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Isopropyl-N(3-chlorophenyl) carbamate (CIPC) induced chromosomal loss in soybean: a new tool for plant somatic cell genetics

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Summary. The properties and uses of tissue culture partial haploid soybean cell lines are explored. Partial haploid lines were prepared by CIPC treatment of a genetic heterozygote, and compared to this heterozygote and to homozygous cell lines corresponding to the parental genotypes from which the heterozygote was derived. Cell lines which lack chromosomes were characterized physiologically and with respect to a variety of isozyme markers. Often the loss of chromosomes revealed a phenotype corresponding to a recessive parental genotype. In some cases, however, new phenotypes were observed indicating a complex genotype and suggests the interaction of several genes. The implications of this for plant breeding are discussed.

CIPC also was used as a tool to dissect a complex phenotype which arose as the result of mutagenesis. A mutant cell line which required asparagine for growth but also had acquired the ability to grow on allantoin as a sole source of nitrogen was treated with CIPC to remove chromosomes. The requirement for asparagine could be separated from the ability to use allantoin, demonstrating that these phenotypes were the result of separate mutations.

Key words: CIPC treatment – Controlled chromosome loss – Soybean – Genetic analysis

Introduction

Conventional genetic analysis of plants is limited by the space and time required to raise and breed plants. In addition, genetic analysis of self-fertilizing plants such as soybean is often laborious and limited by the ability to make genetic crosses. Development of a somatic genetic system using tissue culture would reduce both the space and time required. Such a system would require that chromosomes be combined and segregated in tissue culture. Protoplast fusion may eventually be used as a routine method of combining chromosomes in a single cytoplasm. However, without this tool, cell cultures can be prepared from heterozygous plants in which chromosomes have already been combined in a natural manner. As a substitute for segregation, chromosome loss can be used to reveal genetic attributes otherwise masked by dominant alleles. Such partial haploids could eventually be used in a routine manner to map genes whose phenotypes could be screened or selected in tissue culture.

Controlled chromosome loss has proven a valuable tool for genetic analysis of mammalian cells (Kato and Yosida 1971; Larizza et al. 1974; Larizza et al. 1979; Puck 1981), *Dictyostelium* (Williams and Barrand 1978) and yeast (Wood 1982a, b). In yeast it has been possible to map genes in appropriately marked heterozygous diploids using chromosome loss induced by treatment with Benomyl (Wood 1982a, b). Recently, we described the ability to induce chromosome loss in soybean cell suspensions by treatment with the herbicide CIPC (Roth et al. 1982). In this paper, we examine the results of chromosome loss from a cell line derived from a heterozygous plant. We demonstrate that this chromosome loss reveals a number of heritable phenotypes that also are observed in cell lines derived from the two cultivars which had been crossed to produce the heterozygote. In addition, we describe the use of CIPC treatment as a tool for the genetic analysis of a mutant cell line which exhibits more than one mutant phenotype.

Materials and methods

Soybean cultivars and tissue culture lines

Characteristics of the different cell lines used are described in Table 1. For some experiments we used suspension cultures which were prepared from the following plants: 'Minsoy', 'PI-290136' and an F_1 plant obtained by crossing these cultivars. The plants were grown from seed provided by Dr. Reid Palmer, Dept. of Genetics, Iowa State University. Some properties of the plants have been described (Delannay and Palmer 1982). The preparation of cell suspension cultures also has been described in detail (Roth et al. 1982). Partial haploid cell lines were then prepared from this heterozygote cell line by treatment with CIPC as described below. For other experiments, we used an auxotrophic cell suspension culture (Sln-asn-) incapable of growing on minimal medium containing only inorganic nitrogen. The properties of this line, and the parent cell line from which it was derived, already have been described (Roth and Lark 1982). A prototrophic revertant was prepared from this auxotroph by treatment with UV following the procedure described previously (Roth and Lark 1982). This cell line was again able to grow on unsupplemented, minimal medium. Suspension cultures were stored under liquid nitrogen (Weber et al. 1983).

Cell growth

Shaking, suspension cultures of cells were grown at 33° or 22 °C (Chu and Lark 1976). During use, they were transferred on a regular basis to maintain exponential growth (usually diluted ¼ every 2 days at 33 °C). Cell growth was routinely measured as the packed volume of cells contained in 2.5 ml of suspension (Limberg et al. 1979). For most experiments, cells were grown in B5C – i.e., Gamborg's B5 medium (Gamborg et al. 1968), supplemented with casamino acids (ICN Pharmaceuticals Inc.). Wherever this medium has been altered, the change is noted in the text.

Preparation of partial haploid cell lines by CIPC treatment

In a previous paper we described the preparation of partial haploids from the heterozygous cell line as well as some properties of these partial haploids (Roth et al. 1982). The same procedure was used to prepare partial haploid colonies of Sln, Sln-asn-, and Sln-asn-R with CIPC: Suspension cultures (25 mls) of Sln, Sln-asn-, and Sln-asn-R were diluted to 2×10^5 cells/ml in B5C and incubated at 7 °C on a gyratory shaker. After 8 h, CIPC (Sigma Chemical Co.) dissolved in DMSO (0.1%) was added to the cultures. The cultures then were incubated at 7 °C in CIPC for 48 h on a gyratory shaker. The cells then were washed three times with B5C medium and surviving cells were plated for colonies on B5C "feeder plates" (Weber and Lark 1979). For each dose of CIPC, five plates were used. (The results in Table 4 were obtained using three different CIPC concentrations, 10^{-4}, 10^{-5} and 10^{-6} M, all of which gave identical results. These results were pooled to give the values in Table 4.) After 4 weeks colonies were picked. Each colony was tested on four different "feeder plates" – i.e., B5, B5C, B5 plus asparagine (20 µg/ml), and B5 in which allantoin was the sole source of nitrogen (see 'Analysis of growth phenotypes'). Cell lines derived from these colonies were stored under liquid nitrogen.

