Theor Appl Genet (1992) 84:430–434

THEORETICAL AND APPLIED GENETICS

Modulation of auxin-binding proteins in cell suspensions

II. Isolation and initial characterization of carrot cell variants impaired in somatic embryogenesis

F. Filippini [1], M. Terzi [2], F. Cozzani [2], D. Vallone [1], and F. Lo Schiavo [2]

[1] Department of Genetics, Biology, and Molecular Biology, University of Naples, Naples, Italy
[2] Department of Biology and CRIBI, Via Trieste 75, I-35121 Padova, Italy

Received September 20, 1991; Accepted December 19, 1991
Communicated by P. M. A. Tigerstedt

Summary. Cultured cell lines from carrot (*Daucus carota* L.) with little or no embryogenic potential were examined for the auxin-binding capacity of their membranes. The lines belonged to different classes: (a) wild-type lines kept in culture for different periods (the longer the period, the lower being their embryogenic potential); (b) variants, isolated after mutagenesis, showing normal growth but a lack of embryogenic response; (c) auxin-resistant lines, isolated as colonies on solid media containing 45 μM 2,4-D; (d) a previously described tumorous line (E9) isolated because of its resistance to hypomethylating drugs. All of these lines showed alterations in auxin-induced, auxin-binding capacity (modulation), i.e. in the non-embryogenic lines the addition of auxin increased the auxin-binding capacity to a very small degree, or removal of the hormone did not produce the proper decrease in that capacity, or both defects could be simultaneously present. Both types of defects were shown to be correctable: after treatments designed to increase the amplitude of modulation, embryogenic capacity was restored in a number of lines.

Key words: Somatic embryogenesis – Auxin binding proteins – Auxin resistance – Developmental mutants – *Daucus carota*

Introduction

Lo Schiavo et al. (1991) have shown that, in carrot, embryogenic cell cultures consist of two types of cells: vegetatively proliferating cells that respond to increased levels of auxin in the medium by increasing their level of auxin-binding proteins (ABP) (we call this response ABP modulation) and proembryogenic masses (PEM), i.e., small clusters of cells, that are generated from proliferating cells capable of modulating their level of ABP. PEM once generated, lose the ABP-modulating capacity, and under appropriate conditions they will develop into embryos and regenerated plantlets.

In the study presented here we examined numerous lines with a much reduced, or even absent, embryogenic potential and found all of them to be limited in their capacity to modulate their ABP levels in response to auxin. Hence, the capacity to modulate ABP levels – from high to low levels, and vice versa, in a cyclical way – seems to be a pre-requisite of embryogenic capacity. Cells having either a high or a low level of ABP, but with the inability to change it in response to variations in exogenous auxin, show severe reductions in regeneration efficiency.

We will show that if treatments can be found that cause an increase in the amplitude of modulation, either by raising the level of response to auxin or by decreasing the constitutive level of ABP, embryogenesis will also result in lines that would be considered, under standard conditions, as being without embryogenic potential. This phenomenon may have interesting practical applications.

Correspondence to: M. Terzi

Materials and methods

Plant material and culture conditions

Our wild-type line A^+ derives from germinating seedlings of *Daucus carota* cv 'S. Valery'. The line and its derivatives described in the present work were cultivated in B5 medium (Flow) supplemented with 0.5 mg/l (2.3 μM) 2,4-D and 0.25 mg/l (1.1 μM) 6-benzylaminopurine (BAP). When the embryogenic potential showed signs of decline, the line was rejuvenated by isolating a few somatic embryos and treating them individually with auxin to induce de-differentiation. Each callus so obtained

gave rise to a sub-line; one of which was chosen for continuous cultivation. When this sub-line, in turn, showed signs of decline of the embryogenic potential, it also was rejuvenated in a similar manner. Under these conditions, the embryogenic potential is stable only for about 1 year. In this study line A^+ had been rejuvenated twice (hence designated A^+ T2).

Embryogenesis

Seven-day-old cell cultures consisting of isolated cells and clumps were filtered and resuspended at a low density in basal B5 medium in order to start the embryogenic process (time zero). Further details can be found in Giuliano et al. (1984). When the embryogenic efficiency had to be calculated, filtration was omitted and the number of embryos in the whole culture was scored 20 days after time zero.

