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High frequency, heat treatment-induced inactivation of the phosphinothricin resistance gene in transgenic single cell suspension cultures of *Medicago sativa*

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Summary. One descendant of the *Medicago sativa* Ra-3 transformant T304 was analysed with respect to the somatic stability of the synthetic phosphinothricin-*N*-acetyltransferase (*pat*) gene which was used as a selective marker and was under the control of the 5′/3′ expression signals of the cauliflower mosaic virus (CaMV) gene VI. In order to quantify gene instability, we developed a system for culturing and regenerating individual cells. Single cell suspension cultures derived from T304 and the ancestral non-transgenic *M. sativa* cultivar Ra-3, were established. The cells were regenerated into monoclonal calli. In transgenic calli, the phosphinothricin (Pt)-resistance phenotype was retained after more than 2 months of non-selective growth. In contrast, up to 12% of the suspension culture cells grown under non-selective conditions and at constant temperature (25° C) lost the herbicide-resistance phenotype within 150 days. Surprisingly, a heat treatment (37° C), lasting for 10 days, during the culture period resulted in an almost complete (95%) loss of the Pt resistance of the suspension culture cells. However, the frequency of cell division was identical in cultures grown under normal and heat treatment conditions. A biochemical test revealed that no phosphinothricin-*N*-acetyltransferase activity was present in heat treated, Pt-sensitive cells. The resistance level of the Pt-sensitive transgenic cells was equivalent to that of the wild-type cells. A PCR analysis confirmed the presence of the *pat* gene in heat treated, Pt-sensitive cells. From these results it is concluded that the Pt resistance gene was heat-inactivated at a high frequency in the *M. sativa* suspension cultures.

Key words: Gene inactivation – Heat treatment – Single cell culture – Herbicide resistance gene – *Medicago sativa*

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Introduction

Since the discovery of *Agrobacterium tumefaciens* and its ability to transfer DNA into the plant cell, many different plant species have been transformed using this technique. Plants carrying virus- or herbicide-resistance genes have been constructed and used in field tests (USDA 1990). An example of a gene used in such experiments is the gene conferring resistance to the herbicide phosphinothricin (Pt). Pt is produced as phosphinothricyl-alanyl-alanine (Ptt) by *Streptomyces hygroscopicus* (Kondo et al. 1973) and *S. viridochromogenes* (Bayer et al. 1972). Pt is a structural analogue of glutamic acid, which competitively inhibits bacterial and plant glutamine synthetases (Bayer et al. 1972; Lea et al. 1984). Resistance genes have been isolated from the producer strains (Thompson et al. 1987; Strauch et al. 1988) and transferred to a variety of plant species (e.g. De Block et al. 1987; Wohlleben et al. 1988). Both genes code for a phosphinothricin-*N*-acetyltransferase which inactivates Pt. The amino acid sequence derived from the resistance-conferring phosphinothricin-*N*-acetyltransferase gene isolated from *S. viridochromogenes* (Wohlleben et al. 1988) was used to determine the corresponding DNA sequence which was then adapted to the codon usage pattern of recipient plants (Eckes et al. 1989). The gene was transferred to *M. sativa* Ra-3 to give rise to the Pt-resistant *M. sativa* transformant T304 (Eckes et al. 1989). These plants are intended for release into the environment and use in agriculture. It is consequently of importance to ascertain the somatic stability of transgene expression and the effects of environmental conditions on the transgenic plant.

Most of the transgenic plants analysed to date inherited the integrated genes according to Mendelian laws (e.g. Otten et al. 1981). Nevertheless, Tepfer (1983) reported a lack of opines in some descendants of transgenic tobacco plants that contained a full-length T-DNA. Later, similar phenomena were described by Potrykus et al. (1985), Budar et al. (1986) and Müller et al. (1987). It was assumed that transgenic progeny which failed to

