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Isolation and developmental characterization of temperature-sensitive carrot cell variants

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Summary. Carrot cell lines multiply indefinitely in the presence of the auxin 2,4-D. If auxin is removed, the cells regenerate plantlets in a process that closely resembles embryogenesis in vivo. We isolated a temperature-sensitive variant, *ts 2*, which is unable to regenerate at 31 °C (non-permissive temperature), but does form embryos and plants at 24 °C (permissive temperature). The temperature treatment had no effect on fully differentiated *ts 2* plantlets. In other variants (*ts 5* and *ts 11*) cell proliferation was inhibited at the restrictive temperature. These lines were leaky with respect to the inhibition of embryogenesis at 31 °C.

Key words: Differentiation – Carrot – Somatic embryogenesis – Temperature sensitivity

Introduction

Totipotency, i.e. the ability to regenerate whole plants from single cells – demonstrated in carrot cell cultures by Reinert (1958) and Steward (1958) – offers unique opportunities for the analysis of ontogeny.

In auxin-free medium, single carrot cells give rise to embryoids which, with respect to their development, closely parallel the various embryonic steps of the seed. The minor differences found between the embryoids and the zygotic embryos are the pattern of the first cell divisions (McWilliam et al. 1974) and the somewhat lesser extension of the cotyledons (Halperin 1966). Techniques for the synchronization of embryo induction in culture (Fujimura and Komamine 1979) and for the isolation of large, pure fractions of embryonic stages (Warren and Fowler 1977; Giuliano et al. in preparation) are now available and facilitate biochemical studies.

Abbreviations: EMS = ethylmethanesulfonate; EU = embryogenic unit (see Materials and methods); *ts* = temperature-sensitive; 2,4-D = 2,4-dichlorophenoxyacetic acid

Conditional mutants have proved to be essential tools in the genetic dissection of development of organisms as diverse as *Drosophila* (Suzuki et al. 1976), *Caenorhabditis* (Hirsch and Vanderslice 1976) and *Volvox* (Huskey et al. 1979). Temperature-sensitive (*ts*) carrot lines capable of regenerating plantlets at the permissive temperature of 24 °C, but blocked in various ways at 32 °C, have been described by Breton and Sung (1982) and Sung and Okimoto (1981). Preliminary data about the isolation of similar lines have also been reported from our laboratory (Giuliano et al. 1981; Terzi et al. 1982).

Here we describe in detail the isolation and the characterization of some of the variants, and their use in attempting a temporal dissection of development. The classes of variants recovered both by Breton and Sung (1982) and in our laboratory were essentially two: *ts*-emb$^-$, in which the inhibition is confined to embryonic development, and *ts*-growth$^-$, in which both embryogenesis and cell growth are affected.

Materials and methods

Carrot cells (*Daucus carota* var. 'S. Valery') were grown in suspension in Gamborg's B5 medium, commercially available from Flow. The medium (referred to as B5A) contained 1 mg · l^{-1} 2,4-D (From BDH). For callus induction B5+ medium, containing 0.5 mg · l^{-1} 2,4-D and 0.25 mg · l^{-1} 6-Benzylamino purine (Sigma), was used. For embryoid induction B5 medium devoid of hormones (B5-) was used.

The cultures were kept at 24 °C in flasks put on rotary shakers (80 rpm) under a light intensity of 500 lx. Subculturing was performed regularly every 20 days by inoculating 2 ml packed cell volume (after centrifugation for 5 min at 200 g) in 50 ml fresh medium. Cell growth was checked as described by Vergara et al. (1982).

Regeneration was obtained, starting from the 50 to 80 μm fraction of a culture, in the following way: a culture at its 8th day from subculturing was filtered through a nylon sieve with pore size 80 μm and the filtered fraction was refiltered on a second sieve with 50 μm pore size. The clumps remaining on the sieve (each containing 7 ± 3 cells) were washed three times with B5- and resuspended in the same medium.

For the characterization of *ts* lines, two concentrations (6,000 and 12,000 clumps/ml) and three replicas per concentration were routinely used. Three ml aliquots of the resulting suspensions were put in 6 well (∅ 35 mm) tissue culture trays (Corning) and kept on a shelf under 1,500 lx (continuous illumination). Single Petri dishes (∅ 35 mm), filled with 3 ml of suspension (6,000 clumps/ml), were used in temperature shift experiments with the *ts 2* line. Small volumes and low cell concentrations allowed rapid and precise counting of embryoids in the whole plate without sampling. Counting was done after 15 days under 40× magnification and expressed in terms of clumps giving rise to embryoids (referred to as Embryogenic Units or EU).

