Structure and physiology of the development of roots from microcalli of leaf protoplasts of Ri-transformed and wild type *Nicotiana plumbaginifolia*

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SUMMARY

Data are presented of a comparative study on the structure and the
physiology of the formation of single roots from microcalli, derived
from leaf protoplasts of a wild-type clone (Npwt) and an Ri-
transformed clone (Np99) of *Nicotiana plumbaginifolia*, during culture
in $\frac{1}{2}$V-KM medium containing naphthyl acetic acid (NAA) and zeatin.
Optimum concentrations for root induction and development were
found to be 0·1 to 0·3 mg l^{-1} NAA for Npwt and 0·3 to 1·0 mg l^{-1}
NAA for Np99, both at 0·1 mg l^{-1} zeatin. Root formation in Np99
occurred at higher frequency and reproducibility than in Npwt. The
rate of development and the morphology of the roots were equal
however for both clones. Subculture of rooted microcalli on hormone-
free MS medium resulted in abundant hairy-root growth and shoot
formation in Np99, whereas only occasional shoot formation occurred
in Npwt. Structural research on root initiation and development,
carried out on Np99, revealed the presence of a small, distinct group of
cytoplasm-rich cells, at approximately 10 days after protoplast culture.
This group of cells, which seemed to have a single-cell origin, showed
unipolar growth, resulting in the formation of a root meristem,
followed by the emergence of the young root from the microcallus,
several days later. Ultrastructural studies showed the presence of
osmiophilic droplets and amyloplast development in the tip region of
the young roots.

Key-words: Agrobacterium transformation, *Nicotiana*, physiology,
protoplasts, rhizogenesis, ultrastructure.

INTRODUCTION

In recent years, various disciplines in plant science have shown an increasing interest in
root differentiation and growth *in vitro*. Adventitious rooting of cuttings is extremely
important in vegetative propagation (see reviews in Jackson 1986, Davis & Haissig 1990).
Root clones, obtained through genetic transformation with *Agrobacterium rhizogenes*

T-DNA (Chilton *et al.* 1982, Bercetche *et al.* 1987), form a good source for the production of secondary metabolites (Rhodes *et al.* 1987, Signs & Flores 1990). They are also useful for studying the root-microbe interactions (Mugnier 1988), and can be regenerated into Ri-transformed plants (De Vries-Uijtewaal *et al.* 1989, Ottaviani *et al.* 1990). Rhizogenesis from protoplast-derived calli has been used as an intermediate step towards shoot-bud differentiation in *Prunus cerasus* (Ochatt & Power 1988). Young seedling-roots were successfully employed *in vitro* to study the development of gravitropic sensitivity (Kiss & Sack 1989).

The physiological conditions for new-root formation are generally known. Auxins alone or in specific balance with cytokinins play a crucial role in the development of roots from organized tissue as well as from undifferentiated callus (Skoog & Miller 1957, Thorpe 1982, Davis & Haissig 1990). There are however, only a few reports on light and electron-microscopical investigations on the process of root formation (Peterson 1975, Vigil & Ruddat 1985, Bercetche *et al.* 1987, Kiss & Sack 1989). Two main drawbacks hamper morphological as well as cell physiological, biochemical and molecular biological studies on morphogenesis of undifferentiated cells. First, the fact that only one or a few cells in a great mass of cells are directly involved in the organ initiation, and secondly, the low degree of synchrony in the process of differentiation in such systems (Thorpe 1982). Although there is a lack of suitable experimental systems for such studies (Davis & Haissig 1990), at present some systems exist which meet the requirements i.e. a synchronous differentiation at a high frequency within a very small mass of cells—and are useful for various studies on morphogenesis. Amongst them are cell-suspension cultures of *Daucus carota* (Jones 1974, Wurtele *et al.* 1988) and *Zea mays* (Fransz 1988) for investigations on embryogenesis, cell-suspension cultures of *Cucumis sativus* (Bergervoet *et al.* 1989) for caulogenesis, and protoplast cultures of *Medicago sativa* (Lu *et al.* 1983), *Nicotiana sylvestris* (Facciotti & Pilet 1979) and (Ri-transformed) *N. plumbaginifolia* (Gilissen & De Vries 1987) for embryogenic and rhizogenic studies, respectively.

This article reports about the effect of the ratio between auxin and cytokinin in the culture medium on callus development and on root formation in microcalli from leaf protoplasts of a wild type clone and an Ri-transformed clone of *N. plumbaginifolia*. Further, to study the expression of hormone autotrophy (transformation character), the influence of the culture medium (without hormones) on the growth of regenerated roots in both types of clones was compared. In addition, light and electron-microscopical observations of the early stages of root initiation and development of roots from microcalli of protoplasts of the Ri-transformed clone are described.