Analysis of growth phenotypes

Suspension cells of the seven lines (PI, M, F_1, 2, 5, 8 and 12) were grown in 25 ml batches in 125 ml Delong flasks. Cell growth was determined as packed cell volume (PCV). Cells were diluted to maintain a density of 2×10^5 cells/ml.

Cold sensitivity. Cells growing at 33 °C in suspension were transferred to 22 °C. Growth at 33 °C and 22 °C was determined as packed cell volume.

Growth in allantoin. Cells were grown at 33 °C in allantoin-B5 medium (B5 medium minus KNO_3, and $(NH_4)_2SO_4$, to which was added 25 mM KCl, 3.19 mM $MgSO_4 \cdot 7\,H_2O$, and 30 mM allantoin (Sigma Chemical Co.)). Control cells were grown in this modified B5 medium minus allantoin.

Growth in auxin. Cells growing in B5C medium (which contains 1.0 mg/L 2,4 D) were transferred to B5 medium with 0.5, 1.0, 2.0, 6.0, 12.0, 24.0, and 48.0 mg/L 2,4 D.

Cytosine arabinoside (AraC) toxicity. Cells growing at 33 °C were transferred to 22 °C. After 2 days 30 µg/ml of AraC (Sigma Chemical Co.) was added and the cells were grown at 22 °C for an additional 6 days. The cells were then washed three times and resuspended in B5C lacking AraC at 2×10^5 cells/ml. The cells were then incubated at 33 °C and growth measured.

Table 1. Cell lines used

Cell line	Comments	Reference
'PI-290136' (PI)	2n = 40; derived from root tissue	(Delannay and Palmer 1982)
'Minsoy' (M)	2n = 40; derived from root tissue	(Delannay and Palmer 1982; Roth et al. 1982)
F_1	2n = 40; derived from root tissue of a heterozygous plant (PI 290136 × Minsoy)	(Delanay and Palmer 1982; Roth et al. 1982)
No. 2	"2n" = 25; derived from F_1 (CIPC)	(Roth et al. 1982)
No. 5	"2n" = 26; derived from F_1 (CIPC)	(Roth et al. 1982)
No. 8	"2n" = 23; derived from F_1 (CIPC)	(Roth et al. 1982)
No. 12	"2n" = 29; derived from F_1 (CIPC)	(Roth et al. 1982)
Sln	Originally haploid, now heteroploid	(Weber and Lark 1980)
Sln-asn-	Derived from haploid Sln; requires asparagine or glutamine for growth	(Roth and Lark 1982)
Sln-asn-R	UV induced revertant of Sln-asn-	(Roth and Lark 1982)

5-Fluorodeoxyuridine (FUdR) toxicity. Cells growing at 33 °C were transferred to 22 °C. After 2 days 1 μg/ml of FUdR was added and the cells were grown at 22 °C for an additional 6 days. The cells were then washed three times and resuspended in B5C lacking FUdR at 2×10^5 cells/ml. The cells were then incubated at 33 °C and growth measured.

Ethanol toxicity. Suspension cells were grown at 33 °C in B5C with 0, 0.1%, and 1% ethanol.

Growth of Sln, Sln-asn-, and Sln-asn-R in B5 and in allantoin-B5 media. Suspension cultures of Sln, Sln-asn-, and Sln-asn-R were transferred from B5C to B5 and to allantoin-B5 media. Packed cell columes were measured every 2 days.

Isozymes

Sample preparation. Five mls of a 2 day old suspension culture (8×10^5 cells/ml) were filtered on Whatman paper filters and washed three times with 1 M Tris HCl pH 6.8. The cells were scraped off of the filter paper into a microfuge tube. Four drops of extraction buffer (0.1 M Tris HCl pH 6.8, 0.1% β-mercaptoethanol, 17% glycerol) were added. The sample was frozen on dry ice for 20 min and then sonicated three times, 20 s each time. The sample was then centrifuged 5 min in a microfuge and the pellet was discarded. 12.5% glycerol and 0.01% bromophenol blue were added to the supernatant. Five to ten μl of supernatant was loaded in each well of a polyacrylamide gel.

Polyacrylamide gel preparation. Samples were analyzed by polyacrylamide gel electrophoresis using a slab gel (14 × 12 × 0.1 cm) and the Tris-glycine buffer system of Laemmli (Laemmli and Favre 1973). Sodium dodecyl sulfate was omitted, since these were non-denaturing gels. Gels were run at 7 °C for 1 h at 50 volts (stacking gel) and then for 4 h at 150 volts. They were stained for the appropriate enzyme.

Acid phosphatase (AP). A 12.5% acrylamide gel was used. The gel was stained for 1 h at room temperature using the procedure of Efron (1970).

α-Naphthyl esterase (αNE). A 12.5% acrylamide gel was used. The gel was stained for 1 h at room temperature using the procedure of Shaw and Prasad (1970) except only α-naphthyl acetate was used as substrate.

Leucine amino peptidase (LAP). A 12.5% acrylamide gel was used. The gel was stained for 1 h at room temperature using the procedure of Beckman et al. (1964).

Malate dehydrogenase (MDH). A 7.5% acrylamide gel was used. The gel was stained for 1 h at room temperature using the procedure of Thorne et al. (1963).

Malic enzyme (ME). A 7.5% acrylamide gel was used. The gel was stained for 1 h at room temperature using the procedure of Goodman et al. (1980).

Peroxidase (Per). A 12.5% acrylamide gel was used. The gel was stained for 1 h at room temperature using the procedure of Shaw and Prasad (1970).

Results

Expression of parental growth phenotypes by partial haploid cell lines

In a previous publication, we described the isolation of partial haploid cell lines following treatment of a heterozygous cell line with the herbicide CIPC (Roth et al. 1982). Four partial haploid cell lines were established. These lines, as well as the heterozygote cell line from which they were prepared have been studied in detail. In addition, cell lines were prepared from the cultivars, 'PI-290136' and 'Minsoy', the parental lines from which the F_1 heterozygote was derived. All seven of these cell lines (two parental lines; one heterozygote line; four partial haploid lines) were frozen and preserved in liquid nitrogen.

