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The lysopinedehydrogenase gene used as a marker for the selection of octopine crown gall cells

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Abstract: Plant cells transformed into octopine-synthesizing tumour cells by the bacterium *Agrobacterium tumefaciens* survive when cultured in the presence of homo-arginine (HA), whereas both normal plant cells and nopaline producing plant tumour cells do not. Survival of octopine crown gall cells is due to the activity of the enzyme lysopinedehydrogenase (LpDH) in these cells, which converts toxic homo-arginine into non-toxic homo-octopine. The selective toxicity of homo-arginine for normal cells can be applied for the enrichment of octopine Ti plasmid transformed plant cells *vs* normal plant cells in mixed cultures.

1. Introduction

The bacterium *Agrobacterium tumefaciens* induces tumours, called crown galls on dicotyledonous plants. The bacterial genes that are essential for tumour formation are located on an extrachromosomal element, the Ti plasmid. During tumour induction part of the Ti plasmid DNA becomes stably integrated in the nuclear DNA of the plant tumour cells [6, 19, 20, 23]. This part is called T-DNA and it carries genes that either directly or indirectly cause changes in the activities of the phytohormones, auxin and cytokinin [11]. This is presumably the reason that crown gall cells are able to grow in phytohormone free media.

Recently, a method has been developed for the transformation of plant protoplasts with *A.tumefaciens in vitro* [9, 21] as well as with Ti plasmid DNA [2, Krens *et al.* in preparation]. In the transformation experiments described, selection of Ti plasmid transformed plant cells has exploited their ability to grow on hormone free media, in contrast with normal plant cells. A disadvantage of this type of selection is that it is laborious and slow, for selection on hormone free media only can be started several weeks after the protoplasts have been incubated with *A.tumefaciens* or Ti plasmid DNA. Non-transformed plant cells survive for a long period of time, hence, selection

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is completed only after 12 to 16 weeks. Moreover, plant protoplasts transformed with *A.tumefaciens* Ti plasmid mutants carrying mutations in the genes that control phytohormone autotrophy cannot be selected on phytohormone free media.

In this paper we describe an alternative selection procedure based on the presence of an enzyme called lysopinedehydrogenase (LpDH) in plant cells transformed by octopine Ti plasmid carrying *A.tumefaciens* strains [3, 4, 13]. This enzyme catalyzes the synthesis of opines by the reductive condensation of certain amino acids with pyruvate. Production of LpDH is due to a T-DNA gene [5, 20]. *In vitro* translation of T-DNA specific mRNA that had been isolated from crown gall tissue resulted in a protein that showed precipitation with anti-LpDH antibody [17], suggesting that the structural gene for LpDH is located in the T-DNA.

The enzyme LpDH causes the accumulation of octopine, lysopine, octopinic acid or ornopine and histopine, – derivatives of L-arginine, L-lysine, L-ornithine and L-histidine, respectively – in tumour cells. However, the substrate range of LpDH – at least *in vitro* – is much wider [13]. As plant cells like other organisms, are sensitive to many amino acid analogues [1], we tested whether certain amino acid analogues that are toxic to normal plant cells are not toxic to octopine tumour cells, because of their conversion into non-toxic compounds by the action of LpDH. In this paper we present evidence that this is the case for the toxic arginine analogue homo-arginine. We found that octopine tumour cells can be selected from a mixed population of normal and tumour cells using this compound.

For plant cells – except for the huge Ti plasmid – no suitable vectors exist at the moment. Our findings suggest that the LpDH gene can be used as a selectable marker for plant cells in the construction of smaller plant DNA-vectors.