Isolation of variants

Auxin-resistant lines

Carrot cell cultures were plated on solidified B5 medium (supplemented with 45 μ*M* 2,4-D and 1.1 μ*M* BAP) at 50,000 cell units/dish and scored for growth 2 months later. The frequency of the resistant colonies thus obtained was 6×10^{-5}.

emb^- lines

A culture was mutagenized with ethyl methanesulfonate (Giuliano et al. 1984), and set to embryogenize at 31 °C according to the protocol of Breton and Sung (1982). After the embryos developed at 31° had been discarded, the culture was plated and the colonies capable of growing but, upon subsequent testing, not capable of giving embryos even at the temperature of 25 °C, were isolated and designated MF1–MF7.

Membrane preparation

Cells were collected on the 4th day of sub-culture, washed and homogenized in a mortar with liquid nitrogen. The microsomal fraction was separated by centrifugation and resuspended in extraction buffer without detergents. The protein concentration was determined with the BioRad assay. For more details see Lo Schiavo et al. (1991).

IAA binding assay

This in vitro assay was conducted as described in Lo Schiavo et al. (1991) by incubating 50 μg proteins of a membrane preparation suspended in 500 μl binding buffer (7 m*M* Na_2 citrate, 5 m*M* $MgCl_2$, brought to pH 5.5 with citric acid) with 55.5 kBq[^{3}H]-IAA (1 TBq/mmole, Amersham) at 0 °C in the dark for 15 min, which is the time required for reaching equilibrium. At least three replicates were performed. The amount of binding was not affected by the addition of lipophilic substances, such as benzoic acid, nor of sucrose; similarly omission of sucrose from the extration buffer had no effect, which indicates that our results cannot be due to incorporation of IAA into artificial vesicles formed during membrane purification. The data on non-specific binding were not given as this was always negligible: with a 200× excess cold IAA, non-specific binding was less than or equal to zero.

Results

Embryogenesis and auxin-binding capacity

An embryogenic cell line tends to lose its embryogenic potential upon continuous cultivation. Unless the culture is periodically restarted from a clone (i.e. a de-differentiated embryo), its embryogenic potential tends to be lost in a matter of months, or a few years (Smith and Street 1974).

Table 1. IAA binding assay in vitro (pmoles of IAA bound to 50 μg of membrane protein extracted from cells kept for 4 days in the presence or absence of 2.3 μ*M* 2,4-D)

Cell line	2,4-D		Embryogenic potential[a]
	−	+	
A			
A^+T2	6.0	39.6	++
A^+T1	7.1	36.2	+
A^+	14.6	24.5	−
B			
MF1	3.0	6.6	−
MF2	2.8	7.2	−
MF4	3.2	6.4	−
MF7	2.7	3.1	−
E9	17.7	26.6	±
w1	3.9	10.1	−
w2	4.0	17.0	−
w3	3.7	21.7	−
w4	4.3	14.4	−

[a] Embryogenic potential was grouped as follows: ++ corresponds to an embryogenic efficiency greater than 0.5 embryos formed per cell unit; + means efficiency comprised between 0.05 and 0.4 embryos/cell unit; ± means efficiency on the order 0.001 to 0.04; − means an efficiency always lower than 0.0001 (i.e. embryos never seen under the conditions used)

With our wild-type (WT) embryogenic line A^+, we sequentially isolated subclones; the first one was designated A^+ T1. When the embryogenic efficiency of A^+ T1 began to decrease, we isolated a second subclone, designated A^+ T2. Embryogenic potential has been shown to be correlated with the capacity to modulate ABP (Lo Schiavo et al. 1991); in other words, the capacity to respond to auxin (auxin-binding capacity, ABC) by making more ABP when the auxin concentration in the medium is increased or to decrease ABP following auxin removal seems to be typical of good embryogenic lines. Table 1 shows such a correlation between embryogenic potential and capacity to modulate ABP, expressed as pmoles of IAA bound to 50 μg of membrane proteins, in an in vitro binding assay.