express the transferred marker gene resulted from mutation or suppression of the transgene. In animal cells, the methylation of a gene was shown to be correlated with the suppression of gene expression (e.g. Doerfler 1983). The same was found for transgenes in several plant species (e.g. Gelvin et al. 1983; Hepburn et al. 1983; Amasino et al. 1984; John and Amasino 1989). In many cases, it was found that the presence of multiple copies of the gene (reviewed by Jorgensen 1990) and even the introduction of a second transgene (Matzke et al. 1989; Matzke and Matzke 1990), promoted the suppression of the marker gene. All the changes in transgene expression in plants mentioned above, occurred before or during meiosis and in plants which predominantly contained more than one copy of the transgene. Since the meiotic stability of a transgene is of less importance than its somatic stability in the field, we decided to ascertain whether a transgene is stable during the initial growth period of the plant when herbicides are applied to the field. In order to enhance the stability of the transgene expression, a transformant containing only one copy of the resistance gene should be used.

Using a newly established single cell culture system, we demonstrated that even a single copy of a herbicide-resistance gene was inactivated, at high frequency, in cells of a transgenic suspension culture when the growth temperature increased. This effect occurred during somatic cell division and was quantified under controlled conditions.

Materials and methods

Plants. Seeds of *M. sativa* cv. Ra-3 and seeds of transgenic plants (T304, Pt^r transformant of Ra-3) were obtained from Hoechst AG, Frankfurt/Main. T304 contains one copy of the transgene only (G. Donn, personal communication).

Culture of M. sativa *plants.* The seeds were sterilised with a saturated solution of sodium hypochlorite in water (20 min), washed twice and germinated on MS medium (Murashige and Skoog 1962). All plants were cultivated under sterile conditions, on MS medium with or without phosphinothricin (concentrations as indicated in the text), in a growth chamber (16/8 h light/dark, 25° C, illuminated with fluorescent light). Plants were propagated vegetatively every 1–2 months.

Induction and maintenance of callus and suspension cultures. Callus formation from leaf or petiole fragments was induced on MS medium supplemented with 1 mg/l 2,4-D and 0.1 mg/l kinetin. Fast-growing calli were subcultured every 4–5 weeks on the same medium.

Suspension cultures were induced by transferring approximately 1 g of fast-growing, healthy looking callus (no phenolic areas or yellow appearance) to 20 ml of liquid MSKM medium (50% MS medium, 50% KM medium; Kao and Michayluk 1975) supplemented with 1 mg/l 2,4-D, 0.1 mg/l kinetin and 3% coconut water (MSKMC medium). The cultures were grown in 100-ml erlenmeyer flasks which were placed on a rotary shaker (130 rpm). Cultures were incubated at room temperature or in a growth chamber at specific temperatures. Subculturing was done every 10–20 days by transferring 10 ml of the culture of 10 ml of fresh medium. A large number of vigorously growing, single cells were detected in the culture growing in MSKMC medium. The cell aggregates which were present were separated from the culture by sedimentation. The supernatant contained single cells as verified by microscopy. The growth rate of the culture was estimated by counting the numbers of cells every 10 days. When subcultured as described above, the cultures grew exponentially, having a generation time of approximately 10–14 days.

Estimation of cell growth. Every 3–5 days a 100 µl aliquot of the cell suspension was removed from the culture and the single cells were counted in a counting chamber (Bürker). The cell number of the culture was calculated as the mean value of five cell counts. Cell aggregates were not counted.

Regeneration of single cells from suspension cultures. The suspension culture was removed from the shaker every 5–10 days to separate cell aggregates from single cells. After 10 min, when the aggregates had settled to the bottom of the flask, a 4 ml aliquot of the supernatant, single cell suspension was removed. This was diluted with 4 ml of liquid MSKMC medium. Subsequently, 40 µl drops of the culture were transferred to the bottom of a petri dish and mixed with 20 µl of Seaplaque agarose in KM8 medium (30–40° C, $1–4 \times 10^3$ cells/ml, final concentration). Because of the low cell density, the regeneration medium was enriched with coconut water of *M. sativa* seedling extract. The resulting mini-drop was spread as thinly as possible to facilitate diffusion of gases. The cultures were placed in the dark in a growth chamber at 25° C. The rate of cell division was monitored regularly under an inverse microscope and every 3–4 days 20 µl of fresh medium was added to each culture. When they had reached 1–2 mm in diameter, the regenerating calli were transferred to MSKMC medium supplemented with 1 mg/l 2,4-D and 0.1 mg/l kinetin.