Materials and methods

a. *Bacterial strains and plasmids*

LBA4013 carries octopine Ti plasmid, pTiAch5 and has the Ach-5 chromosomal background

LBA4058 carries nopaline Ti plasmid, pTiT37 and has the Ach-5 chromosomal background.

b. *Plant material*

N.tabacum c.v. petit Havanna, SR1 was obtained from Dr. P. Maliga, Szeged. It is a streptomycin resistant mutant which is maintained as axenic shoot cultures [Maliga *et al.*, 1973]. Shoots were grown on solidified MS medium (0.8% agar) without phytohormones [11]. The SR1 crown gall shoots were a kind gift of Dr. G. Wullems, Dept. of Biochemistry, Leiden. They originated from regenerating crown gall calli that had been obtained after *in vitro*

transformation of protoplasts by the *Agrobacterium* strains mentioned above. The names of the crown gall shoot cultures (SR1-4013-3$^+$; SR1-4013-44$^+$; SR1-4058-38$^+$) refer to the *A.tumefaciens* strains (LBA4013, LBA4058) used for transformation [21]. Shoots were grown on solidified LS medium (0.8% agar) without phytohormones [7].

Protoplast isolation and culture

Protoplasts from SR1 shoot leaves were isolated as described [18]. For isolation of protoplasts from crown gall shoots the cellulase and macerozyme concentrations were raised from 1% and 0.5% respectively to 2.5% and 1% (Onozuka-R10, Yakult Biochemistry, Japan). Protoplasts were plated at a density of 7×10^4 protoplasts ml^{-1} in K_3m.3s medium [18] in 1.5 ml or 3 ml aliquots.

Every week fresh K_3m medium [18] was added 1:1, lowering the sucrose concentration. After 24 to 28 days of liquid culture cells were plated after 1:10 or 1:20 dilution in K_3m.2s solidified with agar (0.8%). Plating efficiency was calculated after six to ten weeks depending on growth rates after colony counting using a Gallenkamp colony counter. Homo-arginine was added from a 10 mM filtersterilized stock solution to the final concentration mentioned in the experiments, L-(+) homo-arginine was purchased from Aldrich-Europe.

Pulse labelling

Pulse labelling with ^{3}H (-methyl)-thymidine (43 μCi mmol^{-1}, Amersham, UK) was performed with five and six days old protoplasts during 1 hr in K_3m.3s medium containing different homo-arginine concentrations. The details of the pulse labelling procedure have been published previously [18].

LpDH tests

The LpDH activity of 5–10 mm calli was measured according to Otten [14]. Extracts from calli were incubated with arginine and pyruvate as substrates and NADPH as a cofactor. LpDH activity was demonstrated by paperelectrophoresis of the incubation mixture and staining the product, octopine, with phenanthrenequinone [24].

Results

a. *Homo-arginine sensitivity of normal and crown gall shoots*

Small normal SR1 shoots and crown gall SR1-4013-3$^+$ shoots were transferred to solidified agar media containing 0, 0.1, 0.5 and 1.0 mM HA. After two to three weeks of growth the SR1 shoots were clearly inhibited compared to the untreated controls (Fig. 1). The crown gall shoots were only slightly (0.5 and 1.0 mM HA) or not at all (0.1 mM HA) inhibited on media containing HA (Fig. 2).

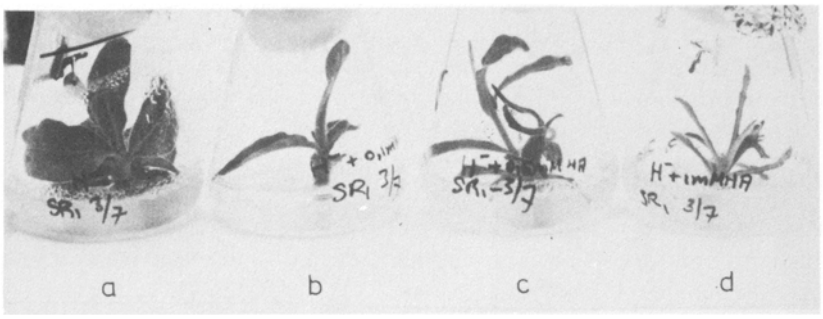

Figure 1. Normal (SR1) shoots grown for three weeks on HA containing medium: (a) no HA; (b) 0.1 mM HA; (c) 0.5 mM HA; (d) 1.0 mM HA.

