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Early phases in in vitro culture of tomato cotyledons: starch accumulation and protein pattern in relation to the hormonal treatment

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Summary. Tomato cotyledon explants, cultured in vitro in the presence of sucrose, were subjected to different hormonal treatments to establish whether the induction of different organogenic programmes could be correlated with differences in starch accumulation and protein electrophoretic pattern. The cytohistological changes in explants over the first 15 days of culture were studied by light and electron microscopy. It was found that starch accumulation occurs under all conditions, though varying in duration and amount. Over the first 2 days of culture the protein electrophoretic pattern changes in a similar way under all conditions, while after 7 days changes take place which are probably related to the different developmental programmes induced by the treatments.

Keywords: In vitro culture; Tomato; Starch accumulation; Protein pattern.

Abbreviations: BOA benzisoxazole-3-acetic acid; 2,4-D 2,4 dichlorophenoxyacetic acid; DTT dithiothreitol; GA_3 gibberellic acid; HF hormone-free; LM light microscopy; LSB Laemmli sample buffer; PAGE polyacrylamide gel electrophoresis; PMSF phenylmethylsulfonylfluoride; TEM transmission electron microscopy; ZR zeatine riboside.

Introduction

Starch accumulation in callus cells seems to be a prerequisite for the development of shoots in vitro (Thorpe and Meier 1974, Ho and Vasil 1983, Thorpe et al. 1986, Stamp 1987). In tobacco, shoots do not develop if mannitol is used in place of sucrose in the culture medium (Mangat et al. 1990), or if starch accumulation is prevented by treating the callus with gibberellic acid (Murashige 1961, 1964; Thorpe und Murashige 1968, 1970). On the other hand, organogenesis in vitro is strictly correlated to the ratio and quantity of growth regulators in the culture medium. In tomato, in the presence of sucrose and optimal concentrations of cytokinin it is possible to obtain shoots, roots, hairy root-like structures or callus, in that order, by progressively increasing the concentration of auxin (Branca et al. 1991).

We have now investigated whether there is a correlation between starch accumulation and the induction of different organogenic programmes. We have studied the cytohistological changes that occur in tomato cotyledon explants plated without growth regulators, or induced to develop either shoots or callus by the administration of different growth regulators in the presence of sucrose. We have also tried to ascertain whether the changes in protein electrophoretic pattern which occur under different culture conditions (Reynolds 1989, 1990) correlate with the different morphogenetic programmes.

Materials and methods

Seeds of *Lycopersicon esculentum* Mill. var. Alice were sown and germinated as described by Gavazzi et al. (1987). Cotyledons were excised, cut in half along the midrib, and plated on MS medium (Murashige and Skoog 1962) containing 0.8% agar and 30 g/l sucrose. They were incubated at 25 °C and 90 μmol/m²/s light intensity, under a 16 h photoperiod. Five groups of 20 plates, each containing 12 explants, were used.

Each group was subjected to a different hormonal condition, as follows: (1) hormone-free; (2) 1 μM GA_3 (GA_3 treatment), which inhibits starch accumulation (Thorpe and Murashige 1968); (3) 1.4 μM ZR plus 20 μM BOA, which induces shoots (Branca et al. 1991); (4) 1.4 μM ZR, 20 μM BOA, and 1 μM GA_3; (5) 1.4 μM ZR plus 20 μM 2,4-D, which induces callus (Branca et al. 1991).

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All growth regulators were sterilised by filtration and added to MS medium after autoclaving.

1, 2, 4, 7, and 15 days after plating a 1 mm wide strip was cut parallel to the cut midrib. The strips were fixed in phosphate-buffered glutaraldehyde, dehydrated in ethanol and embedded in Araldite. Semithin sections (0.5 µm) were stained with toluidine blue and observed with a Zeiss Axioskope. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a Siemens Elmiskop 1 A electron microscope.

Protein content and electrophoretic pattern

2 and 7 days after plating other strips were cut and those of each group were pooled, weighed, immediately frozen in liquid nitrogen and homogenised in extraction buffer (Hawkesford and Belker 1991), containing 0.1 mM PMSF and 1 mM DTT. As controls, strips were taken from cotyledons of 8-day-old seedlings and extracted as above. Protein content was assessed by the Biorad assay method (Bradford 1976) on homogenate samples precipitated according to the method of Renaudin et al. (1991).