Table 2 summarizes several parental growth phenotypes which are expressed in the four partial haploid cell lines. (These phenotypes were observed in connection with experiments to improve selection of mutants and chosen for further study when we found that the phenotypes of the two parental cell lines differed from each other or from the heterozygote) (Roth et al. 1982). Chromosome numbers of the cell lines, also listed, had been determined previously (Roth et al. 1982). It can be seen that the two parental cell lines, 'Minsoy' and 'PI-290136' differ in a number of properties: the ability to grow at 22 °C, in low auxin concentrations, or after treatment with FUdR. In some characteristics, expression of fluorescence, ability to grow on allantoin as a sole nitrogen source, or sensitivity to AraC, neither

Table 2. Expression of parental phenotypes in heterozygote and partial haploid cells. Chromosome numbers and fluorescence were determined by Roth et al. 1982. Growth conditions are described in 'Materials and methods'. Growth or fluorescence is indicated by (+); lack of growth or fluorescence by (–). CS indicates cultures which were cold sensitive, i.e., died at 22 °C

Cell line	F_1	M	PI	2	5	8	12
Chrom. no.	40	40	40	25	26	23	29
Fluorescence	+	–	–	+	–	+	+
Growth:							
at 22 °C	+	+	cs	+	+	cs	cs
in Allantoin	+	–	–	–	+	+	+
in Auxin							
1 mg/L	+	–	+	+	+	+	–
6 mg/L	+	+	+	+	+	+	+
24 mg/L	+	–	–	+	–	+	–
after AraC (at 22 °C) 30 mg/L	–	+	+	+	–	+	+
after FUdR (at 22 °C) 1 mg/L	+	–	+	+	+	–	–
in 0.1% Ethanol	+	–	–	+	+	+	+

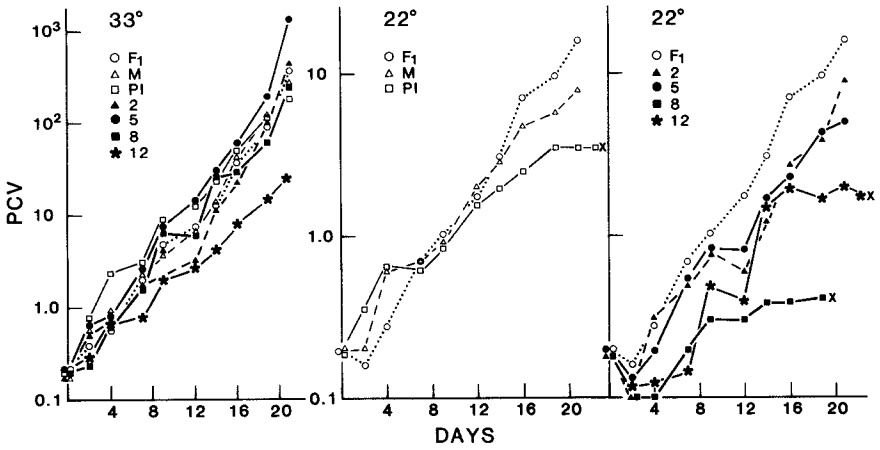

Fig. 1. Growth of different cell lines at 22 °C. Cells growing in suspension culture at 33 °C, were transferred to medium at 22 °C. Growth was followed as packed cell volume (*PCV*). At intervals, the suspension cultures were diluted to maintain a cell density of about 10^5 cells/ml. Cell lines are indicated in accord with the nomenclature used in Table 1. The three cell lines which failed to grow (*PI, 8* and *12*) had died when the growth curves were terminated (X)

parent possesses the phenotype which is expressed in the heterozygote. In the case of fluorescence, this is known to be the result of the expression of two independent, dominant, unlinked characters (Delannay and Palmer 1982). Each parent is homozygous recessive for one of these and homozygous dominant for the other. The F_1 cell line is heterozygous at the two loci.

Superficially, the partial haploid lines appear to possess phenotypes characteristic of either one of the parent cell lines, or of the heterozygote line from which they were derived. The phenotypes summarized in Table 2 have the interesting characteristic that none of them are distributed in the same manner between the different cell lines. Thus, if differences derive from loss of particular linkage groups, one must conclude that all of these characteristics are determined by genes in separate linkage groups. Closer examination of the partial haploids suggests that they often differ from either the parental or heterozygous cell line in the severity of the phenotype expressed.

A detailed examination of the phenotypes follows:

Cold sensitivity. Figure 1 presents the ability of the seven lines to maintain growth at 33 °C or 22 °C. Whereas the heterozygote and 'Minsoy' grow well at both 33 °C and 22 °C, 'PI-290136' eventually dies. Two of the partial haploids (Nos. 2 and 5) grow at 22 °C. Number 12 slowly dies at this temperature expressing a phenotype identical to 'PI-290136'. However, number 8 is much more severely affected by cold and dies rapidly. This suggests a second genotype, present in the heterozygote, which may be masked or suppressed by the presence of some other gene. When this second gene is removed as a result of chromosome loss the severe, cold sensitive phenotype is revealed. The data indicate that at least two genes, present on different chromosomes, may control growth at 22 °C.