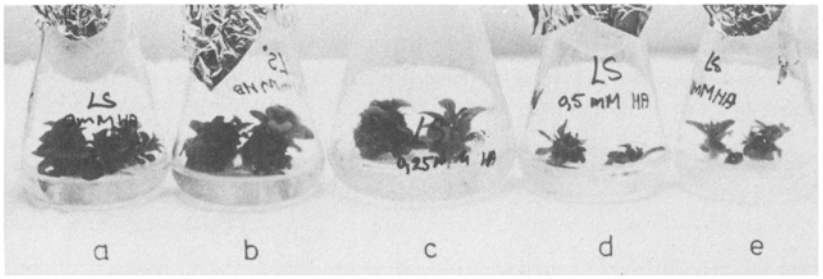

Figure 2. Crown gall (SR1-4013-3$^+$) shoots grown for three weeks on HA containing medium: (a) no HA; (b) 0.1 mM HA; (c) 0.25 mM HA; (d) 0.5 mM HA; (e) 1.0 mM HA.

b. *Homo-arginine sensitivity of normal and crown gall protoplasts*

Normal (SR1) and crown gall (SR1-4013-3$^+$) protoplasts were cultured in K_3m.3s medium containing HA. In one experiment after five days, and in another experiment after six days, protoplasts were labelled with |^{3}H|-(methyl)-thymidine. It was found that the |^{3}H|-thymidine incorporation in normal SR1 protoplasts was strongly inhibited in the presence of HA even at low concentrations (0.25 mM). However, in the case of crown gall protoplasts, |^{3}H|-thymidine incorporation was not affected at HA concentrations up to 0.25 mM and only slightly inhibited in the presence of higher HA concentrations (Fig. 3). Addition of arginine (0.5 mM) to the HA-containing media abolished the inhibitory effect of HA (data not shown), presumably because of competition in uptake by the cells with HA.

c. *Plating efficiency of normal and crown gall protoplasts after culturing in the presence of homo-arginine*

Freshly isolated normal (SR1) and crown gall (SR1-4013-3$^+$) protoplasts were cultured during one week in the presence of 0.25, 0.35 and 0.5 mM HA. In the following weeks fresh medium containing 0.5 mM HA was added, as we

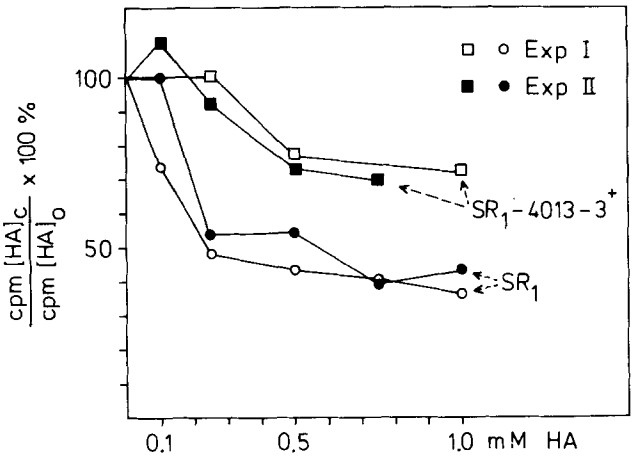

Figure 3. Effect of different HA concentrations on the uptake of [^{3}H]-thymidine in normal SR1 (○, ●) and crown gall SR1-4013-3$^+$ (□, ■) protoplasts after pulse on day 5 (□, ○) or day 6 (■, ●) after isolation.