MATERIALS AND METHODS

Transformation

Wild-type plants of diploid *Nicotiana plumbaginifolia* (Npwt, $2n = 2x = 20$) (kindly supplied by Dr M. Devreux, Ispra, Italy) were used for leaf-disc transformation with *Agrobacterium tumefaciens* strain LBA1020 (= LBA285 with the *A. rhizogenes* plasmid Ril855::Tn5) (Hooykaas *et al.* 1982, Cardarelli *et al.* 1985). Transformation of leaf discs was carried out as described previously for potato-stem internodes (De Vries-Uijtewaal *et al.* 1988). Three- to 4-week-old primary hairy-roots, that had been developed from the leaf discs on hormone-free MS medium (Murashige & Skoog 1962) containing sucrose (3%), agar (0·8%) and cefotaxime (0·02%) at 24°C and 16 h day^{-1} light of 1 klm m^{-2} (TL type FTD58W33), were excised and subcultured monthly under the same culture-conditions.

Spontaneous shoot regeneration occurred in many of the root clones. From the root clone Np99 one shoot was taken and cloned on hormone-free MS medium containing sucrose (2%) and agar (0·8%) under the culture conditions mentioned above. The clone was verified as being diploid by flow-cytometric analysis of nuclear-DNA content. In addition, the clone showed the hairy-root phenotype and the character of hormone autotrophy, both caused by expression of the *rol*-genes of the T_L-DNA, and produced opines due to expression of the genes of the T_R-DNA of the *A. rhizogenes* plasmid (Vilaine & Casse-Delbart 1987).

Protoplast culture

Protoplasts were isolated from the in-vitro cultured, 3- to 6-week-old Npwt and Np99 plant clones. Leaves were cut into pieces of approximately 1 cm^2 and incubated overnight at 28°C on a gyratory shaker (30 rpm) in a 9-cm Petri dish with a mixture of 1% Cellulase 'Onozuka' R10 and 0·2% Macerozyme R10 (Yakult Biochem., Nishinomiya, Japan) in half-strength V-KM medium ($\frac{1}{2}$V-KM) (Bokelmann & Roest 1983). After filtration and washing as described by Bokelmann & Roest (1983), the protoplast suspension was diluted to a density of $5·10^4$ protoplasts ml^{-1} in $\frac{1}{2}$V-KM medium containing various concentrations of naphthyl acetic acid (NAA) and zeatin and plated in 5-cm Petri dishes. Low gelling-temperature agarose (Sea Plague) (0·8%) was added to prevent protoplast agglutination. The cultures were incubated at 24°C in continuous light (1 klm.m^{-2}, TL type F48T12). After 1 and 2 weeks, the growing cultures were diluted with equal volumes of the culture medium containing hormones at the same concentrations. During the latter dilution step, the cultures were transferred to 9-cm Petri dishes. Frequencies of root formation from microcalli (expressed in percentages of total number of microcalli) were calculated from at least 500 microcalli per 9-cm Petri dish in duplicate experiments.

At regular intervals during culture, samples were taken from the cultures for microscopic examination. After 7 weeks, regenerated roots were transferred to hormone-free, solid (0·8% agar) MS medium with sucrose (3%) and cultured at 24°C and 16 h/d light (1 klm.m^{-2}, TL type FTD58W33).