Growth on allantoin. When cell lines are deprived of a nitrogen source they begin to die after a few days. If allantoin is supplied as a sole source of nitrogen (Fig. 2) the different cell lines respond in different ways. The heterozygote grows well, whereas both parents even-

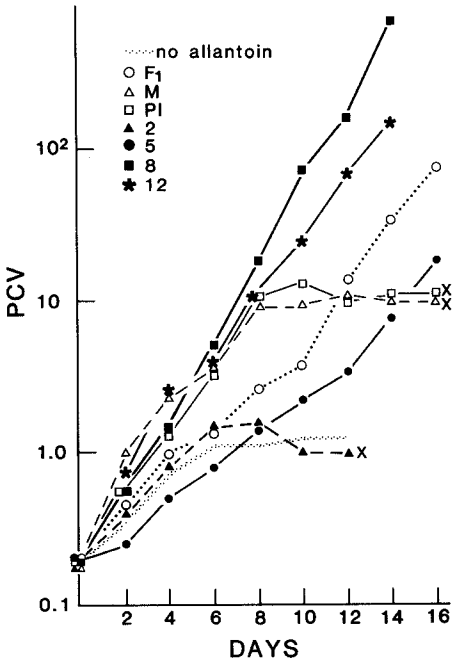

Fig. 2. Growth of different cell lines when allantoin is the sole source of nitrogen. Cells growing in suspension culture, were transferred to B5 medium containing allantoin as the only source of nitrogen. Growth was followed as packed cell volume (*PCV*). At intervals, the suspension cultures were diluted to maintain a cell density of about 10^5 cells/ml. The cell lines are indicated in accordance with the nomenclature used in Table 1. The residual growth in the absence of added nitrogen (no allantoin (·····)) was the same for all cell lines. Cell lines (*M, PI, 2*) which had died when the growth curves were terminated are indicated: (X)

tually slow their growth and die. The partial haploid lines respond differently. Lines 5, 8 and 12 grow well like the heterozygote from which they were derived. Line 2 is unable to utilize allantoin and dies as rapidly as if no nitrogen were present in the medium. It is much more severely affected than either of the lines derived from the parental cultivars. Explanations of this difference in severity of phenotype will be discussed below.

Auxin requirement. As with other tissue cultures, all seven cell lines require auxin to grow as suspension cultures (Fig. 3). Although 'Minsoy' cannot maintain growth unless the auxin concentration exceeds 1.0 mg/L, the heterozygote and 'PI-290136' can both grow on as little as 0.5 mg/L. The heterozygote tolerates high concentrations (24.0 mg/L) somewhat better than either parent (only 12.0 mg/L). The partial haploids demonstrate that the regulation of growth by auxin is genetically complex. Numbers 2 and 8 resemble the heterozygote from which they were derived, although number 8 is somewhat more resistant to high auxin concentration. Number 5 can grow on as little as 0.5 mg/L, but it is inhibited by 12.0 mg/L, showing an increased sensitivity to auxin when compared to either heterozygote or parent lines. Number 12 has an intermediate range of auxin concentrations in which it can grow – 6 to 24 mg/L. These results again suggest that several genes may be interacting to determine the auxin range optimal for cell growth.

Cytosine arabinoside (AraC) toxicity. AraC is an inhibitor of DNA replication, acting as a chain terminator when incorporated into DNA. Thus, once incorporated, its action is irreversible. In attempting to use this agent to select DNA replication mutants, we observed that none of the cell lines were inhibited at 33 °C. However, 30 mg/L was toxic to the heterozygote line when the cells were grown in the inhibitor at 22 °C (Fig. 4). In contrast, the parental lines were not inhibited. Inhibition of one of the partial haploid lines, number 5, also was observed. A simple explanation of this result is that resistance to AraC is the inability to take up the analogue and/or to incorporate it into DNA. Uptake and incorporation would be controlled by dominant alleles. Assuming at least two controlling loci, each parent would be homozygous recessive for one of these and therefore fail to incorporate AraC into