In order to better define this correlation, we decided to measure ABC in a series of poorly embryogenic or non-embryogenic carrot cell variants recently isolated in our laboratory.

emb^- lines

In our laboratory we routinely seek mutants that are temperature sensitive for embryogenesis. When we encounter colonies that show instead the absolute defect

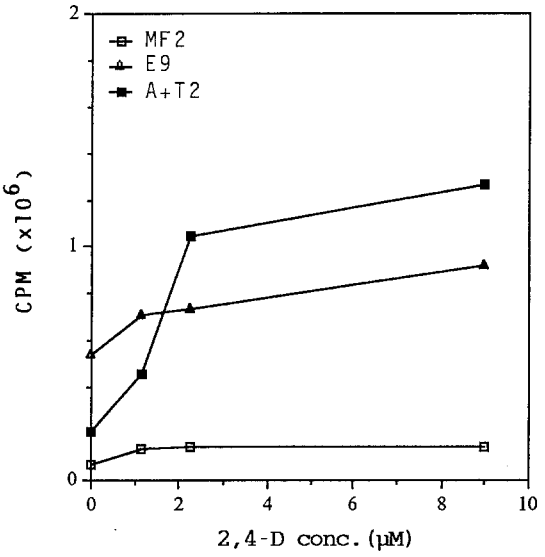

Fig. 1. IAA bound by ABP of cells of wild type (A^+ T2) and two variant lines (E9 and MF2) grown for 4 days in different concentrations (μM) of 2,4-D. The bioassay of ABP was performed as described in Materials and methods

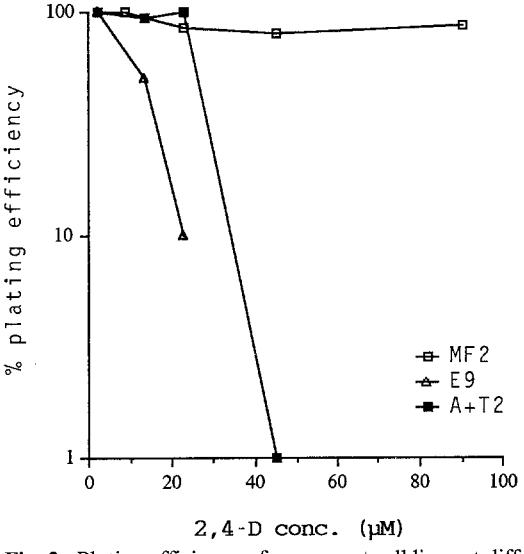

Fig. 2. Plating efficiency of our carrot cell lines at different concentrations of 2,4-D (micromolar). Plating efficiency at standard 2,4-D concentration (2.3 μM) was made equal to 100%

[emb$^-$ according to the terminology of Breton and Sung (1982)], these are isolated and designated MF plus serial number. Four of the MF lines were considered in the present study.

Auxin-resistant variants

They were obtained by plating a cell culture on solidified B5 medium containing 45 μM 2,4-D. Of the 25 colonies that grew, 4 were isolated, purified and designated w1–w4.

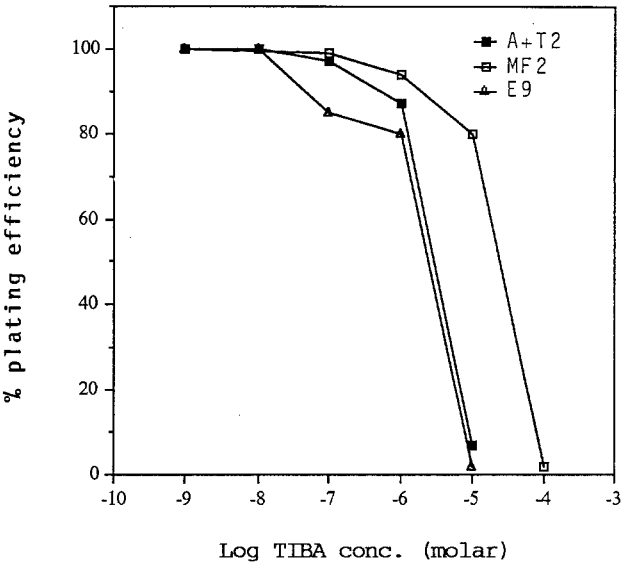

Fig. 3. Plating efficiency of our carrot cell lines in agarized B5 medium supplemented with TIBA at the concentrations indicated on the abscissa

To these two sets of variants we added E9, a semi-habituated tumorous line that is poorly embryogenic (Lo Schiavo et al. 1989). We measured ABP on all these lines, and the results, presented in Table 1, show that the MF and w series are characterized by low ABC after growth in the presence of 2,4-D, whereas the E9 line shows a relatively high ABC that is minimally reduced after removal of 2,4-D. All lines examined showed poor modulation, either because of a poor induction of ABP by 2,4-D (as in the MF and w series) or a poor reduction after 2,4-D removal, as in the case of A^+ and E9 lines.

Can the amplitude of modulation be increased?