Analysis of pat *gene stability in monoclonal calli.* The developing monoclonal calli were divided in two once they had reached approximately 5–10 mm in diameter. One half was placed on MS medium while the other half was transferred to MS medium supplemented with phosphinothricin (50 mg/l). Twenty-one days later, the growth of the calli was analysed. Pt-resistant calli grew vigorously on this medium, whereas Pt-sensitive clones became brown (phenolic) and showed no increase in size. The percentage of resistant calli which later became sensitive to Pt was determined by comparing the number of regenerating calli on the two media. Transgenic calli grew vigorously on media containing more than 300 mg/l Pt while the non-transgenic calli showed retarded growth on media containing as little as 10 mg/l Pt. The rate of cell division was retarded in direct proportion to the increasing Pt concentrations (10–30 mg/l).

At concentrations of 300 mg/l Pt, very little increase in callus weight was detectable in the Pt-sensitive calli.

Biochemical assay of phosphinothricin-N-*acetyltransferase activity (Pat test)*. The Pat activity was ascertained as described by Dröge et al. (1992).

Isolation of DNA from plant tissue. DNA was isolated from callus tissue according to Rogers and Bendisch (1987).

Oligodeoxynucleotide synthesis. The chemicals required for the synthesis of DNA were purchased from Applied Biosystems. The oligodeoxynucleotides were synthesised with an Applied Biosystems DNA Synthesizer, Model 380B, using the phosphoramidite method (Beaucage and Caruthers 1981).

Polymerase chain reaction (PCR). The PCR (Mullis and Faloona 1987) was set up at 4° C. The 50 µl reaction mixture consisted of 200 nM of each oligonucleotide primer, 200 µM d(NTP), 2.5 units of Taq polymerase (BRL), 1 µg linearised template DNA in PCR buffer (67 mM DTT, 0.17 mg/ml BSA). The reaction mixture was overlaid with mineral oil. After the first denaturation, which lasted for 2.5 min at 94° C, 33 cycles of amplification (at 94° C for 90 s, at 55° C for 90 s, at 72° C for 90 s, extending each cycle at 72° C for 1 s) were performed in a thermocycler (Collasius et al. 1989). An aliquot of the PCR product was digested with *Eco*RV or *Sma*I. The digested and undigested PCR products were electrophoretically separated in a 1% agarose gel.

Results

Expression of the synthetic pat *gene is maintained constantly during* M. sativa *callus culture*

In order to detect the suppression of a transgene in single cells, the cells must be cultured individually and separately from each other. Since single cell culture in microdroplets demands much effort (Spangenberg et al. 1986), we established single cell suspension cultures of the *M. sativa* plants Ra-3/1 (Pt^s, wild type) and T304/5 (F_1 descendant of T304, a Pt^r transgenic derivative of Ra-3, Eckes et al. 1989). The regeneration of monoclonal calli from single cell suspensions required a very low cell density and the fixation of the regenerants in a carrier medium which was solidified with Seaplaque agarose.

The occurrence of somaclonal variation in callus cells has been observed recurrently (Damiani et al. 1985). The callus culture conditions must be optimised, therefore, in order to minimize changes in gene expression that may occur during this phase. Single cells of *M. sativa* Ra-3/1 (Pt^s) and T304/5 (Pt^r) were mixed in different ratios. Samples were taken from the mixture, regenerated to monoclonal calli without selection, and analysed with respect to Pt tolerance. If no change in gene expression had occurred, the original number and ratio of sensitive and resistant calli would be recoverable, irrespective of the cultivation time. Since this proved to be the case (Table 1), we concluded that *pat* gene expression was stably inherited during the callus phase. In addition, it indicated that the method could be used to recover even small numbers of sensitive cells from a mixed culture.