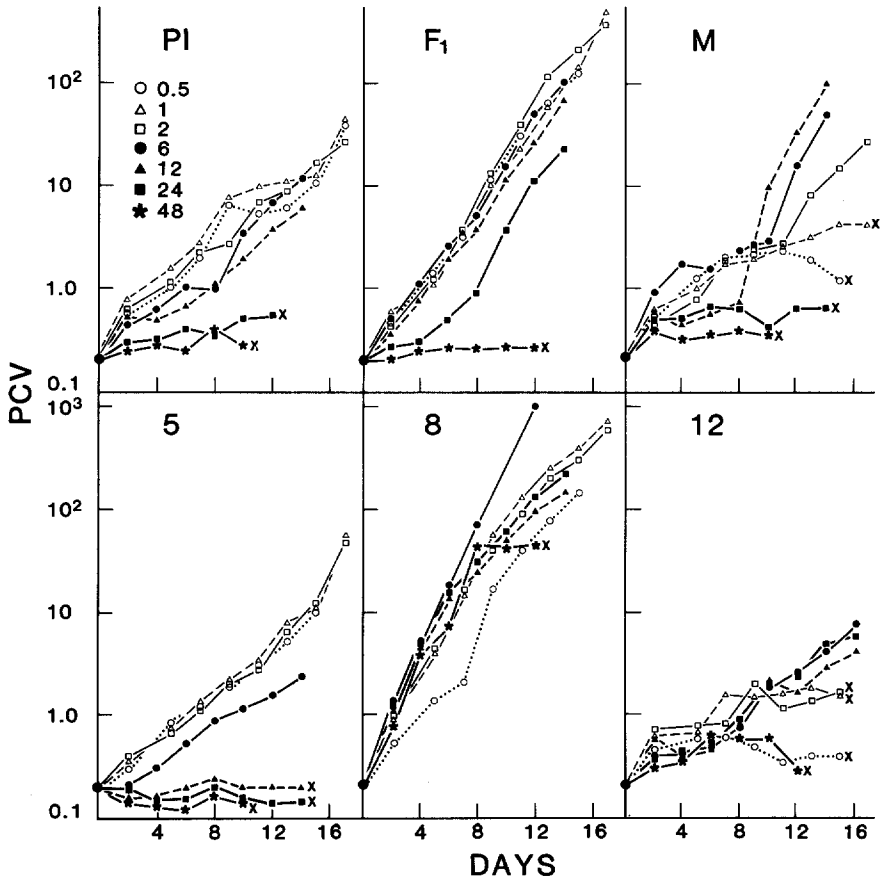

Fig. 3. Growth of different cell lines in different concentrations of auxin. Cells growing in suspension culture at 33 °C, were transferred to B5 medium without casamino acids, but containing different concentrations of the auxin, 2–4 D. Growth at 33 °C was followed as packed cell volume (*PCV*). At intervals, the suspension cultures were diluted to maintain a cell density of about 10^5 cells/ml. Each panel contains a cell line indicated in accordance with the nomenclature used in Table 1. The partial haploid line, number 2, is not shown, but gave results identical with the F_1 line from which it was derived. Auxin concentrations in mg/L are designated in the upper left panel (*PI*). Note that both the F_1 and *PI* lines can grow at low auxin concentrations (*open symbols*), whereas the Minsoy and partial haploid *12* lines cannot. Note the resistance of cell line *8* to high auxin concentrations (eventual death in 48 mg/L followed an initial period of rapid growth). This is in contrast to line *5* which dies in 5 mg/L auxin. Cell lines which had died when the growth curves were terminated are indicated: (X)

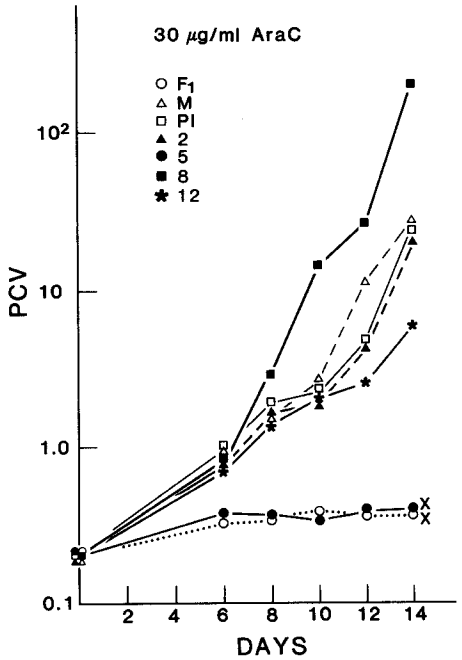

Fig. 4. Growth of different cell lines after treatment with *AraC* at 22 °C. Cells growing in suspension culture at 33 °C, were transferred to B5 medium at 22 °C. After two days 30 mg/L of *AraC* was added. Six days later, the cells were washed and suspended in 33 °C medium lacking *AraC*. Growth was followed at 33 °C as packed cell volume (*PCV*). At intervals, the suspension cultures were diluted to maintain a cell density of about 10^5 cells/ml. The cell lines are indicated in accordance with the nomenclature used in Table 1. The two cell lines which failed to grow after removal of *AraC* (F_1 and *5*) had died when the growth curves were terminated (X)

DNA. Because the heterozygote line would carry one dominant allele at each locus, it could incorporate AraC into DNA. Lines 2, 8, and 12 would be resistant if they lost either one of the dominant alleles.

Fluorodeoxyuridine (FUdR) toxicity. FUdR, an analogue of thymidine, prevents the synthesis of thymidine from deoxyuridine and thereby blocks DNA replication. The compound can produce permanent damage to cells if interruption of DNA replication is lethal, i.e., produces damage to the DNA which is irreparable; or if the analogue is accumulated by the cell and cannot be washed out, thus permanently blocking thymidine synthesis. In the course of experiments in which this compound was used to select for cold sensitive DNA replication mutations, we observed differential toxicity of this compound at 22 °C. The effects of treatment with this compound are shown in Fig. 5. Different cell lines were treated with 1 mg/L of FUdR for 6 days at 22 °C after which the inhibitor was removed and the cells grown at 33 °C. Although the inhibitor does not irreversibly block growth of the heterozygote or PI-290136 cell lines, it does stop growth of 'Minsoy'. Two of the partial haploids, 8 and 12, are also inactivated. The mechanisms of inactivation may be different since the two cell lines differ in the amount of residual growth observed (Fig. 5). This complexity is supported by other experiments now underway (data not shown) which indicate that the mechanism of inactivation is complex, since addition of the thymidine analogue BUdR can rescue 'Minsoy', and partial haploid line 12, from inactivation. Inactivation of number 8, cannot be reversed.

Ethanol toxicity. At a concentration of 0.1%, ethanol is toxic to both parental cell lines. In contrast, the heterozygote line can grow well despite the presence of 0.1% ethanol. All of the partial haploid lines also are resistant to 0.1% ethanol. (These data, (not shown), are reported as an example of a parental phenotype which is not revealed, as yet, by chromosome loss.)

Expression of isozyme polymorphism by partial haploid cell lines

The preceding results suggested that partial haploid cell lines should express isozyme polymorphisms exhibited

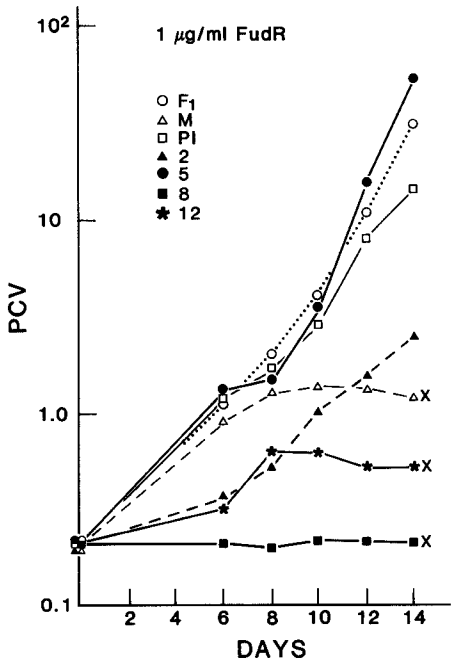

Fig. 5. Growth of different cell lines after treatment with *FUdR* at 22 °C. Cells growing in suspension culture at 33 °C, were transferred to B5 medium at 22 °C. After two days 1 mg/L *FUdR* was added. Six days later, the cells were washed and transferred to medium at 33 °C lacking *FUdR*. Growth at 33 °C was followed as packed cell volume (*PCV*). At intervals, the suspension cultures were diluted to maintain a cell density of about 10^5 cells/ml. Cell lines are indicated in accordance with the nomenclature used in Table 1. The three cell lines which failed to grow (*M*, *8* and *12*) had died when the growth curves were terminated (X)