Light and electron microscopy

At several developmental stages, between 0 and 24 days of culture in $\frac{1}{2}$V-KM medium containing 0·3 mg l^{-1} NAA and 0·1 mg l^{-1} zeatin, samples were used for microscopic examination. Protoplasts were centrifuged at 400 rpm in culture medium before sampling. Protoplasts as well as microcalli with or without a regenerating root were fixed in 3% glutaraldehyde in 0·1 M sodium cacodylate buffer, for 30 min at pH 7·2. Then, the samples were rinsed three times for 10 min in buffer, post-fixed with 1% osmium tetroxide in buffer for 30 min and rinsed again. After dehydration in a graded ethanol-series, the specimens were embedded in Epon. Sections of 1–3 µm and of 70–90 nm were cut using an LKB Ultratome III. In some cases, the sections of 1–3 µm were post-stained with potassium iodide (IKI) to localize starch deposition. Both bright-field as well as phase-contrast photographs were prepared. For transmission electron-microscopy the sections of 70–90 nm were post-stained with uranyl acetate and lead citrate and examined with a Philips TEM 301 electron microscope. For scanning electron-microscopy the samples were critical-point dried. They were sputter-coated with palladium-gold and examined using a Jeol 30C scanning electron-microscope.
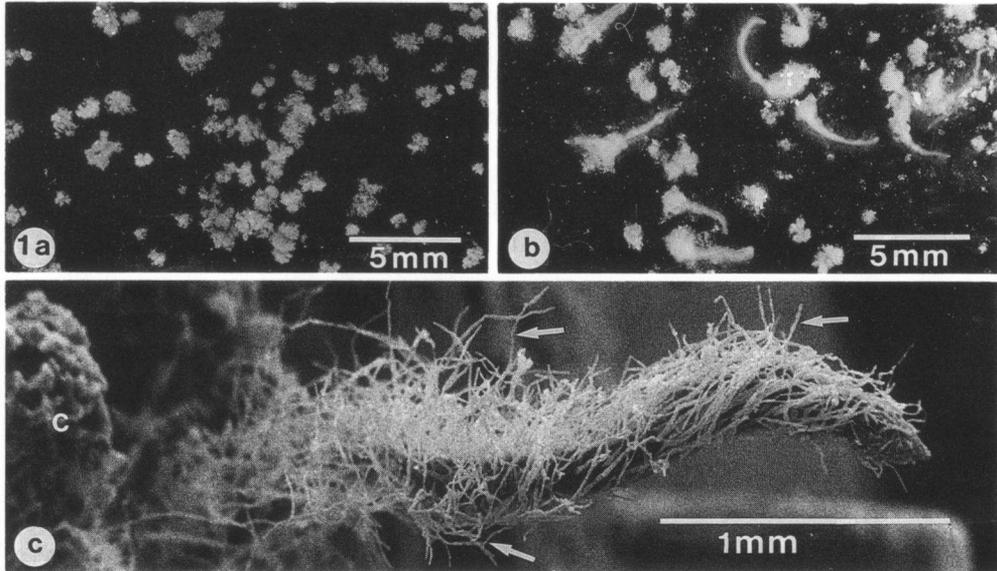
Fig. 1. Three-week-old leaf protoplast-derived microcalli of the wild type clone Npwt (a) and the Ri-transformed clone Np99 of *Nicotiana plumbaginifolia* with developing roots (b). (c) Details of 'b' showing a single root, appeared from callus (white c), with numerous root-hairs (arrows).

RESULTS

Root initiation

A preliminary experiment with the clones Npwt and Np99 showed frequencies of root formation from microcalli of leaf protoplasts after culture in $\frac{1}{2}$V-KM medium containing 0.3 mg l^{-1} NAA and 0.1 mg l^{-1} zeatin of 21.3 and 65.9%, respectively. The morphology and the rate of development of the roots in both clones were identical, i.e. per microcallus a single root with abundant root-hair formation had developed (Fig. 1b, c). Various repetitions of the experiment revealed a considerable variability in the frequency of root formation in the Npwt; in several cases root formation was completely absent (Fig. 1a), or reduced as is shown in Table 1. This table gives summarized data on the development of microcalli from the clones Npwt and Np99, as observed from growth rate, colour, frequency of root formation and presence of shoot primordia. Protoplasts of both clones did not survive in hormone-free $\frac{1}{2}$V-KM medium, indicating that initial growth and development are hormone-dependent. An additional experiment (results not shown) carried out on protoplasts of clone Np99 showed that the presence of NAA alone gave no growth (0.3 and 1.0 mg l^{-1}) or poor growth (0.03 and 0.1 mg l^{-1}). Calli which developed slowly from protoplasts in the latter case had a white colour. By contrast, in media containing only zeatin as hormone (0.03 and 0.1 mg l^{-1}) growth and development of green calli with several shoot-primordia was observed. Table 1 further reveals a broad optimum for all NAA/zeatin ratios between 0.3 and 10 for callus development in Np99. For Npwt, callus growth rate was reduced at the NAA/zeatin ratio of 10. Greening of calli of Npwt and Np99, which was related in most cases to the development of various shoot-primordia per callus, was restricted to zeatin-containing media with a low concentration (0.03 mg l^{-1}) of NAA. White microcalli with green shoot-primordia were found in cultures of Npwt, occasionally together with microcalli showing root formation in the same Petri dish. Root