Table 1. Recovery of phosphinothricin (Pt) sensitive and resistant calli from mixed single cell suspension cultures

Percentage of single cells Pt^s	Number of regenerating calli	Pt^s	Pt^r	Percentage of sensitive calli
100	128	128	0	100
0	152	0	152	0
1	444	7	437	1.6
10	365	26	339	7.1
25	394	91	303	23
50	382	180	202	47
75	467	363	104	77.5

Heat treatment of a transgenic Pt-resistant M. sativa *suspension culture results in a dramatic increase in the incidence of Pt-sensitive cells*

Transgenic *Daucus carota* and *M. sativa* suspension cultures which were maintained for a long period without selective pressure, occasionally showed a high percentage of sensitive cells (W. Dröge et al., unpublished data). Therefore, we decided to quantify this loss of resistance using the single cell system described above. The *M. sativa* T304/5 culture was incubated under non-controlled, room temperature conditions (approximately 23° C) with and without selective pressure. Samples were taken approximately every 10 days. A consistent loss of Pt resistance was observed in 1–2% of cells per generation. After a week-long period of increased temperature in the laboratory (week average 26° C, highest temperature 40° C), 90% of the cultured cells had lost their Pt resistance phenotype. Hence, roughly 40% transgenic cells per generation became sensitive over a period of only 28 days in culture (see Fig. 1).

Since the increase in the incidence of Pt-sensitive cells seemed directly correlated with the increase in temperature, the experiment was repeated under controlled temperature conditions. Single cell cultures were divided into two parts. Both cultures were grown in the growth chamber at 25° C for 60 days. Samples of both cultures were analysed with respect to their content of Pt-resistant and sensitive cells, according to the method described above. Approximately 450–700 calli, each derived from a single suspension culture cell, were examined per sample. Additionally, five samples were taken approximately every 10 days to determine the growth rate of the culture. The growth of the cultures as well as their proportion of Pt-sensitive cells increased at the same rate during this period of time. Thereafter, a heat treatment of 37° C was applied for 10 days to one of

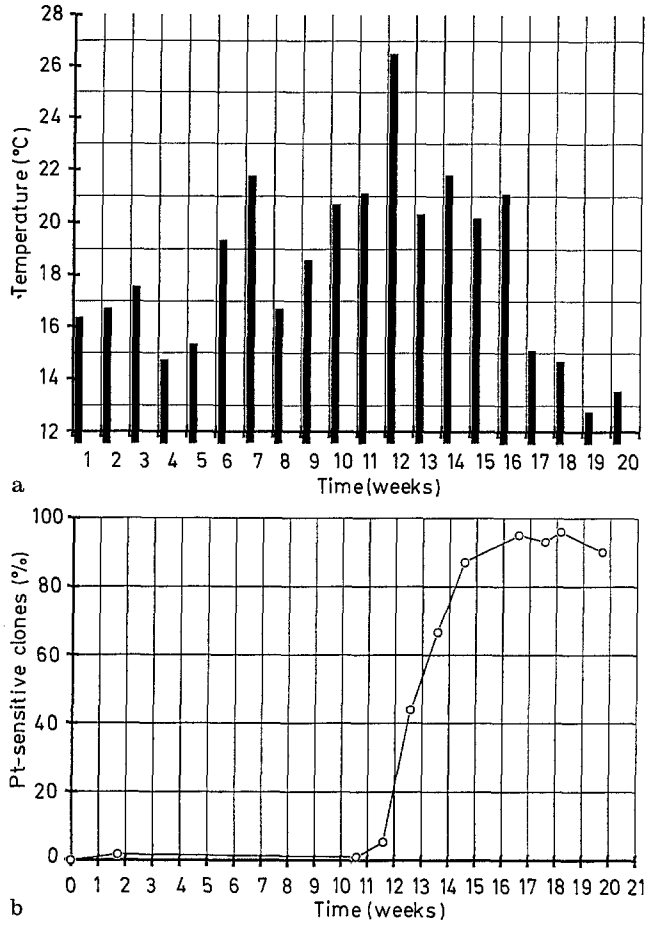


Fig. 1a and b. Correlation between incidence of herbicide-sensitive cells and growth temperature. Percentage of phosphinothricin-sensitive (Pt^s) cells in a T304/5 suspension grown at room temperature (**b**) is compared to the average daytime temperature (calculated for each week) during the experimental period (**a**). The temperature data were obtained from the local weather station