Other samples from the homogenates were used for protein extraction by the method described by Hawkesford and Belker (1991), slightly modified as follows. The pellets obtained after ammonium acetate/methanol precipitation were washed twice with the latter and then with 80% (v/v) acetone in water. After drying the pellets were resuspended in LSB buffer (Laemmli 1970) to the standard concentration of 3 mg/ml protein, heated at 80 °C for 10 min and centrifuged at 5000 rpm in a Beckman JS 75 centrifuge. The supernatants were supplemented with 0.1 mM PMSF and 1 mM DTT and stored at −80 °C. The extracts were used for one dimensional PAGE.

1.5 mm thick gradient acrylamide gels (7.5–15%) were used and 35 µg protein were loaded on top of each lane. Markers of different M_r were run concurrently. Gels were run at 50 mA constant current, stained with Coomassie blue and read with a Molecular Dynamics Personal Densitometer ImageQuant v3.3.

Results

After 1 day in culture, no starch deposition occurred in the GA_3 treatment (Fig. 1 a). In the presence of ZR with BOA and GA_3 or ZR with 2,4-D starch deposition was restricted to the guard cells and a few cells of the palisade layer (Fig. 1b). In HF medium, or in the presence of ZR with BOA, starch grains were observed in the cells of the bundle sheaths of the main veins, in the vascular parenchyma of the midrib and in the guard cells of stomata.

After 2 days, in HF and ZR with BOA media starch grains were present in the vascular parenchyma of the veins and in the palisade and epidermal cells located above them (Fig. 1 c). In the presence of 2,4-D or GA_3 starch deposition was also evident, being observed in the stoma guard cells, in a few palisade cells (Fig. 1 d) and in the bundle sheath and vascular parenchyma cells of the main veins (Fig. 1 e). In HF-cultured explants, cell proliferation (i.e., wound callus formation) was evident in the excision area, while explants treated with ZR and 2,4-D showed meristematic groups associated with the veins (Fig. 1 f).

At this time, the protein electrophoretic pattern changed in a similar way in all treatments (Table 1). Compared with the protein pattern of freshly excised cotyledons, there was an increase in proteins of 94, 91, 88, 83, and 30 kDa, and a decrease in proteins of 54, 44, and 14 kDa.

After 4 days, starch localisation and content were the same in HF, GA_3, or ZR with 2,4-D treatments, while starch increased in the bundle sheath cells of ZR with BOA- or ZR with BOA and GA_3-treated explants. At this time, conspicuous differences were observed between the treatments in relation to the proliferative capacities of the explants. With HF wound callus formation continued, but no meristem formation occurred. In GA_3 no sign of proliferation or meristematic formation was observed, while in ZR with BOA or ZR with BOA and GA_3 treatments the first meristematic groups originating from the phloem parenchyma of the main veins were visible and the first signs of proliferation occurred in the excision area. With ZR and 2,4-D meristem development was more intense, as meristemoids were even observed near the uncut cotyledonary margin (Fig. 1 g, h), and callus formation occurred near the excision area.

After 7 days, HF- or ZR with BOA-cultured cotyledons have conspicuous starch grains with manifest signs of fragmentation in the upper epidermis and palisade layer (Figs. 1 i and 2 a), a well as in the bundle sheaths

Fig. 1. Tomato cotyledon explants cultured under different hormonal conditions. Bars: a, b, d, e, and i, 1 µm; c, 30 µm; f, 40 µm; g, 100 µm. **a** TEM, Chloroplast of palisade cell. No starch is visible after 1 day of GA_3 treatment. **b** TEM, small starch grains in a palisade cell chloroplast, after 1 day of BOA with ZR and GA_3 treatment. **c** LM, starch grains in the cells of the palisade layer and adaxial epidermis above a vein, surrounded by a sheath of cells with starch grains, after 2 days in HF medium. **d** TEM, palisade cell chloroplasts with large starch grains, after 2 days of ZR with BOA and GA_3 treatment. **e** TEM, large starch grains in amyloplasts of a bundle sheath cell, after 2 days of ZR with BOA and GA_3 treatment. **f** LM, meristematic group associated with a vein, after 2 days of ZR with 2,4-D treatment. Xylem elements of the vein. **g** LM, meristemoids associated with different order veins at the uncut cotyledonary margin, 4 days after ZR with 2,4-D treatment. **h** LM, detail of a meristemoid, 4 days after ZR with 2,4-D treatment. **i** TEM, partially fragmented starch grains in the plastids of palisade cells after 7 days of ZR with BOA treatment