by parental cell lines, but masked or altered by heterozygosis. To test this, we examined extracts of the seven cell lines for isozyme polymorphism. Extracts were prepared and electrophoresed through polyacrylamide as described in the materials and methods. These gels were stained to detect 17 different enzyme activities. Those showing polymorphism were: malate dehydrogenase (MDH), leucine amino peptidase (LAP), malic enzyme (ME), peroxidase (PER), α-naphthol esterase (αNE), and acid phosphatase (AP). Those in which no polymorphisms were apparent were: alcohol dehydrogenase, alkaline phosphatase, glucose 6-phosphate dehydrogenase, glutamate dehydrogenase, glutamine oxaloacetate transaminase, isocitrate dehydrogenase, phosphoglucomutase, phosphoglucose isomerase, shikimic acid dehydrogenase, sorbitol dehydrogenase, and xanthine dehydrogenase.

Table 3 summarizes the results of these experiments. Three types of results were obtained: A) The heterozygote gave a pattern identical with one of the parents and at least one partial haploid resembled the other parent (MDH); B) the heterozygote gave a pattern identical with one parent and at least one partial haploid had a pattern resembling a modification of the pattern of one of the parents (LAP, ME); C) the heterozygote was different from either parent (but contained components from each), one or more of the partial haploids represented either a parental type or the heterozygote, and at least one partial haploid was different from the combinations found in either the parental or heterozygote cell lines (AP, αNE, PER).

Table 3. Expression of isozymes in heterozygote, parental, and partial haploid cells. Isozyme assays are described in 'Materials and methods'. Mobility patterns for AP, LAP, and MDH are compared in Fig. 6. (*) indicates that the F_1 pattern differed from both M and PI. D indicates that some of the enzyme mobilities differed from F_1, M, or PI. AP, acid phosphatase; αNE, α naphthyl esterase; LAP, leucine amino peptidase; MDH, malate dehydrogenase; ME, malic enzyme; PER, peroxidase

Cells line	F_1	M	PI	2	5	8	12
Isozymes							
AP	F_1(*)	M	PI	F_1	D	F_1	D
NE	F_1(*)	M	PI	F_1	D	M	F_1
LAP	F_1 = PI	M	PI	F_1	D + M	F_1	F_1
MDH	F_1 = M	M	PI	F_1	F_1	PI	F_1
ME	F_1 = PI	M	PI	D	F_1	F_1	F_1
PER	F_1(*)	M	PI	D	F_1(*)	M	D

Figure 6 presents examples of each of these types of patterns.

The patterns observed in the heterozygote cell line demonstrate different types of interaction when two genomes are present in the same cell: A) The expression of more than one form of the enzyme (as with peroxidase and acid phosphatase; B) The suppression of one or more forms of the enzyme (as with leucine amino peptidase and peroxidase). This mixture of expression and suppression gives rise to complex patterns that will vary not only with the presence or absence of structural genes, but also with the presence

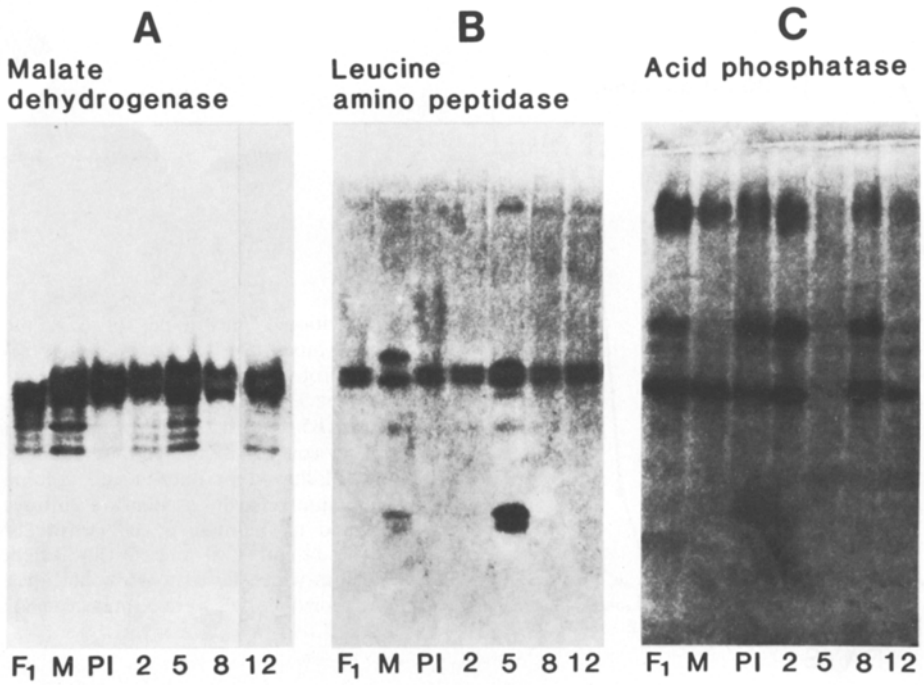

Fig. 6 A–C. Isozyme patterns for the seven cell lines (F_1, *M, PI, 2, 5, 8* and *12*): **A** malate dehydrogenase (MDH); **B** leucine amino peptidase (LAP); **C** acid phosphatase (AP). Extracts (Materials and methods) were run on non-denaturing polyacrylamide gels. Cell lines are indicated (as in Table 1). **A** (MDH): The patterns of F_1, *M, 2, 5,* and *12* are the same. *PI* and *8* resemble each other. **B** (LAP): The patterns of F_1, *PI, 2, 8,* and *12* are the same. The pattern of the partial haploid 5 showed a modification. **C** (AP): The patterns of F_1, *2* and *8* were the same, but different from either parent (*PI* or *M*). However, they contained some bands from each parent. Partial haploid *5* and *12* showed different patterns from the other cell lines

or absence of suppressors. Hence, it is possible to obtain isozyme patterns in partial haploids which differ from either parental or heterozygote cell lines.

