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A transient duplication of the acetolactate synthase gene in a cell culture of *Datura innoxia*

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Summary. A 2.0 kb fragment of the yeast *ILV2* gene, which codes for the target enzyme acetolactate synthase (ALS) of the herbicide chlorsulfuron, was shown to hybridize to the nuclear DNA of a haploid cell culture of *Datura innoxia* P. Mill. Nuclear DNA of a chlorsulfuron resistant line of *D. innoxia,* CSR6, gave a prominent 2.65 kb band when cleaved by either *EcoRI* or *Hind*III. The 2.65 kb band has been shown to hybridize with the yeast *ILV2* probe. A herbicide resistant line descended from CSR6 by continuous culture resulted in the loss of the 2.65 kb restriction fragment. These observations suggest that CSR6 resulted from a large tandem duplication of the ALS gene and that a point mutation for herbicide resistance in an ALS gene repeat unit of the duplication was selected during subsequent growth of the resistant line.

Key words: Gene amplification – *Datura* – Sulfonylurea resistance – Plant cell culture

Introduction

It has been established that mutations conferring resistance to antimetabolites can arise through different mechanisms, among which is a mutation that allows the generation of large amounts of the target protein of an antimetabolite. In many cases, gene amplification is involved in this process, such as methotrexate resistance in cultured animal cells (Alt et al. 1978). Cell lines showing gene amplification are usually selected by growth in increasing concentration of the antimetabolite, and the resistant mutants are phenotypically stable or unstable in the absence of selection pressure, depending on the form of the duplicated gene sequence. For the stable mutants, the repeated DNA sequence can be found within the chromosome, the region of occurence referred to as a homogeneously staining region. Unstable gene ampifications often take the form of double-minute chromosomes which lack a centromere. Recently, Schimke et al. (1986) suggested that DNA overreplication-recombination in eukaryotic cells might produce the above repetitious structures.

The isolation of plant cell lines with resistance to antimetabolites has been reported; the resistance in some of these lines resulted from an enhanced production of the target proteins (Barg et al. 1984; Nafziger et al. 1984; Donn et al. 1984). Plant cell lines tolerant to the herbicides L-phosphinothricin (Donn et al. 1984) and glyphosate (Shah et al. 1986) were shown to result from low copy number gene duplication.

Chlorsulfuron (CS) and sulfometuron methyl (SM) are members of the sulfonylurea family of herbicides. These herbicides inhibit the biosynthesis of isoleucine and valine by binding to and inactivating the enzyme acetolactate synthase (ALS, EC. 4.1.3.18). Mutants resistant to sulfonylurea herbicides have been isolated from the bacterium *Salmonella typhimurium* (La Rossa and Schloss 1984), the yeast *Saccharomyces cerevisiae* (Falco and Dumas 1985), and the higher plant *Nicotiana tabacum* (Chaleff and Mauvais 1984; Chaleff and Ray 1984). Resistance in these different organisms was inherited as single dominant or semidominant mutations that produced ALS which showed in vitro resistance to the sulfonylurea herbicides.

In this report, we document a transient high copy number tandem duplication of the ALS gene in a cell line of *Datura innoxia* P. Mill. that resulted in resistance to CS and SM.

Materials and methods

Cell culture and maintenance

A wild type, predominantly haploid (ca. 90%) cell culture of *Datura innoxia* (P×4) was grown in liquid B5 medium (Gamborg et al. 1968) plus 0.5 g · l^{-1} $(NH_4)_2SO_4$ and 1 mg · l^{-1} 2,4-D (designated B5A). Cells from 3-day-old suspension cultures were filtered through a 500 µm nylon screen, collected and mutagenized with EMS (ethyl methane sulfonate) for 2.5 h. Following mutagenization, the cells were collected, washed 5 times with glass distilled water and grown for 10 days in B5A medium for growth recovery. The cells were then plated at a density of ca. 8.2×10^6 cells per plate, on a medium (25 ml) containing CS at a concentration of 5×10^{-8} M, and the cultures maintained in the dark at 28 °C. Surviving clones were picked up after 4 weeks and maintained on and off pressure.

For the prupose of nuclear DNA isolation, soybean (*Glycine max* L.) line SB-1 (originally provided by Dr. F. Constabel, Plant Biotechnology Institute, Saskatoon, Sask., Canada) and *Brassica napus* (provided by Dr. W.A. Keller, Agriculture Canada, Ottawa, Canada) cell suspension cultures were subcultured weekly and biweekly, respectively.