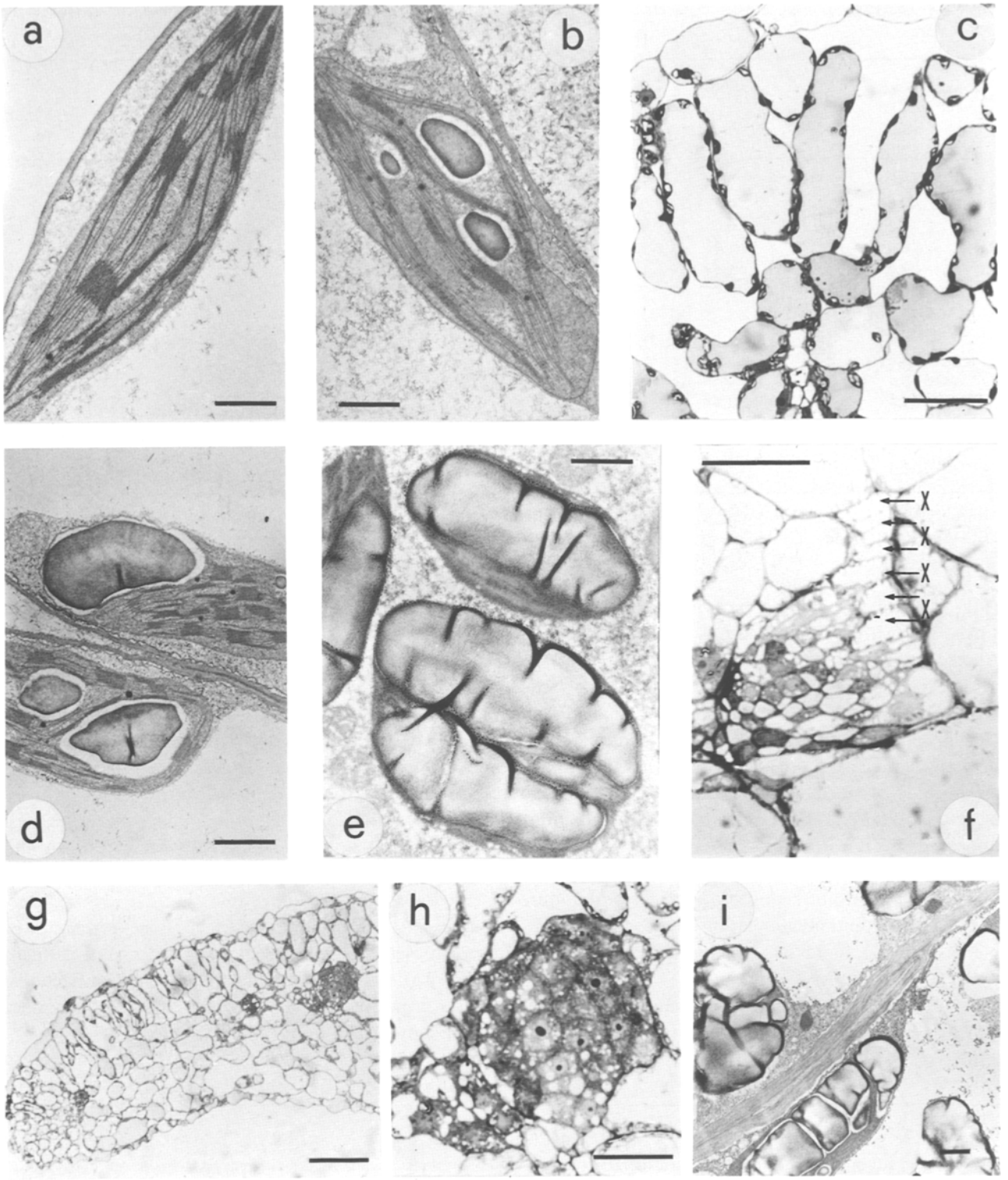
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Table 1. Protein changes in tomato cotyledon explants after 2 or 7 days in culture under different hormonal conditions, compared with the excision time (T_0)