Chromosome counts were performed on cell cultures 5 days after subculturing. Before fixation in Carnoy (ethanol-acetic acid = 3 : 1), the cultures were treated with colchicine (0.05% in distilled water) for 4 h. The staining was done with Feulgen (Darlington and La Court 1962).

Mutagenesis was performed with EMS 0.8% (v/v) for 1 h at room temperature in a screw-cap tube. The culture was then centrifuged and washed three times with fresh medium. This treatment kills about 80% of the colony formers.

For temperature shift experiments embryoids were isolated by hand under a dissection microscope and transferred to fresh B5-medium. Plantlets were put on B5-medium fortified with agar (Oxoid Technical Agar N°3, 0.8% w/v).

Embryoids usually grew into plantlets within 20 days, while plantlets developped in the same time from about 2 mm to about 1–2 cm in length. Temperature sensitive embryoids, on the other hand, were arrested in development at various stages (Terzi et al. 1982).

Temperature was kept at 24° ± 0.5 °C or 31° ± 0.5 °C in air forced circulation rooms (Bertagnin, Bologna). Good temperature control was crucial for reproducibility.

Results

Isolation of ts variants

An essentially diploid (original line, Fig. 1) culture was mutagenized with EMS and subcultured in fresh B5A medium for five days to allow phenotypic expression of the mutations. It was then filtered and the 50 to 80 μm fraction was induced to differentiate at 31 °C under continuous agitation. Cell density was 5×10^3 cell clumps/ml.

After 14 days, the cultures were filtered through a nylon screen (250 μm mesh) which removed 20% of the globular embryos, 60% of the heart-shaped embryos and all the embryos in later stages of development, while undifferentiated cell clumps remained in the filtrate. The filtrate was then cultured at 24 °C for 5 days (experiment 1) or 9 days (experiment 2, performed independently). At the end of this period the newly formed plantlets and some of the torpedoes were collected on a screen (300 μm mesh). This procedure eliminated about 90% of the embryoids which were initially present. Each experiment began with 5×10^5 clumps (approximately 3.5×10^6 cells).

From the embryoids, calli were induced on B5+ medium (polyembryos were discarded) and tested for the *ts* phenotype as follows: from the calli cell suspensions were established in B5A medium and mid-exponential phase cells were pelleted and resuspended at low density in B5-medium. Twenty ml aliquots in 90-mm Petri dishes were incubated at 24 °C and 31 °C. Candidates inhibited partially or completely in embryogenesis were regenerated into plantlets. From these plantlets, suspension cultures were re-established and re-tested for the *ts* phenotype.

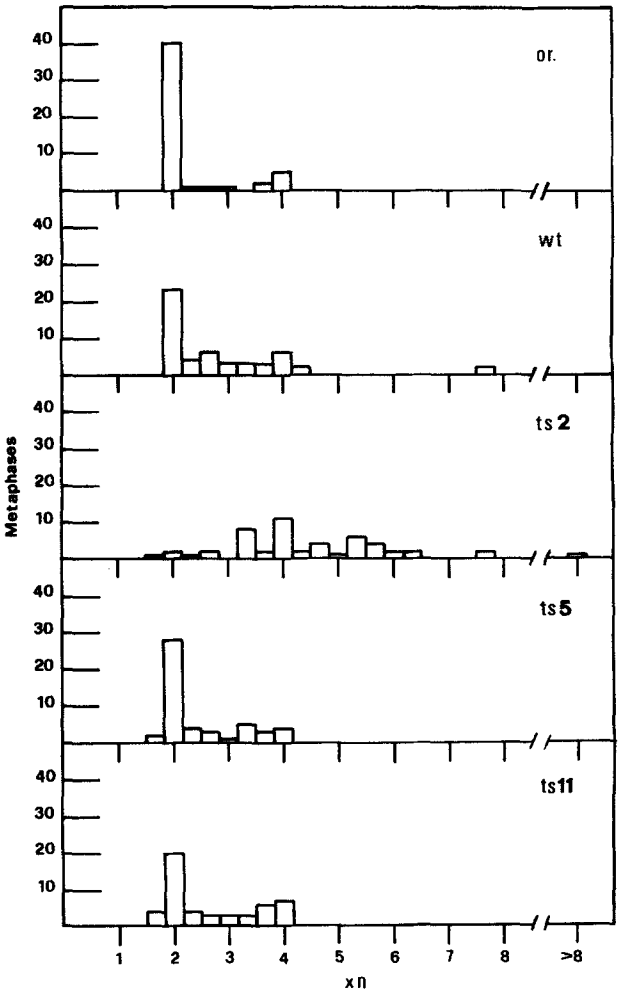

Fig. 1. Frequency distribution of chromosomal numbers expressed as degree of ploidy (diploid number 2n = 18). *Or* = starting culture

Experiment 1 yielded 80 lines, five of which were *ts*. Experiment 2 yielded 115 lines, 8 of which *ts*. Most lines were "leaky", with respect to the *ts* phenotype (Table 1). Line *wt*, which was subjected to the regeneration-dedifferentiation cycle but not to the mutagenesis and filtration-enrichment steps, is used as control.