The use of chromosome loss of analyze mutational change

Frequently, mutations which result from intense mutagenic treatment of cells display multiple phenotypes. The question then arises whether these phenotypes result from a single mutation or from multiple changes in the genome. We have used CIPC induced chromosome loss to answer this question.

In a previous publication, we described a UV induced mutation to auxotrophy resulting in a growth requirement for asparagine. Later studies determined that this auxotroph could also utilize allantoin as a sole nitrogen source, a phenotypic characteristic not found in the parent strain from which the auxotroph was derived. Using UV mutagenesis of this auxotroph, we derived another cell line which no longer required asparagine for growth, but could still utilize allantoin as a sole source of nitrogen. The phenotypes of the mutant, the revertant and the parent cell lines are shown in Fig. 7.

Two explanations of these data were: A) The requirement for asparagine and the ability to utilize allantoin were the result of two separate mutations; B) These phenotypes were the result of a single pleiotropic mutation and in the revertant a second mutation suppressed the requirement for asparagine. To distinguish between these alternatives we induced chromosome loss in the three strains by subjecting them to CIPC treatment. Surviving colonies were isolated and characterised for: growth on allantoin, growth on B5 (ammonia and nitrate) as a nitrogen source, growth on B5 plus asparagine, and growth on B5 plus casamino acids. The results are shown in Table 4a–c.

Table 4. Phenotypes and frequencies of colonies resulting from CIPC treatment of parental[1], Sln-asn-[2], and Sln-asn-R[3] cell lines. Treatment of the three cell lines with CIPC is described in Materials and methods. Growth (+) or lack of growth (–) is indicated. All colonies were originally selected for growth on casamino acid supplement. asn = asparagine.

Cell line and treatment	Phenotype = growth on:			No. of colonies	%
	B5	B5 + asn	Allantoin		
a) Parent[1]:					
– CIPC	+	+	–	81	100
+ CIPC	+	+	–	220	100
b) Mutant[2]:					
– CIPC	–	+	+	100	100
+ CIPC	–	+	+	200	79
	–	+	–	25	10
	–	–	+	16	6
	–	–	–	12	5
c) Revertant[3]:					
– CIPC	+	+	+	90	100
+ CIPC	+	+	+	263	90
	+	+	–	13	4.5
	–	+	+	10	3.5
	+	–	+	2	0.6
	–	–	–	2	0.6
	+	–	–	1	0.3
	–	–	+	1	0.3

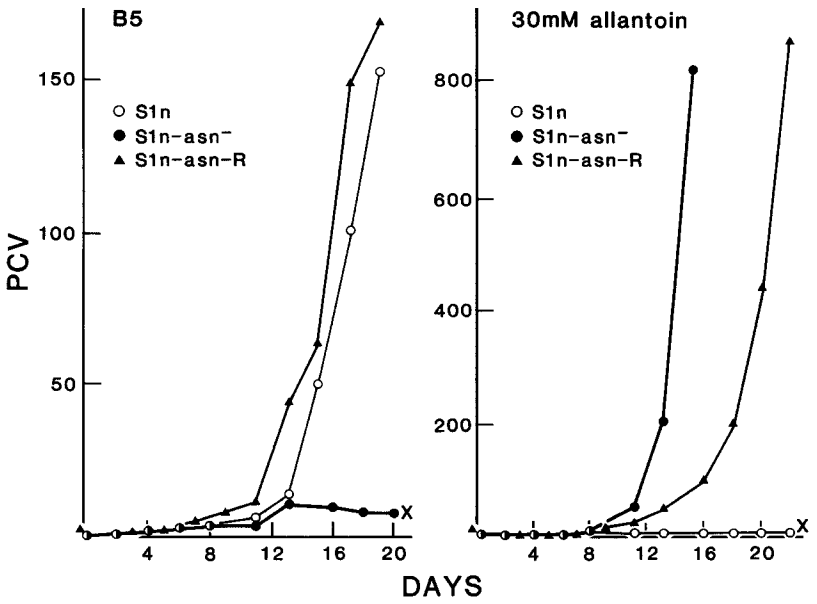

Fig. 7. Nutritional phenotypes of *Sln*, the asparagine auxotroph *Sln-asn* and a revertant to prototrophy, *Sln-asn-R*. The three cell lines (see Table 1) were transferred to B5 medium or B5 medium containing allantoin as the sole source of nitrogen. Growth at 33 °C was followed as packed cell volume (*PCV*). At intervals, the suspension cultures were diluted to maintain a cell density of about 10^5 cells/ml. Cell lines are indicated. The cell lines which failed to grow had died when the growth curves were terminated (X)

CIPC failed to alter the phenotype of the parental line (Table 4a). Moreover, none of the CIPC treated auxotrophic clones had become prototrophs, (Table 4b) a result which is expected if the primary effect of CIPC is to induce chromosome loss. However, 10% of the colonies surviving CIPC treatment had lost the ability to grow on allantoin, demonstrating that this genotype was separable from asparagine auxotrophy. Presumably the two phenotypes are determined by genes which are unlinked.

Additional information about asparagine utilization was obtained from this part of the experiment (Table 4b). The occurrence of colonies able to grow on casamino acids, but no longer able grow on the asparagine supplement, suggests that asparagine auxotrophy is a complex requirement involving other genes. If these are lost, asparagine can no longer be utilized.