Table 1. Callus development and root initiation after 5 weeks following leaf-protoplast isolation from Ri-transformed (Np99) and wild-type (Npwt) clones of *Nicotiana plumbaginifolia* on $\frac{1}{2}$V-KM medium containing various concentrations of NAA and zeatin (Z)

| NAA (mg l^{-1})	Z (mg l^{-1})	Ratio NAA/Z	Callus development				Frequency (%) of root formation[b]	
			Growth rate[a]		Colour			
			Np99	Npwt	Np99	Npwt	Np99	Npwt
0·0	0·0		−	−	−	−	0	0
0·03	0·1	0·3	+ +	+ + +	green	green[c]	0	1·0
0·1	0·1	1·0	+ + +	+ + +	white	white[c]	0·2	11·4
0·3	0·1	3·0	+ + +	+ + +	white	white[c]	80·6	8·1
1·0	0·1	10	+ + +	+	white	white	65·9	1·4

[a]Callus growth-rate; −: no cell division; +: moderate; + +: good; + + +: abundant.
[b]Frequencies were calculated from at least 500 microcalli in duplicate experiments.
[c]Several green shoot-primordia present after 8 weeks of culture.
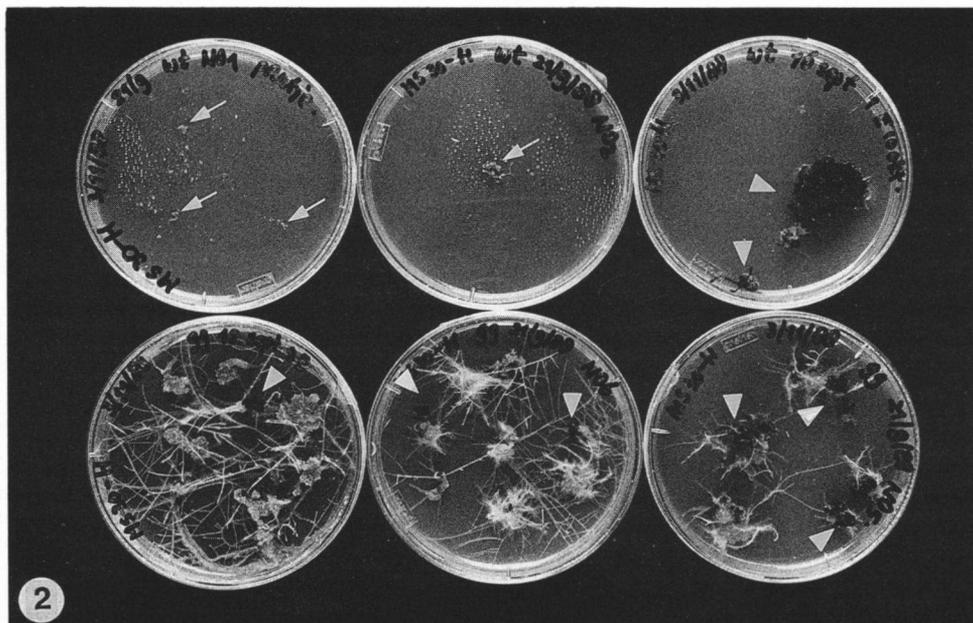
Fig. 2. Subculture on hormone-free MS30 medium of leaf protoplast-derived rooted microcalli of the wild-type clone Npwt (arrows, upper row) and the Ri-transformed clone Np99 (bottom row) of *Nicotiana plumbaginifolia*. Shoot primordia, when once induced on the