Isolation of plant nuclear DNA

Isolated nuclei were used to prepare DNA samples from plant cells (Bendich et al. 1980). Isolation of nuclei followed the method of Saxena et al. (1985) with modifications in the composition of the protoplast and nuclei isolation media. Briefly, 3-day-old cells from suspension culture were collected by passing through one layer of Miracloth. Cells were incubated in an enzyme solution (2 g cells per 10 ml enzyme) containing 2% Cellulase Onozuka "R-10", 2% Driselase, 0.5% Macerozyme "R-10", 0.5 M mannitol, 3 mM MES (pH 5.2) and 5 mM $CaCl_2$. Incubation varied from 1.5 to 3.0 h, until most of the cell wall was removed. The resulting protoplast suspensions were passed through a 85 µm nylon screen, sedimented (100 g for 4 min) and purified by density floatation over a 0.5 M sucrose solution. The plasmalemma was removed by disrupting the protoplasts in 10 volumes of nuclear isolation buffer (0.2 M sucrose, 0.02% Triton X-100 and 10 mM MES, pH 5.0) in a glass homogenizer and the nuclei were purified by filtration through a 15 µm filter. After the addition of trace amounts of $CaCl_2$ to stabilize the nuclear membrane, the nuclei were collected by centrifugation at 100 g for 5 min and were incubated in 1 ml of the lysis buffer (1% Sarkosyl, 10 mM EDTA and 10 mM Tris-HCl, pH 8.0) in an ice-bath for 5 min with gentle shaking. Following the addition of 5 ml $CsCl_2$ solution (10 g $CsCl_2$ dissolved in 10 ml TE buffer containing 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA), the mixture was centrifuged at 10,000 g for 20 min and the supernatant used for $CsCl_2$-EtdBr equilibrium gradient centrifugation. Large molecular weight nuclear DNA was extracted from the gradient and stored at −20 °C for further analysis.

Restriction digestion and Southern hybridization

Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. The 2.0 kb fragment of an *Eco*RI digestion of plasmid pCP2-4 (Falco and Dumas 1985) which contains the majority of the yeast *ILV2* gene coding sequence, was recloned into the *Eco*RI site of the plasmid pUC9 (Vieira and Messing 1982). One of the resulting plasmids, pWX14, was digested by *Eco*RI and the 2.0 kb band was isolated from a low-melting-temperature agarose gel (Weislander 1979). This DNA fragment was labelled with deoxycytidine 5′-(α-^{32}P) triphosphate (Amersham, 3,000 ci/mmol) by nick translation (Bethesda Research Lab). Southern transfer and hybridization was adapted from Maniatis et al. (1982). The filter was incubated with ^{32}P-labelled probe at 42 °C in 50% formamide for 20 h and washed in 1×SSC and 0.1% SDS for 3 h at 68 °C. The treated nitrocellulose filter was exposed to x-ray film (Kodak, X-Omat AR) with an intensifying screen at −70 °C. *Eco*RI digested pCP2-4 was always run along with other samples as a control in the Southern hybridization.

Results

Chlorsulfuron resistant mutants of Datura

The mutant CSR6 is one of more than 20 independently isolated cell cultures of *D. innoxia* having resistance to CS and SM. The resistance of cell lines was characterized by a growth rate study using the filter paper growth assay (Horsch et al. 1980) on a herbicide supplemented medium, showing that these isolated cultures had a variable degree of resistance. However, of these, CSR6 showed the maximal resistance in terms of growth rate in the presence of herbicide; the cells were able to grow in the presence of 5×10^{-8} M CS or SM. By contrast, the wild type cells were unable to grow at 3.16×10^{-10} M. Data listed in Table 1 document the variation in stability and herbicide resistance level among individual cell lines.

Plant nuclear DNA isolation

Nuclear DNA was isolated from three species, *D. innoxia, Brassica napus* and *Glycine max*. Under optimal conditions, about 0.1 mg of nuclear DNA per g fresh weight of cells was routinely obtained. The method should apply to cell cultures of most plant species, although DNA yield may vary depending upon the nature of the cells and protoplasts being used. In our experience, the preparation of protoplasts is the most critical step for the yield and purity of the nuclear DNA. Different combinations of appropriate cell wall digesting enzymes need to be tested to obtain high yield of protoplasts. Even with different cell lines of the same species (e.g. *D. innoxia*), the time for enzyme incubation varied considerably. For the purpose of DNA isolation, the pH value of enzyme solution was reduced to 5.2 in order to maximize the enzyme activity. Electron microscopic observations showed that nuclei prepared by this method were essentially free of mitochondria and plastids (Saxena et al. 1985), thereby eliminating cytoplasmic DNA contamination.