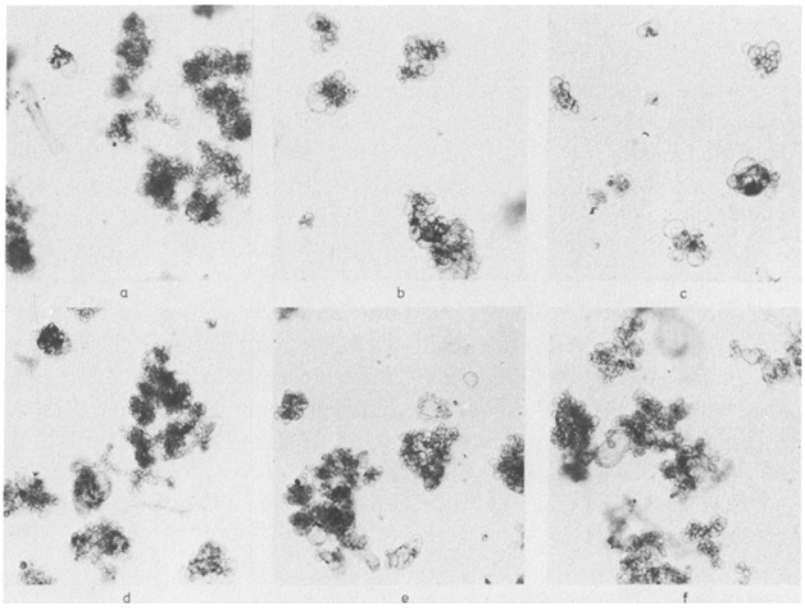

Figure 4. Microscopic view on three weeks cultures of normal (SR1) protoplasts in medium containing: (a) no HA; (b) 0.25 mM HA; (c) 0.35 mM HA and of crown gall (SR1-4013-3$^+$) protoplasts in medium containing: (d) no HA; (e) 0.25 mM HA or (f) 0.35 mM HA (160 X).

found that HA sensitivity of protoplasts decreased with age (data not shown). To the control cultures only fresh K_3m was added 1:1. After three weeks a clear difference was observed (microscopically) in growth of normal cells compared to crown gall cells (Fig. 4). In all seven experiments hardly any difference was observed in the crown gall cultures grown with or without HA. However, in the SR1 cultures, in five out of seven experiments, only few living cell clumps were present after three weeks of HA treatment. In two other experiments SR1 survival was estimated 10% after three weeks growth in the presence of the lowest (0.25 mM) HA concentration.

Table 1. Effects of homo-arginine on the plating efficiency of normal (SR1) and crown gall (SR1-4013-3$^+$) protoplasts after five weeks of culture

Homoarginine* (mM)	Plating efficiency % of the control	
	SR1	SR1-4013-3$^+$
0	100	100
0.25	0–30	100
0.35	0–10	100
0.5	0	75–100

*Homoarginine concentrations refer to the first week of liquid culture of the protoplasts. After one week and once more after two weeks, cultures were diluted 1:1 with K_3m medium containing 0.5 mM HA.

To test the influence of HA on plating efficiency, 3½ week old crown gall and normal cell clumps were plated in 0.25 or 0.5 mM HA-containing agar solidified medium (0.8%). This inevitably resulted in a stop of growth for both types of cells (data not shown). Therefore, in further experiments 3½ weeks old cell clumps were plated in agar medium lacking HA after which plating efficiency of normal and crown gall calli was compared (Table 1). In five out of seven experiments no calli of normal cells were obtained, consistent with the previous microscopic evaluation of the three weeks old cultures, whereas the plating efficiency of crown gall calli was usually 100%. After two weeks in HA-free agar medium the 1.5–3 mm calli could be transferred onto 0.5 mM HA containing agar medium plates. Growth inhibition was only observed for the smallest SR1 calli. Both SR1 and SR1-4013-3$^+$ calli bigger than 0.5 cm always grew without any significant inhibition. This shows, that although normal protoplasts are sensitive, calli are insensitive to HA..