Npwt callus, were able to develop shoots in hormone-free medium (arrowheads, upper right Petri-dish), in contrast to roots that did not show any growth within 4 to 6 weeks of subculture on hormone-free medium. Abundant root-growth and shoot-development (arrowheads, bottom row) occurred from the rooted microcalli of Np99. Petri dish diameter: 9 cm.
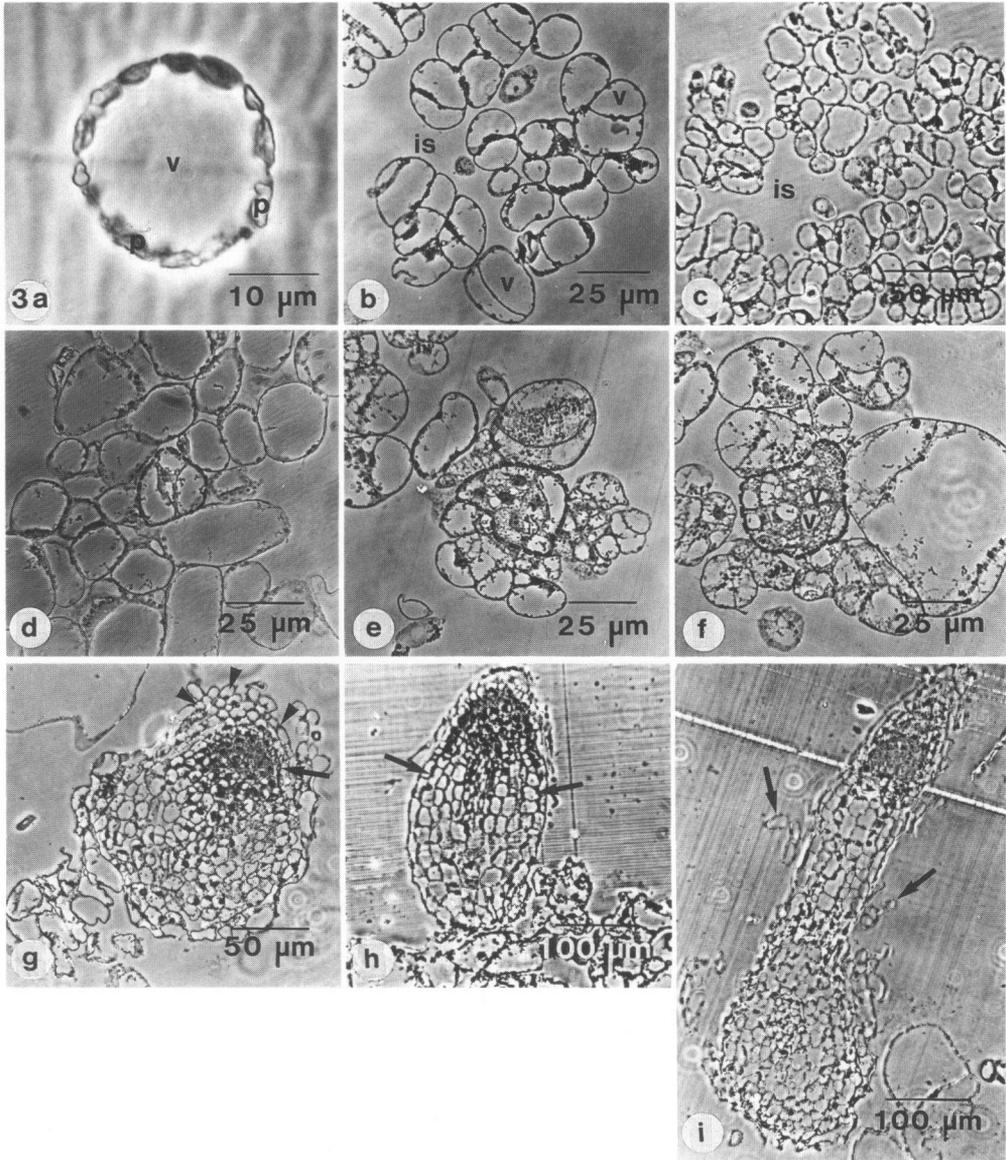
Fig. 3(a–c). Microcallus development from leaf protoplasts of the wild-type clone Npwt of *Nicotiana plumbagini-folia*. (a) Leaf protoplast with plastids (p) and large central vacuole (v). Cross-sectioned 10-day-old (b) and 24-day-old (c) microcallus showing large intercellular spaces (is). (d–i). Cross-sectioned microcalli from leaf protoplasts of the Ri-transformed clone Np99 of *N. plumbaginifolia* showing root initiation and development after 10, 12, 15, 18, 21 and 24 days of culture, respectively. The group of root-initial cells in Figs 3d–f is outlined. (f) Note the gradient in vacuolation (v). (g) Formation of root meristem (arrow) and root-cap cells (arrowheads). (h) Appearance of elongated cells (arrows). (i) Root with some cross-sectioned root hairs (arrows).

