenerable, as shown by the large number of regenerated plants from the wild-type, SC50 and SC100 cultures. CS resistance was not lost during embryogenesis, since a large percentage of regenerated

plants maintained the resistance trait. The achievement of regeneration in SC1000 will allow us to verify the degree of gene amplification at the whole plant level.

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