kDa	T_0	HF		GA_3		ZR + BOA		ZR + BOA + GA_3		ZR + 2,4-D	
		2d	7d	2d	7d	2d	7d	2d	7d	2d	7d
94	0.6	1.4	1.1	1.3	1.0	1.7	1.7	1.8	2.3	1.8	0.7
91.5	0.6	0.9	0.7	0.7	0.5	1.1	0.0	0.8	0.6	0.9	0.0
91	0.2	0.5	0.6	0.5	0.7	0.5	1.0	0.4	0.8	0.5	0.6
88	0.6	1.7	1.4	1.7	1.6	2.5	2.2	2.1	2.4	2.0	0.6
83	1.0	1.5	1.4	1.4	1.6	2.0	1.9	2.0	1.6	1.8	1.0
61	3.1	3.1	7.9	3.0	8.8	2.9	4.3	3.2	5.8	3.1	2.5
54	27.3	16.2	13.3	23.5	11.2	18.3	8.7	22.1	7.6	18.8	7.3
44	3.6	2.3	3.1	1.9	2.3	2.1	1.8	3.0	1.6	1.6	1.4
33	1.6	1.4	5.1	1.5	6.9	1.7	1.4	1.6	3.4	1.7	1.7
30	0.8	2.1	2.0	1.2	1.9	1.5	2.7	1.4	2.6	1.7	2.9
27.5	1.8	1.6	2.8	1.7	2.6	1.8	2.7	1.9	2.6	1.8	4.1
24	1.0	0.9	0.9	1.1	1.5	0.9	0.4	1.0	0.5	1.0	0.0
16	1.0	1.1	1.5	1.0	1.0	0.9	2.7	1.0	1.5	0.9	4.6
14	7.3	3.5	2.5	4.6	2.2	3.1	2.1	3.0	2.1	3.0	1.4

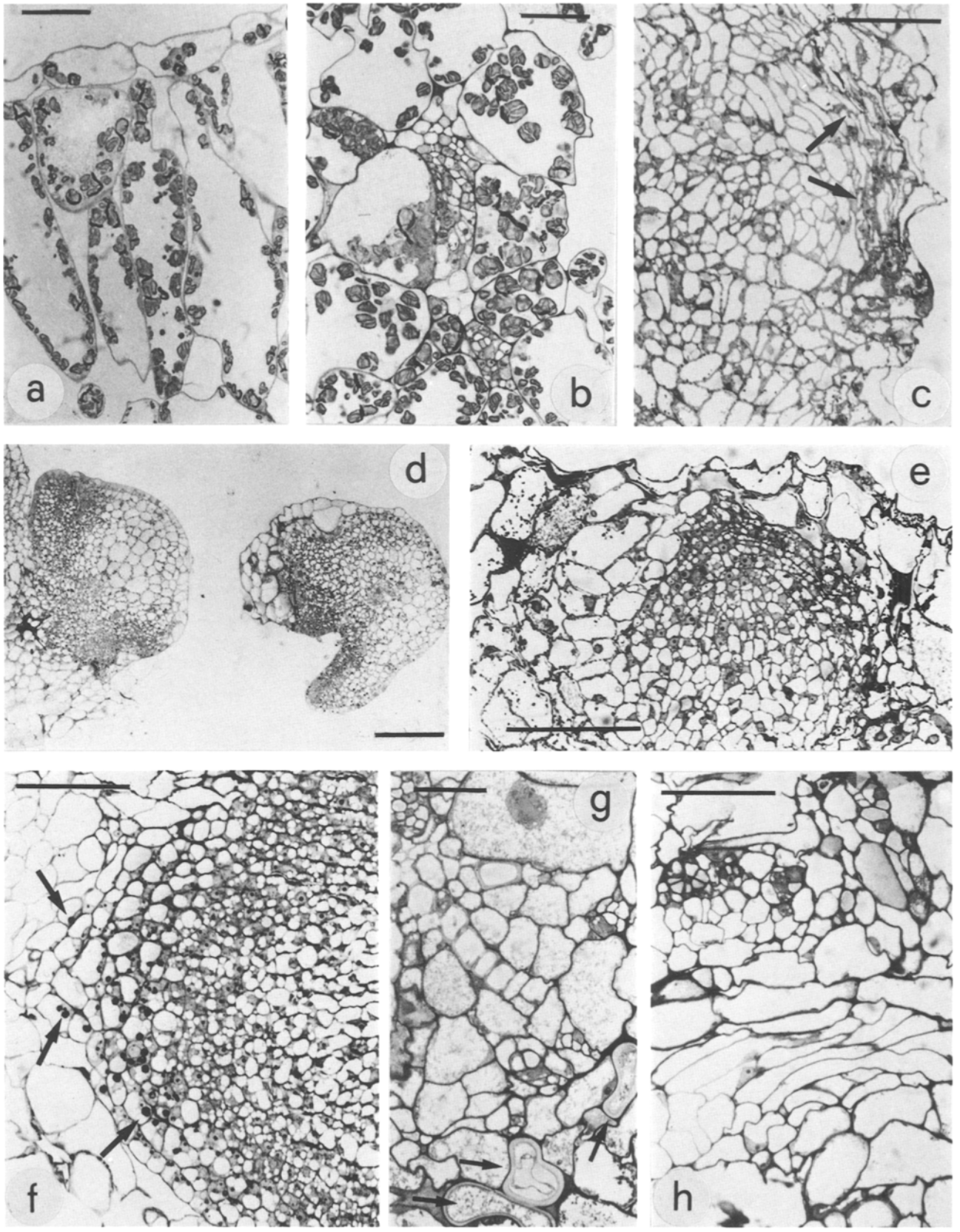
Proteins were separated by 1 D-PAGE. The gels were scanned and the volume of each band determined (volume = optical density × area). Data expressed as means of 3 separate experiments where the S.E. was less than 0.2