formation was much more pronounced in Np99 than in Npwt. Optima for Np99 were found at concentrations of 0·3 or 1·0 mg l^{-1} NAA in combination with 0·1 mg l^{-1} zeatin. An increase of the absolute amounts of both hormones at NAA/zeatin ratio of 3 resulted
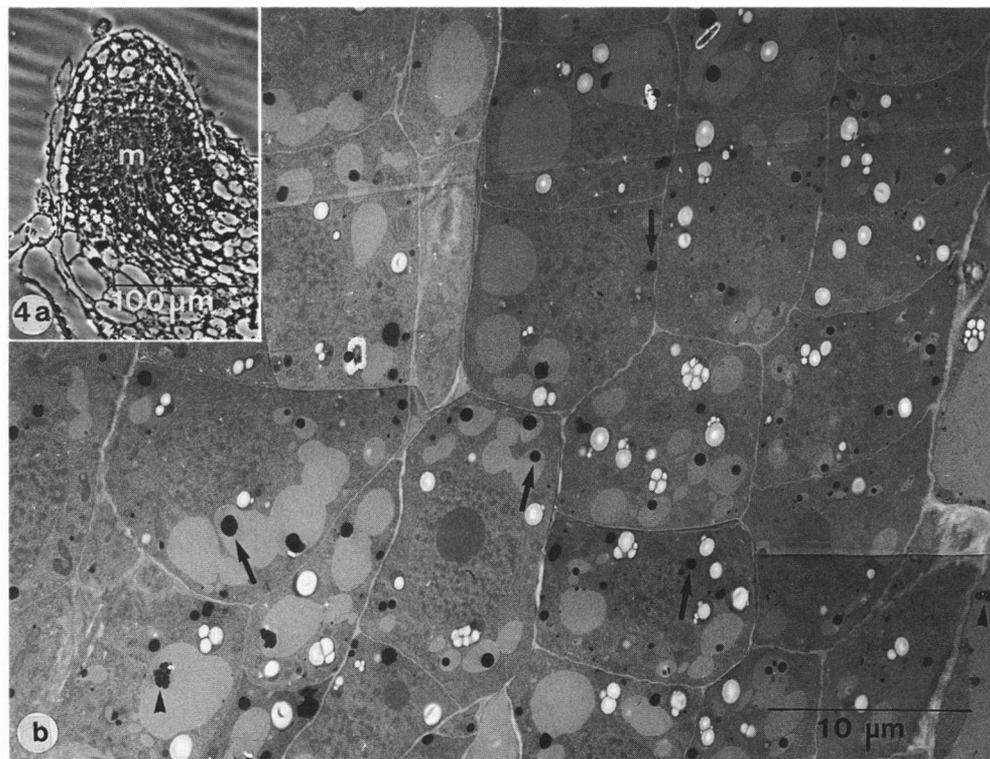
Fig. 4. Details of the meristematic region (m) of a 24-day-old root developed from a leaf protoplast-derived microcallus of the Ri-transformed clone Np99 of *Nicotiana plumbaginifolia*. The cells contain osmiophilic droplets (arrows) in their cytoplasm and vacuoles, and sometimes granular deposits (arrowheads).