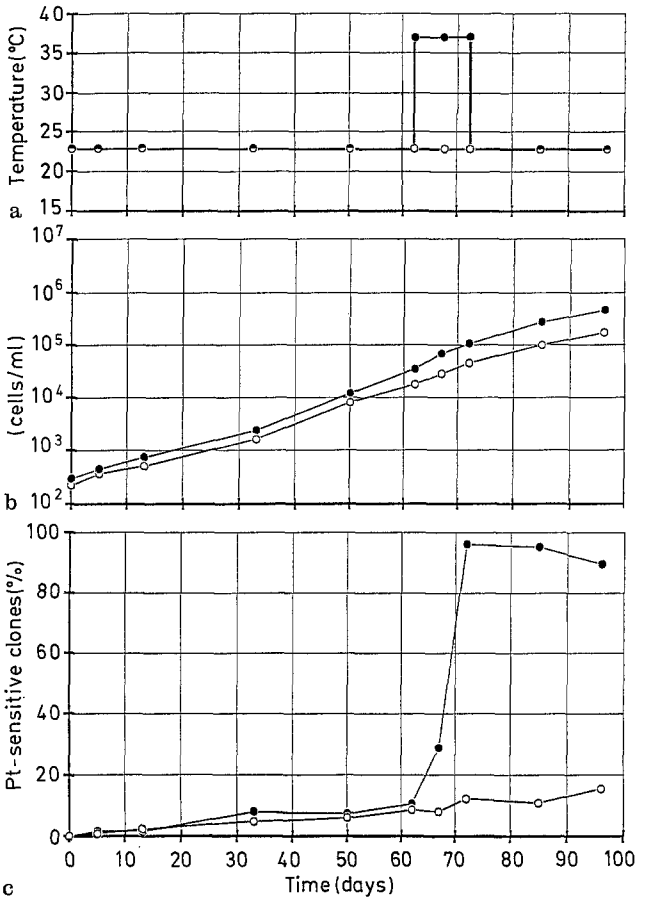
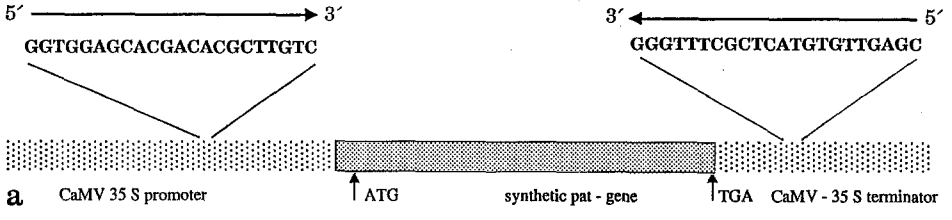


Fig. 2a–c. Heat treatment of suspension cultures. **a** Incubation temperature of the two cultures with and without heat treatment. **b** Growth curve of *Medicago sativa* T304/5 single cells suspension cultures with and without heat treatment. The heat treatment was applied after 62 days of culturing. **c** Percentage of Pt^s cells in a T304/5 suspension grown under controlled temperature conditions (*filled circles*, with heat treatment; *open circles*, without)