Datura nuclear genome

Nuclear DNA of herbicide-sensitive cell lines of *Brassica napus, Glycine max* and *Datura innoxia* were

Table 1. Herbicide resistant phenotypes of independent *D. innoxia* cell lines. Cell lines were isolated individually from plates containing CS or SM. After establishing cell suspension cultures in the absence of herbicide, 2 replicates each of 25 microcalli were transferred to different solid media and incubated at 28 °C for 14 days in the dark. The microcalli were then grouped by their size compared to those of the wild type growing in MS medium (Murashige and Skoog 1962) without SM: same as wild type (+ + +), < 1/3 wild type (+) or no growth (−). The percentage of calli in each category is presented

Cell line	Medium				
	MS + 10^{-8} M SM			MS	
	+ + +	+	−	+ + +	−
CSR2/472	0	20	80	100	0
SMR8/473	0	20	80	100	0
SMR1/452	44	40	16	94	6
CSR2	100	0	0	100	0
CSR3	44	36	20	100	0
CSR6	100	0	0	100	0
P×4	0	0	100	100	0

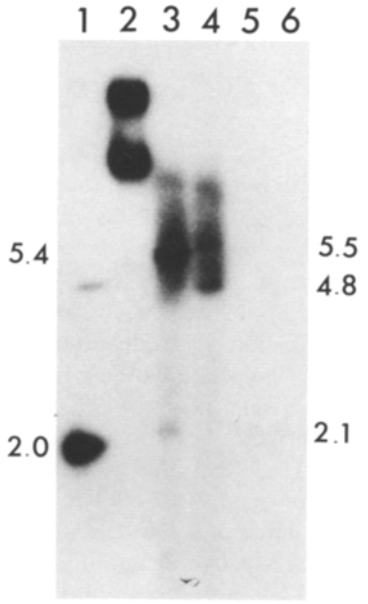

Fig. 1. Hybridization of the yeast *ILV2* probe with plant nuclear DNA. Plant nuclear DNA (10 μg) separated on an agarose gel was transferred to a nitrocellulose filter, hybridized with a ^{32}P-labelled yeast *ILV2* probe and visualized by autoradiography. *Lane 1* pCP2-4 DNA (5 ng) digested with *Eco*RI; *lane 2 Datura innoxia* P×4 DNA untreated; *lane 3 Eco*RI digested; *lane 4 Hind*III digested. Lanes 5 and 6 contain *Eco*RI digested *Brassica napus* and *Glycine max* DNA. The faint and diffuse label which appears in *lanes 3 and 4* is due to extensive overexposure to maximize the possibility of detecting autoradiographic bands in *lanes 5 and 6*. The shorter exposure time used in Fig. 3 (*lane 1*) shows the position of the hybridization bands after *Eco*RI digestion

evaluated for sequences capable of hybridizing to the yeast *ILV2* probe. Under high stringency conditions, both native and restriction enzyme digested nuclear DNA from the wild type P×4 strain of *D. innoxia* showed hybridization bands to the yeast *ILV2* probe (Fig. 1). Undigested DNA showed two diffuse high molecular weight areas. *Eco*RI digested DNA showed a prominent hybridization band at 5.4 kb and a minor band at 2.1 kb; *Hind*III digested DNA showed two prominent bands at 5.5 and 4.8 kb. The Southern hybridization results indicate that the yeast *ILV2* homologous sequence in the *D. innoxia* genome is present in single or low copy number. Neither the EtdBr stained gels nor the yeast *ILV2* probe hybridization exposure gave prominent bands in the 2 to 4 kb range of either digestion. In sharp contrast, *B. napus* and *G. max* DNA did not hybridize with the yeast *ILV2* sequence in the same experiment (Fig. 1, lanes 5 and 6). Low stringency hybridization (filters were washed in a 3×SSC solution at 60 °C) revealed that DNA from these two species shows homology to yeast *ILV2* at about 6 kb after *Eco*RI digestion (data not shown).