We know from previous work (Lo Schiavo et al. 1991) that the amount of ABP correlates with the amount of 2,4-D in the medium over a certain range. It is thus possible to have an ABC higher than the one observed in standard B5 medium (see Fig. 1). But in order to choose the proper concentration, one has to know at what conducted with A^+ T2, E9 (as a poor reducer) and MF2 (as a poor responder). The results, presented in Fig. 2 show that (a) E9 is less resistant and (b) MF2, unexpectedly, is more resistant to auxin than WT.

The reason for the greater sensitivity of E9 could be that this line is semi-habituated and shows alterations in IAA metabolism (Lo Schiavo et al. 1989). Assuming that a high endogenous level of auxin is what characterizes E9, we tried to reduce this level with 7-azaindole, an inhibitor of IAA synthesis (Kochba and Spiegel-Roy 1977). The presence of 100 μM azaindole in basal B5 reduced ABC from 17.7 to 12.7 pmoles and, concomi-

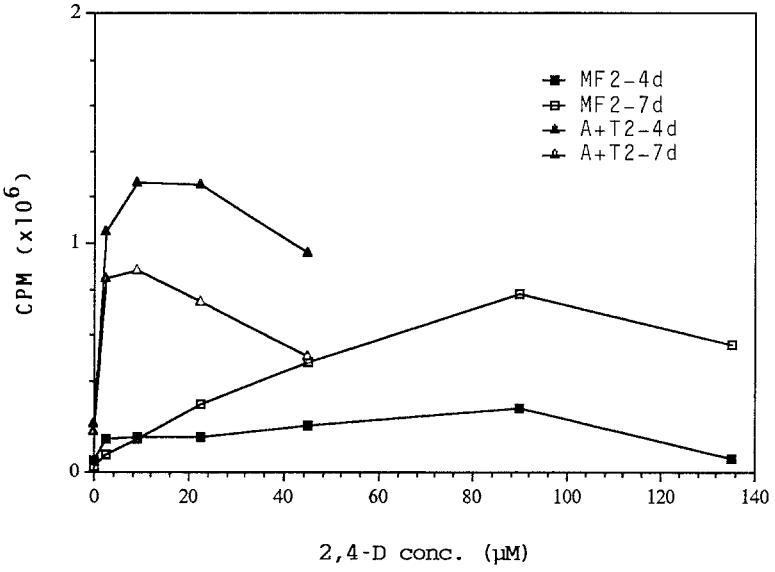

Fig. 4. IAA bound by ABP of A^+T2 or MF2 cells grown for 4 days (*closed symbols*) or 7 days (*open symbols*) in the presence of different concentrations of 2,4-D

Table 2. Effect of senescence on ABP

Cell line	Age (days)	IAA bound[a] (% added)
A^+T2	4	76
	12	22
E9	4	50
	12	27
MF2	4	11
	12	12

[a] Auxin-binding capacity can be inferred from the ratio of the amount of IAA bound over the amount of IAA added into the incubation mixture (reported in the last column)

Table 3. Correlation between ABC and embryogenicity measured at different 2,4-D concentrations

Lines	2,4-D concentrations (μ*M*)					
	2.3		45		90	
	ABC	EE[a]	ABC	EE	ABC	EE
A^+T2	39.3	++				
MF2	7.2	–	16.4	–	26.6	+
w1	10.1	–	22.8	+		
w2	17.0	–	30.5	+		
w3	21.7	–	29.5	+		
w4	14.4	–	26.1	+		

[a] ABC reported as pmoles bound by 50 μg membrane preparations; EE stands for embryogenic efficiency, measured as in Table 1

tantly, improved fivefold embryogenic efficiency (from 0.13% to 0.63%).

Line MF2 showed resistance to exogenous auxin, which might result from a lowered uptake of 2,4-D. That this occurs is indicated by the fact that triiodobenzoic acid (TIBA), which is known to block auxin efflux from the cells (Hertel and Leopold 1963), is less toxic to MF2 cells than the WT cells (see Fig. 3). We hypothesized that increasing the auxin concentration in the media up to 90 μ*M* might promote embryogenesis. This concentration of 2,4-D increased the level of ABP, but did not stimulate embryogenesis. If, however, the cells were exposed to this high concentration of 2,4-D for 7 days (instead of 4, the time used in the standard protocol for measuring ABP) the ABP level went even higher (Fig. 4), and embryogenesis was finally obtained. Figure 4 shows that 7 days are needed for MF2 to express ABC at the highest level; WT cells (which reach full induction in 2 h see Lo Schiavo et al. 1991) show higher levels of ABP induction at 4 days. This decrease with time might be an effect of senescence (Libbenga 1978) and is also shown by E9, but not by MF2 (Table 2).