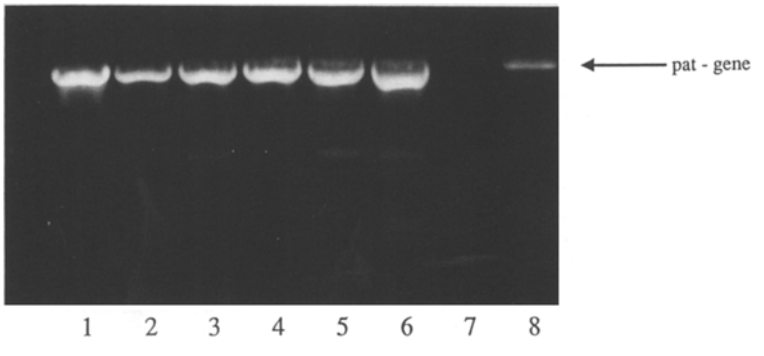


Fig. 3a and b. Polymerase chain reaction (PCR) analysis of secondary PT^s T304/5 callus clones. **a** The modified synthetic *pat* gene. Positions and sequences of the oligonucleotides synthesised for PCR are given. **b** The agarose gel in which the PCR products were electrophoretically separated. Lanes 1–5, PCR products of secondary Pt^s calli; lane 6, PCR product of the Pt^r transgenic T304/5; lane 7, PCR product of the wild-type Ra-3/1; lane 8, plasmid pOCA/AC (Eckes et al. 1989) carrying the modified synthetic *pat* gene

the cultures in order to study the impact of the temperature on the stability of *pat* gene expression (see Fig. 2a). Samples were analysed as described above. Interestingly, the growth rate of the heat-treated culture was not affected, the cell generation time did not differ from the control (see Fig. 2b).

In contrast to this observation, however, the percentage of Pt-sensitive cells in the culture changed dramatically. In comparison to the control culture, in which 12% of the cells lost the Pt-resistance phenotype, 95% of the cells derived from the heat-treated culture were found to be Pt-sensitive (see Fig. 2c).

When the high temperature was decreased, a slight decrease in the number of sensitive cells was detected. We considered this decrease in cell number to be insignificant, because few assessments were possible after the heat treatment since the regeneration capacity of the culture was adversely affected by the age of the culture.

Pt-sensitive calli derived from transgenic heat-treated M. sativa *suspension culture cells contain a silent* pat *gene*

The loss of the Pt-resistance phenotype could be due to a deletion or other mutation in the *pat* gene. Therefore, DNA was isolated from callus clones and the state of the *pat* gene was investigated using the PCR. Oligonucleotides of 22 bp, homologous to the cauliflower mosaic virus (CaMV) 35S promoter or terminator region, were synthesised. The *pat* gene, together with the annealing position and sequence of the oligonucleotides, is shown in Fig 3a.

As shown by the PCR, the *pat* gene was present in all Pt-sensitive clones tested (Fig. 3b). The restriction sites in the synthetic *pat* gene were retained in the PCR products and the sizes of smaller fragments were also identical to those of the wild type. Deletions or mutations were not detectable (data not shown). Nevertheless, point mutations not revealed by agarose gel electrophoresis could have been present.

The Pt-sensitive phenotype could also be caused by reduced activity of the *pat* gene. Therefore, the resistance level of transgenic Pt-sensitive clones was compared to that of wild-type calli. Sixteen different Pt concentrations were applied to 12 transgenic and 12 wild-type calli. Their growth rates were determined by weight. No difference in the resistance levels could be detected between transgenic Pt-sensitive and wild-type cells (see Fig. 4).

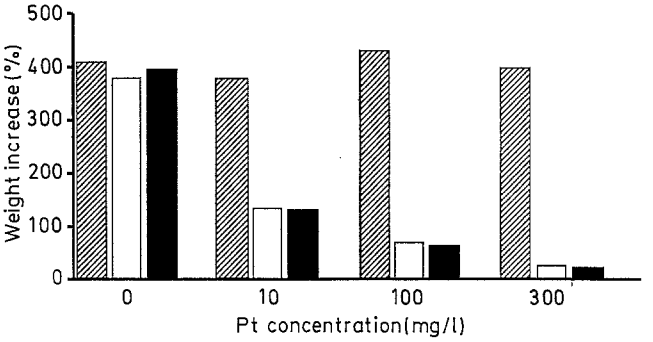


Fig. 4. Growth increase of different *M. sativa* callus clones on media with various concentrations of Pt. Pt^r T304, *hatched box*; Pt^s Ra-3, *open box*; secondary Pt^s clones from T304, *filled box*

In addition, the phosphinothricin-*N*-acetyl transferase activity was measured in transgenic and wild-type calli using the Pat assay (Dröge et al. 1992). No acetylation of Pt was detectable in transgenic, Pt-sensitive clones (Fig. 5). In contrast, Pt-resistant clones showed high turnover rates of Pt.