Chlorsulfuron resistant strain CSR6 DNA

Chlorsulfuron resistant lines were used to monitor nuclear DNA cleavage length polymorphism associated with the resistant phenotype. In the presence of herbicide, CSR6 was different from other CSR lines and wild type P×4 in that CSR6 microcalli were brownish with a smaller cell size. In addition, the cell wall was difficult to remove by the combined enzyme digestion and the yield of nuclear DNA from CSR6 was therefore extremely low. CSR6 nuclear DNA gave a prominent 2.65 kb fragment when cleaved by either *Eco*RI or *Hind*III. As seen in Fig. 2, EtdBr fluorescence of electrophoresed CSR6 DNA clearly revealed the presence of this band. The wild type P×4 did not show this band when 10 μg of DNA was loaded onto the agarose gel, nor did other CSR lines (data not shown). Since we did not observe any bands in undigested CSR6 DNA, this striking result can be most simply explained by assuming a large repeated tandem duplication of the ALS gene in CSR6.

The 2.65 kb band hybridization

CSR6 nuclear DNA was digested with *Eco*RI and *Hind*III. Restriction fragments were transferred to a nitrocellulose filter and incubated with the ^{32}P-labelled yeast *ILV2* probe. As seen in Fig. 3, the 2.65 kb DNA band produced by either *Eco*RI or *Hind*III digestion hybridized to the yeast *ILV2* probe. After prolonged exposure to X-ray film, two faint autoradiographic bands for each enzyme digestion (5.4 kb and 2.1 kb for

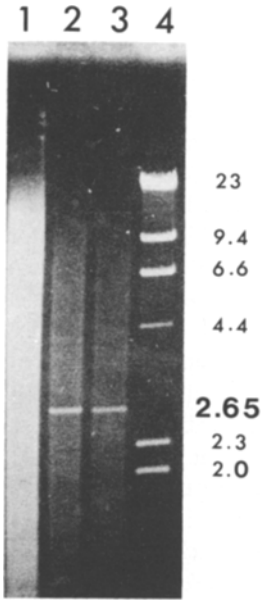

Fig. 2. Ethidium bromide staining of *Datura innoxia* P×4 and CSR6 DNA. Wild type P×4 DNA (10 μg) and chlorsulfuron resistant CSR6 DNA (0.5 μg) was run in an agarose gel and visualized by EtdBr fluorescence. *Lane 1 Eco*RI digested P×4 DNA; *lane 2 Eco*RI digested CSR6 DNA; *lane 3 Hind*III digested CSR6 DNA; *lane 4 lamda* DNA digested with *Hind*III to produce molecular markers (indicated in kilobase pairs). Lower concentrations of P×4 DNA (0.5 μg) digested with *Eco*RI or *Hind*III did not show the 2.65 kb fragments evident in that of digested CSR6 DNA

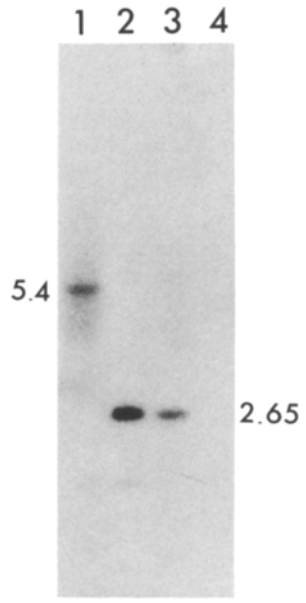

Fig. 3. Yeast *ILV2* hybridization to *Eco*RI and *Hind*III 2.65 kb fragments of CSR6 DNA. A yeast *ILV2* probe was hybridized to wild type P×4 and CSR6 DNA and subsequently visualized by autoradiography. The nitrocellulose filter used for Southern hybridization was prepared from the gel shown in Fig. 2. Longer exposure of the X-ray film revealed the positions of two minor bands each in *lane 2* (5.4 kb and 2.1 kb) and *lane 3* (4.8 kb and 5.5 kb) which were identical to those in Fig. 1, *lanes 3 and 4*, respectively

*Eco*RI, 5.5 kb and 4.8 kb for *Hind*III) appeared. If we take these bands as representing single copy border fragments, the relative comparison of hybridization intensity indicates that the 2.65 kb band contains over 100 copies of homologous DNA. The duplication is unlikely to be an unrelated sequence since it showed hybridization under conditions requiring a high degree of homology (see "Materials and methods"). In contrast, the wild type P×4 DNA control has no evidence of a band at 2.65 kb even after a prolonged exposure. Additionally, *Eco*RI cleaved *D. innoxia* genomic DNA of both P×4 and CSR6 has been hybridized to the plant ALS gene (Dr. B. Mazur, personal communication). After transfer of genomic DNA to "GeneScreen Plus" and incubation with a labeled internal coding sequence of the *Arabidopsis* ALS gene, the filter was rinsed under increasing stringent conditions. The 2.65 kb DNA band continued to hybridize to the plant ALS probe under conditions of very high stringency (0.05× SSPE, 68 °C for 3 days).