Further information was obtained from the CIPC treated revertants (Table 4c). The ability to utilize allantoin was lost, while prototrophy was not. Conversely, asparagine auxotrophy was obtained without loss of the ability to utilize allantoin. This reinforces the conclusion that the two properties, asparagine dependence and allantoin utilization, are controlled by unlinked genes. Moreover, the occurrence of a few clones, prototrophs or auxotrophs, which are unable to grow if asparagine is added to the medium suggests additional complexities involving the metabolism of this compound i.e., in the absence of certain genes asparagine or its metabolites become toxic.

Discussion

Spontaneous chromosome loss from interspecific hybrids has long been used as a genetic tool in mammalian somatic cell genetics (Kato and Yosida 1971; Larizza et al. 1974, 1979; Puck 1981). Induced chromosome loss during cell growth in plant tissue culture has not been used as a genetic tool. An advantage of CIPC treatment is that it is rapid and therefore cells which differ in their chromosome loss can be readily cloned under non-selective conditions immediately after loss has occurred. This avoids prolonged growth of mixed populations in which particular cell types may have a selective advantage.

In the results presented above, we have demonstrated the power of this technique as a genetic tool. We have used chromosome loss to analyze a heterozygote containing two, widely disparate, parental genomes (Roth et al. 1982). We also have analyzed the genetic complexity of a UV induced, auxotrophic, mutant (Roth and Lark 1982).

Studies involving partial haploids derived from the heterozygote cell line

Soybean presents a rich germplasm for use in genetic analysis. This is largely due to the obligate self fertilizing nature of the plant, which isolates different cultivars and rapidly selects new homozygous variants. At any time, a particular cultivar will have tended to optimize its selection of alleles based on its past selective history. Combining these alleles in a heterozygote and then preparing partial haploids presents an opportunity to examine how each allele functions within a different genetic environment. For this purpose, we have chosen two parental cultivars with quite different evolutionary histories (Delannay and Palmer 1982).

All of the growth parameters examined can be explained in terms of dominant and recessive alleles in which the recessive trait, masked in the heterozygote, is revealed in one or more of the partial haploids. The effect of the genetic background is readily seen in the severity of the expressed recessive phenotype. A striking result is that each of the 14 growth or isozyme phenotypes studied is distributed in a different manner between the parents, the heterozygote and the four partial haploid cell lines. This strongly suggests that chromosomes are not being lost selectively and that the chromosome complement which is retained represents a random selection from the initial group of forty.

Three of the phenotypes studied (fluorescence, resistance to AraC and the isozyme patterns observed for MDH) behave as would be expected of simple dominant-recessive allele combinations in which the dominant allele is removed by chromosome loss. All of the other phenotypes are more complex suggesting interactions with genes on other chromosomes, which when lost change the phenotype. We have already discussed the cold sensitive phenotype which becomes much more severe in one partial haploid than in the cold sensitive parent. Similarly, although the parental cell lines use allantoin inefficiently as a source of nitrogen, the heterozygote uses it as efficiently as nitrate. However, one partial haploid cell line could not use this nitrogen source at all. This line behaves as if it had lost some other gene which, when present in the parent, allowed a partial use of this nitrogen source. Finally, the responses to auxin and to FUdR-BUdR treatment suggest that partial haploids can be different from either the parent or the heterozygote cell lines.

This also is apparent when one examines the patterns of five of the isozymes described in Table 3 (AP, αNE, LAP, PER and ME), in which mobilities found in extracts from partial haploid cell lines differ from either parent or heterozygote cell lines. One explanation of these results is that the phenotypes observed reflect the interaction between several genes. In the case of the

isozymes, these genes might result in a modification of the size or charge of the enzyme resulting in altered rates of migration through the poly-acrylamide gel. When the modifying gene is lost, the rate of migration would change to produce a new gel pattern. Similarly, different cultivars might be expected to have different modifiers and, therefore, the heterozygote cell line could have bands with mobilities not easily related to the mobilities found in the parental types. Thus, the presence of such modifiers could explain the result obtained with acid phosphatase where the parents, the heterozygote and at least one of the partial haploid cell lines have enzyme patterns which are each different from one another.

Just as modifier genes can change the mobility of particular enzymes, suppressor genes can alter other phenotypes, such as cold sensitivity, response to auxin, allantoin utilization, or rescue from cell death after treatment with FUdR. Such suppressors are well known in bacteria.

A possible consequence of this type of gene interaction would be the absence of some types of segregant offspring when heterozygous plants are inbred to produce new homozygous offspring. This would occur whenever particular gene interactions (or lack thereof) proved lethal. Recently, such segregation distortion has been observed in the progeny of crosses between 'Minsoy' and 'PI-290136' (R. Palmer, personal communication).

Studies of the asn- mutant and its revertant

Second site suppressors are often encountered in complex metabolic systems. Different metabolic pathways can interact and the product from one may be utilized in another, sometimes producing deleterious results. We have seen that two phenotypes resulting from mutagenesis can be resolved by using chromosome loss to delete one of the phenotypes, allantoin utilization. A second result was that asparagine utilization by the prototrophic revertant of the asn- mutant can lead to death (Table 4c) in some of the cell lines obtained after CIPC treatment of this cell line. One explanation of this result is that asparagine utilization may rely on a metabolic pathway in which asparagine is converted to some intermediate which is toxic if allowed to accumulate. If steps in this pathway are missing as a result of chromosome loss, the toxic intermediate accumulates leading to cell death. Thus, asparagine would be toxic to this prototrophic cell line. If this explanation were correct, the function which is lost could be viewed as a suppressor of asparagine toxicity. This hypothesis can be tested by analyzing the cell lines described in Table 4c for the accumulation of asparagine metabolites.

The experiments described in this paper suggest that CIPC treatment may prove to be a valuable tool for examining metabolic or physiological change in the context of different genetic backgrounds.

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