Discussion

The instability of gene expression in transgenic plants is well documented (e.g. Mittelsten Scheid et al. 1991). In the cases described, the spontaneous silencing of genes was primarily dependent on a high copy number of the transgene and was observed after meiosis or after regeneration from tissue culture (e.g. Matzke et al. 1989). Since one aim of constructing transgenic herbicide-resistant plants is to release them into the environment, it is important that the desired phenotype be stably maintained in the crop. In order to obtain quantitative and reproducible assessments of gene stability, it is convenient to use a test system that enables every possible event of gene silencing in a single plant cell to be investi-

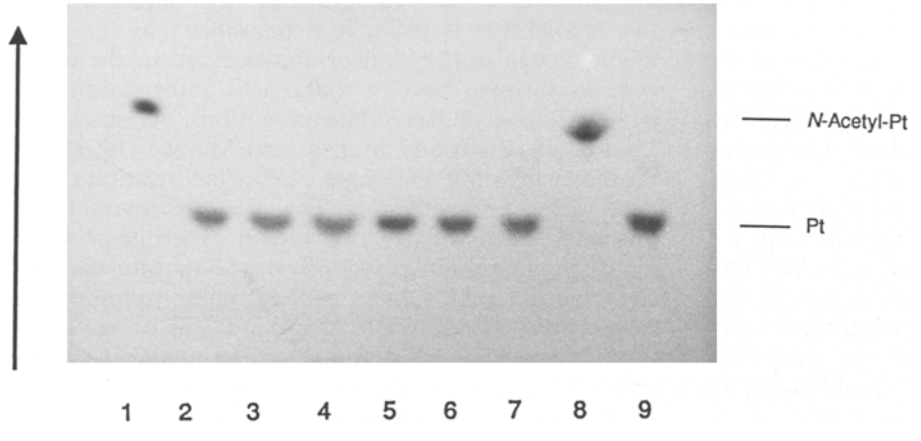


Fig. 5. Thin layer cellulose chromatography of transgenic *M. sativa* extracts incubated with ^{14}C-labelled Pt. Lane 1, *N*-acetyl Pt; lanes 2–7, extracts from secondary Pt^s clones; lane 8, extract from a Pt^r clone; lane 9, extract from a Pt^s wild-type clone

gated. We therefore decided to use suspension cultures which can be cultured as single cells and regenerated to monoclonal calli. However, suspension culture, which is a very artificial system for culturing plant cells, is associated a high rate of somaclonal variation (Van Brunt 1985; Evans 1989). It may represent a 'worst case' system, because changes in DNA expression may occur at higher frequencies, but it cannot replace experiments done on the plant itself.

In order to quantify gene silencing in suspension culture, we first demonstrated that a defined number of sensitive and resistant cells could be recovered from a suspension using the method described (Table 1). We therefore concluded that the system presented here, which quantitatively detects gene silencing events in single cells, gives reliable results.

The test system described here enables environmental factors to be varied under controlled conditions. Therefore, it was possible to identify the cause of gene inactivation. Since all cells are derived from one callus, no tissue specificity or position effects interfere with the analysis.

We decided to use the phosphinothricin-*N*-acetyltransferase gene (*pat*) primarily because of its proposed application in agriculture, and secondly because phosphinothricin proved to be an effective selective agent for *M. sativa* (C. Walter, unpublished data).

Using the test system described above, we detected a high percentage of Pt-sensitive cells in the heat-treated transgenic suspension culture. Since the phenomenon persisted over several cell generations when the growth temperature was reduced to 25° C, it was considered to result from an inactivation of the modified *pat* gene in the *M. sativa* suspension culture cells. Nevertheless, we cannot exlude the possibility that the alterations observed are due to induced and lasting secondary effects that influence the stability of the PAT mRNA or the PAT enzyme. Inactivation events have been described in transgenes (e.g. Jorgensen 1990) in which gene silencing was accompanied by the methylation of the transgene. However, this pertained mostly to the inactivation of multiple copies of the transgene (e.g. 'co-suppression') which might not be applicable to cases where only one copy of the gene is present.