Transient ALS gene amplification in CSR6

A subculture of CSR6 grown in the absence of the herbicide for 2 months still maintained resistance to CS or SM. However, the microcalli morphology of this new strain and the appearance of cell suspension culture became similar to the wild-type cell line. Agarose gel electrophoresis and Southern hybridization with yeast *ILV2* showed that the 2.65 kb band was no longer present, indicating that this callus was a deamplification revertant of CSR6. The acetolactate synthase of the CSR6 subcultures showed a relatively low level of activity compared with the original CSR6 (in preparation). All these results suggest that the ALS gene amplification in CSR6 was transient.

It was not possible to determine the exact time required for the above changes. Gene deamplification and the resulting changes in cell morphology and ALS activity occurred within 2 months. We are currently inducing more ALS gene-amplified plant cell lines and are investigating the mechanism of gene deamplification.

Discussion

There are three lines of evidence supporting the belief that a plant DNA sequence homologous to the yeast *ILV2* probe encodes the plant ALS gene. First of all,

studies have indicated that the ALS gene is highly conserved in different species, including bacteria, cyanobacteria, green algae, yeast, and higher plants. Sequence comparisons of the yeast *ILV2* gene with *Escherichia coli* genes coding for ALS II and III isozymes shows large segments of homology (Falco et al. 1985). Using yeast *ILV2* gene as a probe, putative ALS genes have been cloned from *Anabaena, Chlamydomonas reinhardii, Arabidopsis thaliana* and *Nicotiana tabacum* (Mazur and Chui 1985). Our study also indicates that *Datura innoxia* nuclear DNA has a homologous sequence with the yeast *ILV2* gene. Secondly, ALS from different organisms is the common target protein of sulfonylurea herbicides (La Rossa and Schloss 1984; Falco and Dumas 1985; Chaleff and Mauvais 1984). Thirdly, our experiments demonstrate that the amplification of a yeast *ILV2* and plant ALS gene homologous DNA fragment in *Datura* (CSR6) resulted in an increased resistance to known inhibitors (CS and SM) of ALS activity.

Comparison of the yeast ALS with the large subunit of *E. coli* isozymes ALS II and ALS III showed three regions of highly conserved amino acid sequence (Falco et al. 1985). The Southern hybridization results obtained in this study indicate that plant ALS genes from different species may have different degrees of homology to yeast *ILV2*. For example, *D. innoxia* DNA showed higher homology to yeast *ILV2* than the DNA of *B. napus* and *G. max*. However, the evolutionary relationships of the ALS gene in plants remains to be determined.

Overproduction of the target protein resulting from gene amplification has been shown to cause drug resistance in various organisms. Two herbicide resistant plant cell lines have been shown to result from genomic DNA amplification. In the case of L-phosphinothricin resistance, there is an 8.6 kb DNA repeat which was stably inherited over 1 year (Donn et al. 1984), whereas the genomic DNA from glyphosate tolerant *Petunia* line MP4-G revealed multibands when hybridized with a cDNA clone (Shah et al. 1986). Southern hybridization with cDNA probes indicated that the structural genes for L-phosphinothricin and glyphosate target proteins contained introns. The complex structure of these genes resulted in a relatively large duplication repeat unit. Their duplication copies were 8–12 and 20, respectively. In contrast, CSR6 had a relatively higher copy number and a smaller repeat unit, 2.65 kb; this size is consistent with the yeast *ILV2* gene that is limited in a 2.7 kb fragment (Falco et al. 1985).