in a decrease of the frequency of root formation: a frequency of only $11 \cdot 7\%$ was observed at $1 \cdot 5$ mg l^{-1} NAA with $0 \cdot 5$ mg l^{-1} zeatin (result not shown). In Npwt the optimum concentrations for root formation were found at $0 \cdot 1$ and $0 \cdot 3$ mg l^{-1} NAA, both in the presence of $0 \cdot 1$ mg l^{-1} zeatin.

Transfer of rooted microcalli to hormone-free medium

Transfer of 7-week-old root-bearing microcalli of Npwt from $\frac{1}{2}$V-KM medium with $0 \cdot 3$ mg l^{-1} NAA and $0 \cdot 1$ mg l^{-1} zeatin to hormone-free MS medium resulted in the arrest of callus and root growth (Fig. 2, arrows). Growth continued only when shoot primordia were present in the callus (Fig. 2, arrow heads). However, abundant root and callus growth from rooted microcalli of Np99 was observed on hormone-free medium. Many calli also showed spontaneous shoot-formation (Fig. 2, lower row). These results clearly indicate the expression of the character of hormone autotrophy in the Ri-transformed clone Np99.

Structural and ultrastructural observations

Protoplasts of both Npwt (Fig. 3a) and Np99 showed a large vacuole surrounded by cytoplasm with many plastids at the onset of culture. After 10 days of culture, protoplasts of Npwt, had developed microcalli, which had a friable appearance (Fig. 1a), due to the
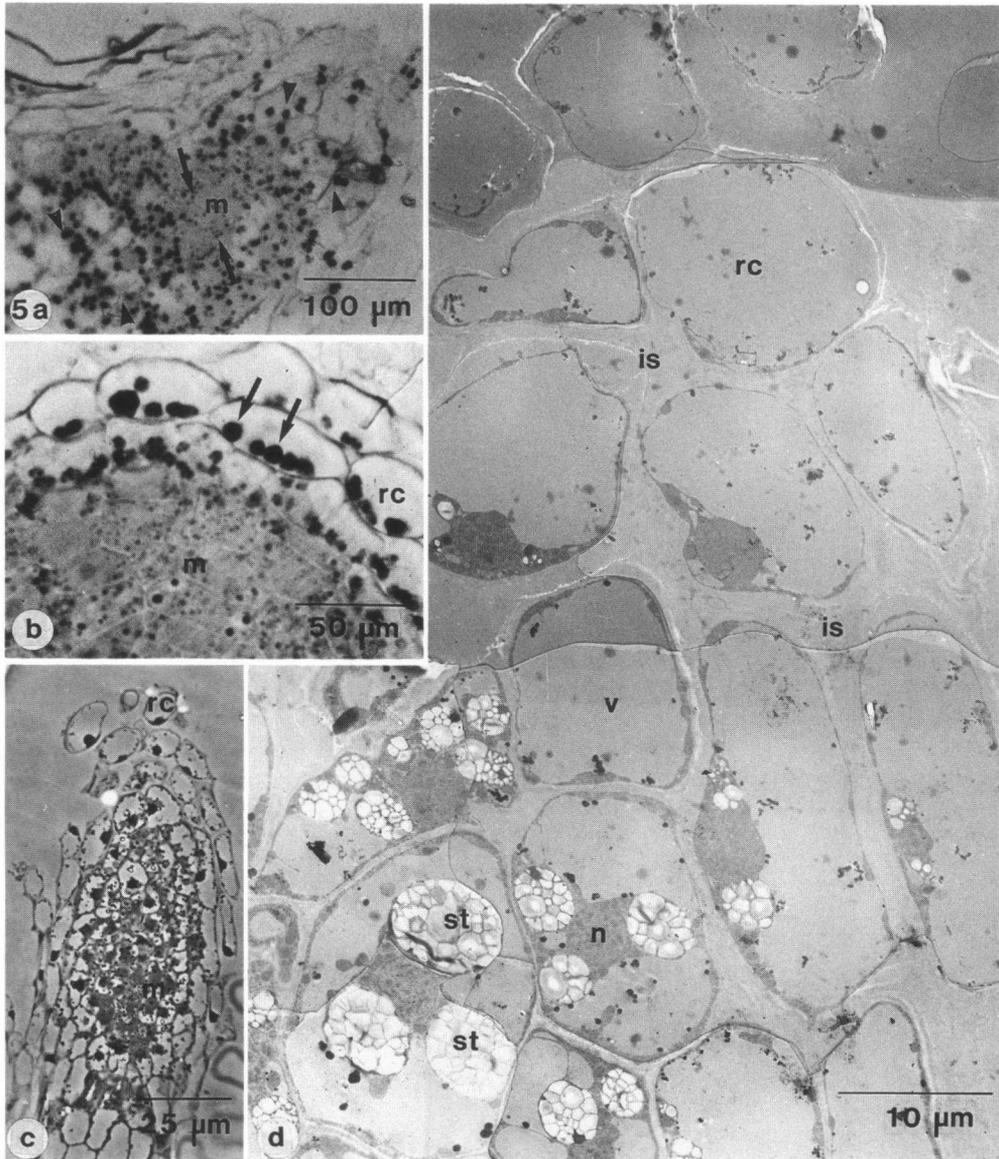
Fig. 5. Distribution of starch in developing roots from leaf protoplast-derived microcalli of the Ri-transformed clone Np99 of *Nicotiana plumbaginifolia* after staining with IKI (a,b) and as observed using electron microscopy (d as details of c). (a) Twenty-day-old root with small starch-grains in the meristematic region (m) and increasing size of the grains in the root-cap cells (arrowheads right) and elongated cells (arrowheads left). (b) Tip region of a 24-day-old root. Starch grains in the root-cap cells (rc) are located at the inward cell-wall. (c, d) Outer root-cap cells (rc) contain large vacuoles (v); the size of the starch grains (st), which are located closely to the cell nucleus (n) decreased in the outward direction. Between the root-cap cells, the intercellular space (is) seems to be filled with mucilaginous substance.

formation of large intercellular spaces (Fig. 3b). The individual cells were similar to each other in shape and size, all containing a large central-vacuole and a thin layer of cytoplasm bordering the cell wall. During prolonged culture, no changes in cell differentiation and

appearance of the calli occurred (Fig. 3c). In the same medium however, different develop-
ment from protoplasts of Np99 was observed: many microcalli showed cell differentiation
(Fig. 3d–i). In 10-day-old microcalli a group of a few cells which seemed to have originated
from one single cell was found (Fig. 3d), as was deduced from serial sectioning. Generally,
only one such group of cells per microcallus was found. After 12 days, the number of cells
in the group had increased; the individual cells were cytoplasm-rich (Fig. 3e). Then, after
15 days, polarity became apparent in the group of cells as a gradient in vacuolation
(Fig. 3f). Unipolar growth and development resulted in the formation of a root meristem
(Fig. 3g, arrow) that formed root-cap cells (Fig. 3g, arrowheads) and later, many
elongated cells appeared in the opposite direction (Fig. 3h, arrows). At this time, part of
the developing root emerged from the callus. After approximately 24 days, many
microcalli in the culture showed one small root (Fig. 3i; see also Fig. 1b), which developed
many root hairs (Fig. 3i, arrows; see also Fig. 1c). Note that, in the case of Fig. 3i, many
root hairs have been torn off, due to the preparation of the material for transmission
electron-microscopy, especially during the embedding steps.