In Fig. 1 the frequency distributions of chromosomal counts of some of the lines analyzed are presented. Among 7 lines analyzed (of which 3 are shown) only

Table 1. Variants resulting from the screening

Cell line	% inhibition of embryogenesis[a]	% inhibition of growth[b]
wt	0	27
ts 1	95	26
ts 2	100	33
ts 3	47	20
ts 4	48	45
ts 5	22	86
ts 6	96	58
ts 7	59	65
ts 8	71	47
ts 9	56	not determined
ts 10	58	30
ts 11	52	90
ts 12	80	56
ts 13	84	87

[a] (EUs at 24 °C – EUs at 31 °C)/EUs at 24 °C scored after 15 days. For further explanation see materials and methods

[b] The doublings of cell volume in a 20 day growth cycle were measured as described in Vergara et al. 1982. The % inhibition was obtained by the formula (Doublings at 24 °C – Doublings at 31 °C)/Doublings at 24 °C

ts 2 showed significant deviation from diploidy – being predominantly tetraploid.

Line ts 2

This line had a completely penetrant *ts*-emb$^-$ phenotype, suitable for developmental analysis. Growth in B5A medium was unaffected at the non-permissive temperature (Table 1). Therefore a characterization with temperature shifts was performed. Differentiating cultures in B5 – were put at both the permissive and non-permissive temperature and then shifted either up (24 °C → 31 °C) or down (31 °C → 24 °C) after increasingly longer periods of time. In addition, embryoids originating at 24 °C were isolated by hand and shifted upwards. The results, shown in Fig. 2, were essentially the following:

a) Embryo development is arrested in a culture whenever the temperature is shifted upwards between day 2 and day 8 after induction of embryogenesis. Arrested embryos present morphological abnormalities (not shown).
b) *ts* inhibition is perfectly reversible after shift down in temperature.
c) *ts* inhibition is abolished after completion of embryonic development (*ts 2* plantlets develop normally after the shift up).

Lines ts 5 and ts 11

ts 5 and *ts 11* are *ts-growth*$^-$ (Table 1, Fig. 3). Inhibition of embryogenesis is leaky in these lines, especially in

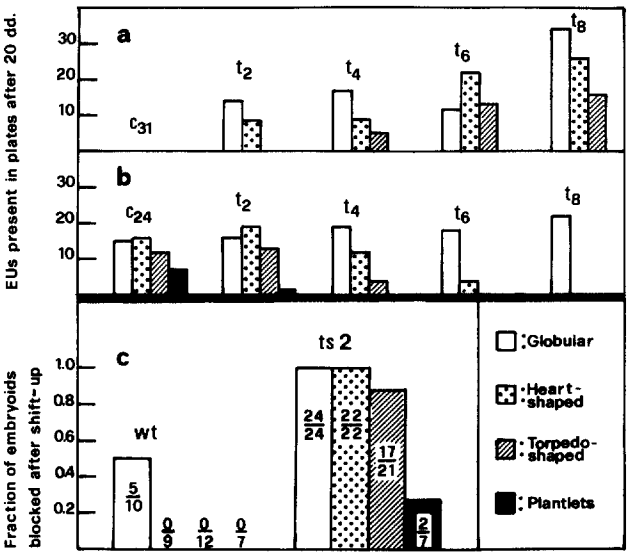

Fig. 2a–c. Temperature shift experiments with line *ts 2*. In the shift-up experiments (**a**) C_{31} represents the control plate kept at 31 °C all the time, whereas plates t 2, t 4 . . . were shifted at t = 2 days, 4 days etc. In the shift-down (**b**) C_{24} is the control plate differentiated at 24 °C. Plates were scored at t = 20 days. The fraction of embryoids of *ts 2* blocked after manual isolation and shift-up is shown in **c** (actual numbers inside bars) together with *wt* as control. 7/13 *wt* globular embryoids, manipulated in the same way and left at 24 °C, were blocked in development, thus showing sensitivity to manipulation rather than temperature