d. *Mixed cultures of normal and crown gall protoplasts*

As a model for selection in protoplast transformation experiments crown gall protoplasts were mixed with a large excess of normal protoplasts (1:10 and 1:100). Mixed cultures were started in K_3m medium containing 0.25 mM HA during the first week. Fresh 0.5 mM HA containing K_3m was added after that

Table 2. Selection of crown gall calli from mixed cultures of normal (SR1) and crown gall (SR1-4013-3$^+$) protoplasts: LpDH activity of the selected calli from two independent experiments I and II

Exp. nr.	Number of protoplasts. ml^{-1}		LpDH$^+$ calli (%)
	SR1	SR1-4013-3$^+$	
Ia	10^5	10^3	72.3
Ib*	10^5	10^4	94.7
IIa	2.7×10^5	2.5×10^3	70.5
IIb	2.7×10^5	10^4	98.1

*In experiment Ib after 2 weeks K_3m was added 1:1 without HA instead of K_3m containing 0.5 mM, because of a rapid decline in protoplast survival.

time as described above. Plating efficiencies that were scored after six weeks of culture were denoted in Table 2 for experiments with protoplasts from two different isolations.

A large number of the six to eight weeks old calli were transferred onto 0.5 mM HA containing K_3m agar medium plates in order to obtain calli which were big enough to be tested for LpDH activity. From each experiment about 200 calli were tested. It was found that 72–98% of the calli were LpDH$^+$.

In two other experiments there were no living calli left after five weeks of culture.

e. *Experiments to test whether LpDH activity is needed for HA resistance*
To find out whether the HA resistance of the octopine type crown gall cells is dependent on the presence of LpDH, one other LpDH positive and one LpDH lacking, NpDH positive crown gall shoot cell line were compared with the SR1-4013-3$^+$ cell line. The origin of these two cell lines (SR1-4013-44$^+$ and SR1-4058-38$^+$) has been described in the Materials and methods section. The LpDH-lacking shoots (SR1-4058-38$^+$) were regenerated from protoplasts transformed by a nopaline Ti plasmid-carrying *A.tumefaciens* strain. These shoots contain the enzyme nopaline dehydrogenase (NpDH) which does not use homo-arginine as a substrate [15, 16]. The results of the culture of protoplasts from shoots of these two cell lines in the presence of HA at different concentrations is presented in Table 3. The protoplasts derived from nopaline shoots were not resistant to homo-arginine. Protoplasts from the alternative octopine line (SR1-4013-44$^+$) were more resistant to homo-arginine than normal SR1 protoplasts but less resistant than protoplasts derived from the other octopine cell line (SR1-4013-3$^+$). Although a milder HA concentration regime was used (two weeks low HA concentrations instead of one week) 41.5% or 0% plating efficiency was scored for SR1-4013-44$^+$ in cultures where SR1-4013-3$^+$ plating efficiency was still 100%.

Discussion

Selection of crown gall cells in experiments in which plant protoplasts are transformed by *Agrobacterium tumefaciens*, has been accomplished by making

Table 3. Effects of homo-arginine on the plating efficiency of protoplasts of the normal SR1 cell line compared to that of protoplasts of an octopine type ($LpDH^+$) crown gall cell line (SR1-4013-44$^+$) and a nopaline type ($NpDH^+$) crown gall cell line (SR1-4058-38$^+$) in two and three experiments respectively

Homo-arginine* (mM)	Plating efficiency % of the control		
	SR1	SR1-4013-44$^+$	SR1-4058-38$^+$
0	100; 100	100; 100	100; 100; 100
0.1	–; 58	–; 100	0; 0**; 0
0.25	0; 32	41.5; 0	0; 0; 0
0.35	0; 0	13.6; 0	0; 0; 0
0.5	0; 0	0; 0	0; 0; 0