   At the ultrastructural level, many osmiophilic droplets were found in cells, especially in
the meristematic regions of the root in 24-days-old microcalli (Fig. 4a, b). These droplets
were present in the cytoplasm as well as in the small vacuoles of these cells (Fig. 4b,
arrows). In some cases the vacuoles also contained dark, granular deposits (Fig. 4b,
arrowheads).

   The occurrence of polarity in the group of root-initial cells was not only visible in the
gradient of vacuolation (Fig. 3f) but also in the gradient of the proportion and number per
cell of starch grains (not visible in this figure). This became clear in a longitudinal section
of a developing root in a 20-day-old microcallus after staining with IKI (Fig. 5a). Here, the
cells of the meristematic zone (m) showed very small starch-grains (Fig. 5a, arrows), in
contrast to the other differentiating cells above and below this zone, which contained
much larger grains (Fig. 5a, arrowheads). In undifferentiated callus-cells, surrounding
the base of the root initial, such starch grains were absent (Fig. 3g–i). Initially, large
intercellular spaces were present between, the outer root-cap cells, possibly filled with a
mucilaginous substance (Fig. 5c, d). These cells showed no, or a few small starch-grains
following a gradient in an inward direction. If present, these starch grains were located
near the nucleus at the inward wall of the cells (Fig. 5b, arrows and 5d). This specific
location of well-developed starch-grains was most clearly visible in root-cap cells of older
stages (Fig. 5b, arrows).

DISCUSSION

The morphology and rate of development of roots which were formed from microcalli of
leaf protoplasts were equal for the wild-type clone (Npwt) and the Ri-transformed clone
(Np99) of *N. plumbaginifolia*. However, striking clone-specific differences were found in
the frequency and reproducibility of the root formation, which both were low in Npwt and
high in Np99. This feature made the latter clone very suitable for structural and ultrastruc-
tural analyses of root formation from cultured leaf-protoplasts. This particular process of
root formation appeared at the microcallus stage from a small group of cytoplasm-rich
cells, which seemed to have a single-cell origin. Similar developments from leaf proto-
plasts, including root formation, were described for *N. sylvestris* (Facciotti & Pilet 1979)
and *Medicago sativa* (Lu *et al.* 1983). In those investigations however, root formation
was considered to be the result of unbalanced development in the process of direct

embryogenesis from protoplasts. The question becomes relevant now whether the roots from the leaf protoplast-derived microcalli of *N. plumbaginifolia* originated from mono-polar bud-formation, or from direct somatic-embryogenesis in which normal development of the shoot apex was suppressed, due to maintenance of the specific hormone-ratio in the medium.

Ultrastructural data of roots in general, and of *Nicotiana* in particular, are scarce. This is the first time that osmiophilic droplets and granular material are described in meristematic cells of young roots, as observed in Fig. 4b. In our opinion, similar structures were visible in the cells of tobacco roots (see Figs 6–8 and Fig. 17, respectively, in Vigil & Ruddat 1985). These latter authors however, reported only the development of proteinoplasts in detail. Figure 5 shows clear differences in amyloplast development in the various cells of the root tip. Cells with well-developed amyloplasts surround the root meristem that is poor in starch. The well-developed amyloplast in the root-cap cells which are generally found in higher-plant roots, may function as statoliths (Hensel 1984, Kiss & Sack 1989, Ottaviani *et al.* 1990). Large accumulations of plastid starch were also observed in cells during early stages of differentiation: in developing shoot meristemoids of *N. tabacum* (Ross & Thorpe 1973) and in embryogenic-cell clusters of *Daucus carota* (Wurtele *et al.* 1988). Considering this relationship of specific starch-accumulations with differentiation and growth, research on amyloplast development in the present system of *N. plumbaginifolia* for root formation might reveal the time and place of the induction of polarity and the initiation of the root meristem. In addition, the system might have general value for application in further detailed physiological and ultrastructural research concerning the induction and initiation of roots.

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