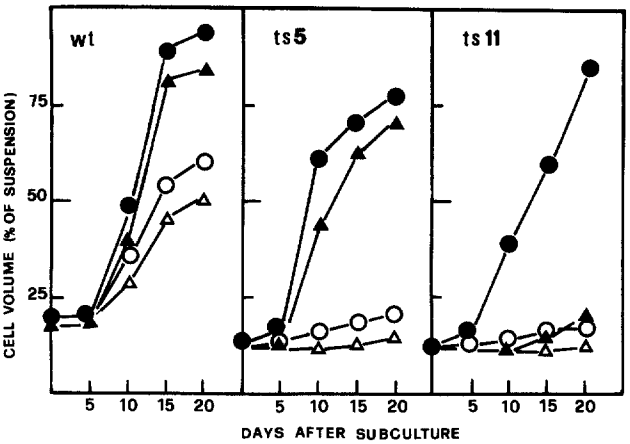

Fig. 3. Growth curves of cell suspensions of the *ts*-growth$^-$ lines at 24 °C (*closed symbols*) and 31 °C (*open symbols*). Cell volume was measured by sedimentation (Vergara et al. 1982). Triangles represent growth of cultures pre-grown for one cycle at 31 °C. Circles represent cultures that were pre-grown at permissive temperature

ts 5. To see if growth inhibition resulted in cell death, cells pregrown for 20 days at 31 °C were reinoculated in fresh medium at 24 °C. *ts 5* cells regained immediately their growth capacity, while *ts 11* showed virtually no recovery (Fig. 3). In addition, a considerable inhibition

Table 2. Effect of preculture at permissive and non-permissive temperature on embryo differentiation (EUs after 15 days normalized)

Preculture at:	24 °C		31 °C	
Temperature during culture in differentiation medium:	24 °C	31 °C	24 °C	31 °C
wt	1.00 (209 ± 18)	0.97 (202 ± 11)	0.89 (187 ± 12)	0.95 (198 ± 8)
ts 5	1.00 (176 ± 10)	0.78 (137 ± 13)	0.24 (42 ± 1)	0.10 (18 ± 1)
ts 11	1.00 (75 ± 3)	0.48 (36 ± 1)	0.49 (37 ± 2)	0.28 (21 ± 1)

Cells pregrown for 8 days at permissive and non-permissive temperature were induced to differentiate at both temperatures at a concentration of 12,000 cell clumps/ml. EUs were counted after 15 days and the results normalized, assuming those in the first column (24 °C + 24 °C) to be = 1 (actual nos. within brackets)

of embryogenic potential was observed in cells from both lines pregrown for 8 days at 31 °C (Table 2).

Discussion

In the preceding sections we have described the isolation and developmental characterization of *ts*-emb$^-$ and *ts*-growth$^-$ carrot cell lines. Negative selection with 5-Bromodeoxyuridine, used by Malmberg (1979) to isolate plant cells *ts* for growth, was not practical here, due to the inhibition of embryogenesis by this substance (Dudits et al. 1979). We used, therefore, a filtration-enrichment procedure. The enrichment used was not more than 10-fold. The selectivity of the procedure could, in principle, be increased by repeated removal of embryoids at 31 °C or by the use of thinner mesh screens. However, no *ts* variants were recovered among the 40 lines obtained after a more radical (150 μm mesh) filtration at 31 °C (Terzi et al. 1982).

We believe that the *ts* phenotype is due to a mutation since it was maintained through repeated cycles of plant regeneration.

In our cultures the embryogenic efficiency ranged between 10^{-3} and 10^{-2} (EU's/total clumps); this compares well with other reported domestic carrot efficiencies (10^{-5}–10^{-3} embryoids/cells) (Hari 1980; Breton and Sung 1982) although higher efficiencies were also reported with particular conditions and strains (Fujimura and Komamine 1979).

The *ts*-emb$^-$ phenotype of *ts 2* is of great interest since the *ts* function is expressed only during embryogenesis: neither growth in B5A nor adult development are affected. The inhibition is reversible if the treatment is applied before the onset of embryogenesis. This suggests that the function of *ts 2* is that of a regulator of the developmental pattern rather than being a "housekeeping" function. Once embryogenesis has started, shifting the temperature upwards places the embryoids in a developmental dead end: blocked embryoid forms bear morphological abnormalities (Terzi et al. 1982) and are no longer capable of reverting to normal development if shifted to permissive temperatures.

We recovered another interesting variant, *ts 1*, in which embryogenesis proceeded at 31 °C until the globular stage, but was then completely blocked at that stage. This line was lost (it grew very slowly at both temperatures). More *ts* lines would be essential for a detailed temporal dissection of embryogenesis.

Lines *ts 5* and *ts 11* are both *ts*-growth$^-$ types. They differ in that *ts 5* cells survive at 31 °C, while *ts 11* cells die. Functions affected in these lines have yet to be clarified.

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