The effect of treatments with high auxin was also examined using variants of the w series, and an increase in embryogenic efficiency was obtained in all cases (Table 3).

Discussion

Vegetatively proliferating carrot cell cultures respond to quantitative variations in exogenous auxin by varying their auxin-binding capacity. This capacity to modulate, which is a pre-requisite for embryogenesis, is lost by the embryos at their earliest stage (PEM).

We isolated lines incapable of embryogenizing: one of them was characterized and proven to be auxin resistant. We also isolated auxin-resistant lines, and they proved to

be incapable of embryogenesis. In all these cases, an increase in the concentration of auxin or in exposure time, or both, increases embryogenic efficiency to a remarkable extent, which, under standard conditions, was virtually nil.

Conversely, alterations were obtained in the embryogenic efficiency of E9, a line that, possibly because of a high endogenous concentration of auxin, was incapable of reducing the ABP level below a certain physiological threshold following removal of exogenous auxin. This result (a fivefold increase in embryogenic efficiency) was obtained with azaindole, an inhibitor of IAA biosynthesis. The possibility of determining the highest amplitude of modulation and of using those conditions for obtaining regeneration of recalcitrant lines may have important practical applications. This is especially true if modulation capacity proves to be of importance in the regeneration of other species as well.

We may ask, together with Guern (1987), if the ABP function as auxin receptors. We think they do since one of the effects of auxin that can be measured inside the cells, i.e. DNA methylation, correlates with the ABP level rather than the amount of auxin in the medium. This is true for E9 (Lo Schiavo et al. 1989), which is recalcitrant to hypomethylation and also cannot reduce the ABP level, and is also true for MF2, which has a very low level of ABP at auxin concentrations as high as 23 μM and also has low levels of methylation (unpublished data). More important perhaps is the correlation between auxin-binding capacity and embryogenic potential, which has been discussed throughout this paper and which seems to indicate a morphogenic receptor role for the ABP.

Our data do not provide evidence on whether there is just one auxin receptor or whether there are more receptors with diverse physiological roles. The fact that MF2 with low levels of ABP requires high auxin levels for embryogenesis but normal levels of the hormone for growth might favour the hypothesis of diverse receptors. The fact that in *Arabidopsis* auxin-resistant mutants have different genes (Lincoln et al. 1990 and references therein) points in the same direction. Extending the analysis we performed in MF2 to the other carrot cell variants we have isolated (and others we may obtain) could provide useful indications to that end.

Acknowledgements. This work was supported by EEC contract BRIDGE (T project on regeneration) and by MAF (Sviluppo technologie innovative).

References

Breton AM, Sung ZR (1982) Temperature-sensitive carrot cell variants impaired in somatic embryogenesis. Dev Biol 90:58–66

Giuliano G, Lo Schiavo F, Terzi M (1984) Isolation and developmental characterization of temperature-sensitive carrot cell variants. Theor Appl Genet 67:179–183

Guern J (1987) Regulation from within: the hormone dilemma. Ann Bot 60 [Suppl]:75–102

Hertel R, Leopold AC (1963) Versuche zur Analyse des Auxin-Transports in der Koleoptile von *Zea mays* L. Planta 59:535–550

Kochba J, Spiegel-Roy P (1977) The effects of auxin, cytokinins and inhibitors on embryogenesis in habituated ovular callus of the "shamouti" orange (*Cytrus sinensis*). Z Pflanzenphysiol 81:283–288

Libbenga KR (1978) Hormone receptors in plants. In: Thorpe T (ed) Frontiers in plant tissue culture. IAPTC, Calgary, pp 325–333

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell 2:1071–1080

Lo Schiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor Appl Genet 77:325–333

Lo Schiavo F, Filippini F, Cozzani F, Vallone D, Terzi M (1991) Modulation of auxin-binding proteins in cell suspensions. I. Differential responses of carrot embryo cultures. Plant Physiol 97:60–64

Smith SM, Street HE (1974) The decline of embryogenic potential as callus and suspension cultures of carrot, *Daucus carota* L., are serially subcultured. Ann Bot 35:233–241