Since the duplicated genes in CSR6 as well as the other two cases are most probably arranged in tandem array, unequal sister chromatid exchange could be involved in the gene amplification. The process has been very well demonstrated in other organisms such as yeast. Yeast *Saccharomyces cerevisiae* rDNA consists of approximately 140 copies of a 9 kb repeat unit in tandem array. Szostak and Wu (1980) found that unequal crossing over naturally occurred within this sequence during haploid mitotic growth, resulting in a reciprocal deletion and duplication of rDNA copies in daughter cells. Similarly, plant rDNA copy number change in stress environments has been reported (Cullis 1979; Landsmann and Uhrig 1985), suggesting that this mechanism is operative in higher plants. According to the hypothesis proposed by Schimke and his colleagues (1986), segmental DNA overreplication is a common phenomenon that results in various chromosome aberrations. One of the major consequences of overreplication is gene amplification, whether or not accompanied by gene deamplification. We suggest that this process produced a high copy number transient duplication of the ALS gene in CSR6 which resulted in overproduction of ALS and tolerance to CS. Point mutations for herbicide resistance (Chaleff and Ray 1984) in the multicopy region would then be expected to be relatively frequent. Unequal sister chromatid exchange would generate both repeats and deletions of the tandem duplication. In the presence of herbicide, selection was directed and resulted in loss of multiple copies of the wild type ALS sequence and eventual fixation of a single copy of the herbicide resistant gene. This mechanism explains the generation of the tandem duplication in CSR6 and its subsequent loss, which may apply to other systems that confer resistance/tolerance to cellular inhibitors or environmental stresses. Such transient gene amplification may or may not lead to the eventual fixation of a single copy mutant allele, depending on the physiological consequences of the particular point mutation.

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References

Alt FW, Kellems RE, Bertino JR, Schimke RT (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J Biol Chem 253:1357–1370

Barg R, Peleg N, Perl M, Beckmann JS (1984) Isolation of methotrexate-resistant cell lines in *Petunia hybrida* upon stepwise selection procedure. Plant Mol Biol 3:303–311

Bendich AJ, Anderson RS, Ward BL (1980) Plant DNA: long, pure and simple. In: Leaver CJ (ed) Genome organization and expressions in plants. Plenum, New York

Chaleff RS, Mauvais CJ (1984) Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science 244: 1443–1445

Chaleff RS, Ray TB (1984) Herbicide resistant mutants from tobacco cell culture. Science 233: 1148–1151

Cullis CA (1979) Quantitative variation of ribosomal RNA genes in flax genotrophs. Heredity 42: 237–246

Donn G, Tischer E, Smith JA, Goodman HM (1984) Herbicide-resistant alfalfa cells: an example of gene amplification in plants. J Mol Appl Genet 2: 621–635

Falco SC, Dumas KS (1985) Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. Genetics 109: 21–35

Falco SC, Dumas KS, Livak KJ (1985) Nucleotide sequence of the yeast *ILV2* gene which encodes acetolactate synthase. Nucleic Acids Res 13: 4011–4027

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158

Horsch RB, King J, Jones GE (1980) Measurement of cultured plant cell growth on filter paper discs. Can J Bot 58: 2402–2406

Landsmann J, Uhrig H (1985) Somaclonal variation in *Solanum tuberosum* detected at the molecular level. Theor Appl Genet 71: 500–505

La Rossa RA, Schloss JV (1984) The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolatate synthase in *Salmonella typhimurium*. J Biol Chem 259: 8753–8757

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, New York

Mazur BJ, Chui CF (1985) Isolation and characterization of plant genes coding for acetolactate synthase, the target for sulfonylurea herbicides. 1st Int Congr Plant Mol Biol, p 41

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nafziger ED, Widholm JM, Steinrucken HC, Killmer JL (1984) Selection and characterization of a carrot cell line tolerant to glyphosate. Plant Physiol 76: 571–574

Saxena PK, Fowke LC, King J (1985) An efficient procedure for isolation of nuclei from plant protoplasts. Protoplasma 128: 184–189

Schimke RT, Sherwood SW, Bill AB, Johnston RN (1986) Overreplication and recombination of DNA in higher eukaryotes: potential consequences and biological implication. Proc Natl Acad Sci USA 83: 2157–2161

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG, Fraley RT (1986) Engineering herbicide tolerance in transgenic plants. Science 233: 478–481

Szostak JW, Wu R (1980) Unequal crossing over in the ribosomal DNA of *Saccharomyces cerevisiae*. Nature 284: 426–430

Vieira J, Messing J (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19: 259–268

Weislander L (1979) A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal Biochem 98: 305–309