*The homo-arginine concentrations refer to those in the medium in week 1 and 2. After two weeks fresh K_3m medium was added 1:1 containing 0.5 mM homo-arginine. After 5 weeks of liquid culture calli were plated in agar solidified K_3m without HA.
**In one experiment a lot of SR1-4058-38$^+$ micro calli were observed in the 0.1 mM HA petri dishes. However, counting of these micro calli with only a size of normal 2–3 weeks calli was impossible.

use of the ability of the tumour cells to grow in a phytohormone free medium [9, 21]. As this selection procedure is slow, it seemed useful to find out whether another T-DNA marker could be used for the selection of crown gall cells. In this paper we have shown that the LpDH gene, that is specific for octopine crown gall cells [3, 4, 13], can be used for this purpose. A major advantage of this marker is that the enzyme is expressed in all dicotyledonous plants tested thus far. In our experiments we have tested whether homo-arginine (HA), that was shown to be toxic for plant cells, could be used to select octopine crown gall cells since it had already been shown that this compound is converted into the non-toxic compound homo-octopine by the enzyme LpDH [15, 16]. The enzyme NpDH, which is present in nopaline tumour cells, does not use homo-arginine as a substrate [15, 16].

Indeed we have found that crown gall cells containing LpDH are resistant to HA, but that normal cells and crown cells containing NpDH – and lacking LpDH – are sensitive. Microscopic observation showed that HA selection can be accomplished within three weeks after which further growth of the calli is necessary if a test on LpDH activity is desired. This selection period is much shorter than in the case of a selection procedure that is based on phytohormone independent growth.

For mixed cultures of normal and crown gall cells, HA selection was not always successful. In two experiments all cells, including the crown gall cells, died in the presence of HA. This probability was not due to the action of HA but merely to a bad viability of the protoplasts in these few experiments. Plating efficiencies of control cultures of normal SR1 protoplasts in the experiments that did not result in any protoplast survival were indeed very low (data not shown).

In two experiments surviving calli were obtained from mixed cultures consisting of normal and crown gall protoplasts that were initially present at a ratio 100:1 and 10:1. Of these calli that survived the HA treatment, 70%

and 98% respectively, consisted of crown gall cells. Whether or not the HA selection procedure will be useful in protoplast transformation experiments will depend on the frequency of transformation. In bacterium protoplast transformation experiments the frequency of transformation can be as high as 10^{-2} [22]. Without any selection, therefore, one can expect to find 2 calli among 200 calli consisting of crown gall cells. In a model experiment simulating such a transformation experiment we found that, after HA treatment, among 200 calli tested 40 consisted of crown gall cells. From this observation it can be concluded that the procedure described here is in principle suitable for the selection of crown gall cells in bacterial transformation experiments.

By comparing the results of the culture of $LpDH^+$ and $NpDH^+$ crown gall protoplasts in the presence of HA, we conclude that HA resistance is specific for crown gall cells transformed by an octopine Ti plasmid.

With this new selection procedure a novel class of Ti plasmid transformed plant cells may be isolated efficiently, *viz.* cells showing LpDH activity, but lacking the ability to grow on phytohormone free medium [22]. T-DNA analysis of such cells will show whether or not the LpDH gene always is linked to the part of the T-DNA carrying the genes that promote phytohormone activities in crown gall cells.

Multiple copies of part of the T-DNA may be integrated into the plant's nuclear DNA [10]. Plant cells with multiple copies of the LpDH gene could possibly be identified by their higher level of resistance to HA. However, difference in HA resistance – as shown in the SR1-4013-3^+ cell line and the SR1-4013-44^+ cell line – may also be due to differences at the level of gene expression. At the moment there are no data suggesting differences in copy number of the LpDH gene in these cell lines [G. Ooms, personal communication].

Genetic engineering of plant cells will require the construction of suitable vectors. Provided that an efficient DNA transformation procedure for plant protoplasts will be developed, our results suggest that vectors carrying the LpDH gene as a selective marker may become useful for the genetic engineering of plants.

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