As in our experiment, Meyer et al. (1992) tried to enhance gene stability by using only one copy of the transgene per cell. After a period of high temperature, the expression of one copy of the maize *A1* gene in hemizygous *Petunia hybrida* was repressed, accompanied by the methylation of the promoter region of the silenced transgene. However, the repression of the *A1* gene resulted in reduced pigmentation only, and not in the complete loss of colour. The percentage of white or variegated plants did not change. Since control plants in the greenhouse showed less reduction in pigmentation, although the temperature was even higher, this effect has to be considered to be mainly field dependent, and not solely temperature dependent. In our experiments, the phosphinothricin-*N*-acetyltransferase activity was totally repressed; no cells with reduced expression could be detected. No differences between gene expression in transgenic cells that had lost the Pt-resistance, with or without heat treatment, could be observed. Since controlled experimental conditions were used, we can be certain that the increase in culture temperature led to the repression of the *pat* gene expression. The differences between our results and those of Meyer et al. (1992) may also be due to factors that are plant- or suspension culture-specific. Furthermore, the reduction in the *A1* gene product may have been influenced by *Petunia*-specific sources of instability in the genome (Farcy and Cornu 1979).

As found in all other cases of gene silencing described to date, the transgene was still present in the secondary Pt-sensitive cells. No changes in fragment sizes were observed after restriction analysis of the PCR product. The occurrence of single basepair mutations could not, however, be excluded. Since no reactivation of the silenced genes was observed, it was not possible to prove that the DNA sequence had remained unaltered. On the other hand, the probability of a single basepair mutation occurring spontaneously in 40% of the cells in one generation is very low. Therefore, we assume that the phenotypic inactivation of the marker is due to a suppression of gene expression and not to a spontaneous mutation of the transgene. Whether the gene silencing was caused by methylation or other factors, and whether the promoter or coding region was affected, remains to be analysed.

One important question that arises from these data is whether the plant cell specifically recognizes foreign DNA and consequently represses its expression when stress is applied, or whether the plant suppresses a whole set of genes under these conditions, leaving only those genes active which are necessary for the plant to survive changes in the environment. The drastic effect of heat treatment on *pat* gene expression, together with the lack of effect on the growth of the culture, indicates that at least some genes necessary for cell division were not subjected to the same suppression as the *pat* gene. Hence, some mechanism must exist to distinguish between genes necessary for growth and others of lesser importance. Faugeron et al. (1990) have proposed a model to explain how recognition of numerous copies of transgenes takes place during meiosis. In our case, a single copy could have to be recognized during somatic cell division as no meiosis took place. To date, there is no model to account for such recognition. If the hypothesis of selective recognition is valid, it is plausible that the plant might recognize the viral promoter fused to the transgene as forming part of the genetic information that is superfluous under certain conditions. As shown by Linn et al. (1990) and Matzke and Matzke (1991), the promoter region is the target site for methylation correlated with gene inactivation. The influence of viral DNA on suppression will be analysed in experiments using plant-specific promoter regions such as that for the gene encoding the small subunit of the ribulose-bisphosphate carboxylase SSU_{pro}. Since the expression of the small subunit is essential for photosynthesising plants (Lorimer 1981), the fused transgene should not be suppressed when the promoter region is the predominant signal for

the recognition of unnecessary genes. The possible influence of the coding sequence on the susceptibility of the transgene to inactivation might be reduced by the high AT content of the synthetic sequence, and will also be analysed using other, plant-specific coding regions.

It can thus be concluded that, at least in suspension cultures grown at 25° C, inactivation of the transgene can occur at higher frequencies than in other types of cell culture. In our system, there is no loss of gene expression in the callus phase. No data are available for the influence of high temperature on gene stability in calli and leaf cells. If the frequency of loss of expression remains as low as that measured without heat treatment, the impact on plants in the field would be undetectable. Nevertheless, the influence of a heat treatment may increase the number of inactivation events in cells of an intact plant. Therefore, similar controlled heat treatment experiments should be done with plants. However, the heat-correlated inactivation of a herbicide-resistance gene is a problem that must be considered when transgenic plants are used in the field, since periods of high temperatures (37° C) do occur, even in Europe.

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