(Fig. 2 b) and vascular parenchyma of the veins. In GA_3-, ZR with BOA and GA_3-, or ZR and 2,4-D-treated cotyledons starch was very rare or completely absent from proliferating tissues and for the meristematic areas (Fig. 2 c), or in the original explant tissue. Cell proliferation at the excision site continued and also appeared in GA_3-treated cotyledons (Fig. 2 c).

At this time, other changes were observed in the protein electrophoretic pattern. For instance, band 91.5 disappeared in ZR with BOA and ZR with 2,4-D treatments and band 24 in ZR with 2,4-D treatment. Other bands which had not changed after 2 days of culture increased differentially in relation to the various treatments (Table 1). Bands 54 and 14 were further reduced under all conditions.

After 15 days, in HF-cultured cotyledons starch content was very low. Occasionally, but very rarely, one root emerged from the explants, when minute starch grains were present in the root cap. In ZR with BOA treatment too starch content decreased, but various sized grains were still present, mainly in the wound callus zone, from which vegetative buds emerged (Fig. 2 d). In ZR with 2,4-D treatment root primordia were observed (Fig. 2 e). In this case starch presence was restricted to the "de novo" formed root cap cells (Fig. 2 f), rarely observed in the other explant regions. No starch was present in GA_3- or ZR with BOA and GA_3-treated explants, either in the vascular bundles (Fig. 2 g) or in the callus area (Fig. 2 h). Nonetheless differentiation programmes, such as neoformation of xylem elements in the main veins in GA_3 treatment (Fig. 2 g), or formation of highly anomalous vegetative buds primordia in ZR with BOA and GA_3 treatment (not shown) also occurred in these explants.

Fig. 2. Starch, cell proliferation and organ formation in tomato cotyledon explants cultured under different hormonal conditions. Bars: a, b, and g, 30 μm; c and h, 40 μm; d and e, 200 μm; f, 60 μm **a** LM, starch grains with signs of digestion in the cells of the adaxial epidermis and palisade layer, after 7 days in HF medium. **b** LM, secondary vein surrounded by cells filled with starch grains undergoing digestion, after 7 days inf HF medium. **c** LM, strands of cambium-like cells (arrows) at the periphery of a proliferation zone, originating from the excision area. No starch is present after 7 days of GA_3 treatment. **d** LM, irregular shaped vegetative buds, near the surface of the proliferated area, after 15 days of ZR with BOA treatment. **e** LM, root primordium surrounded by callus cells, after 15 days of ZR with 2,4-D treatment. **f** LM, tip of root primordium, after 15 days of ZR with 2,4-D treatment. The root cap cells contain starch grains (arrows). **g** LM, xylogenesis at the boundary of the midrib, after 15 days of GA_3 treatment. Arrows show newly formed tracheary elements. **h** LM, wound callus without starch, after 15 days of ZR with BOA and GA_3 treatment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Discussion

Our results show that starch deposition occurs in all treatments, although it starts at different times and varies in its extent. It seems that, regardless of the treatment, amylogenesis is more intense in stomatal guard cells and bundle sheath cells. It is also evident that both GA_3 and ZR with 2,4-D only partially inhibit starch deposition, and that this inhibition does not prevent starch accumulation in cells able to store starch, such as bundle sheath (Avery 1933, Coleman and Greyson 1977) and root cap cells.

While some proteins undergo a conspicuous increase in all explants after 2 days in culture, irrespective of their culture conditions, when wound callus formation and differentiation have begun the electrophoretic pattern undergoes various changes according to the culture conditions. It seems therefore that the increase in protein observed over the first days of culture is non-specific and probably due to plating-induced stress, while the later changes are probably related to the different differentiation programme induced by the hormonal treatments.

To conclude, starch accumulation occurs within the first days of culture, irrespective of the future development of the explants, and therefore the culture regime does no influence amylogenesis. Changes in the protein pattern, probably attributable to the hormonal treatments, only become apparent at a relatively late stage, when cell differentiation is visible.

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