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Cell-wall regeneration in pollen-tube subprotoplasts

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SUMMARY

Pollen-tube subprotoplasts of *Nicotiana tabacum* L. regenerate a new cell-wall with a random texture and a relatively high content of β 1–3 glucan (callose). The subprotoplasts become vacuolized during culture, and both the cytoplasm and wall deposition are limited to a single site of the subprotoplast. After 8 h of culturing, wall formation becomes restricted to a very small area and a fibre-like structure evolves. Fibre formation seems to result from a spontaneous clustering of multiple wall-synthesizing sites. These sites probably give rise to the different strands seen in the fibre. Wall deposition and fibre formation take place independent of the presence of a nucleus or microtubules and actin filaments.

Key-words: Fibre formation, *Nicotiana*, protoplast, wall formation.

INTRODUCTION

Subprotoplasts (SPPs) from in-vitro grown pollen-tubes remain viable for relatively long periods. They can regenerate a cell wall and may grow out by a series of repeated bud-like extrusions, thus forming a variety of long tube-like structures (Kroh & Knuiman 1988, Zhou 1989). The SPPs quickly re-establish a microtubular and actin skeleton (Rutten & Dersen 1990, 1991). Outgrowths occur on both karyoplasts and cytoplasts and are independent of the presence of microtubules and/or actin filaments. After prolonged incubation (8 h or more) part of the SPPs start to produce long extracellular fibre-like wall structures similar to those observed in cultures of *Nicotiana tabacum* leave epidermal protoplasts (Fannin & Shaw 1983). The present paper reports on the development and ultrastructure of wall and fibres on the SPPs.

MATERIALS AND METHODS

Pollen-tube SPPs of *Nicotiana tabacum* L. cv Samsun were prepared and isolated as described earlier (Rutten & Derksen 1990). In order to allow the SPPs to recover from the preparation shock, the time lapse between preparation and isolation was kept to 16 h. After isolation, the SPPs were resuspended in the high calcium W5 medium ($CaCl_2$ 125 mM, NaCl 154 mM, KCl 5 mM, glucose 5 mM; Siderov *et al.* 1981) and incubated in the dark on a shaker (80 rpm) at 28°C for up to 30 h. The presence of cellulosic wall-material

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Abbreviations: AB aniline blue; CW calcofluor white; DIC differential interference contrast: H_2O_2/HAc hydrogen peroxide/glacial acetic acid; PIPES piperazine-*N*,*N*'-bis[2-ethane-sulphonic acid]; SPPs subprotoplasts.

was monitored with calcofluor white (CW) (American Cyanamid Co., Wayne, New Jersey, USA), in a final concentration of 0·01%. Callose was detected with decolourized aniline blue (Merck, Darmstadt, FRG). Preparations were examined in a Leitz Orthoplan Vario Orthomat (Leitz, Wetzlar, FRG) combination, equipped for fluorescence or differential-interference-contrast (DIC) microscopy. Birefringence of the cell wall was studied in a Leitz HM Pol polarization microscope. Both untreated cell-walls and walls from which matrix material was extracted were used. Extraction was with hydrogen peroxide/glacial acetic acid, H_2O_2/HAc, (1:1, v/v) during 4 h at 90°C. Micrographs were made on Kodak T-max ASA 400 professional film.

For transmission electron microscopy, SPPs were fixed in 3% (w/v) glutaraldehyde in 50 mM piperazine-*N*,*N*'-bis [2-ethane sulfonic acid] (PIPES) buffer pH 6·8 for 1 h, postfixed for 1 h in 1% (w/v) aqueous OsO_4, and stained in 0·5% aqueous uranyl acetate for 1 h. The specimens were dehydrated in an ethanol series and embedded in epon. Ultrathin sections (70 nm) were made on a Sorvall MT 5000 microtome (Sorvall Inc., Norwalk, USA). The sections were stained with Reynold's lead citrate (8 min). To study the wall texture, H_2O_2/HAc extracted cell-walls were placed on formvar-coated grids and shadowed with platinum at an angle of 45°. The specimens were examined in a Jeol 100 CX II electron microscope (Jeol Ltd., Tokyo, Japan).

In order to disrupt the cytoskeleton, colchicine and cytochalasin D (both from Sigma Chem. Co., St Louis, MO., USA) were used. The drugs were added to the medium immediately after isolation of the SPPs. Colchicine was used in a final concentration of 10^{-3} M, cytochalasin D was added to the suspension in a final concentration of 10^{-5} M. Cells were cultured in the presence of the drug for up to 16 h.

RESULTS

Using CW staining we could not detect wall regeneration during SPP recovery. A new cell-wall formed within one hour after isolation. The first wall-material appeared in small spots spread over the entire surface of the SPP. Gradually a large vacuole appeared. The cytoplasm became concentrated at one site of the SPP, the vacuole occupying the remaining space. Hardly any cytoplasm was present between the vacuole and the plasma membrane. The wall was very thick near the cytoplasm but very thin or absent at the site of the vacuole (Fig. 1a, b). As the volume of the vacuole further increased, the cell wall was ruptured and a bud-like protrusion was formed at the site of the vacuole. In about 20% of the growing SPPs, the plasma membrane detached from the cell wall and moved forward until finally, a spherical SPP was located on top of an empty tube-like wall (Fig. 1c). The process of plasma-membrane withdrawal, followed by deposition of a new wall, was repeated several times and sometimes piles of 20 or more disc-shaped walls were formed (Fig. 1d). The walls reacted with AB revealing the presence of callose. Cell walls extracted with H_2O_2/HAc revealed a network of randomly oriented cellulose-microfibrils (Fig. 2). After prolonged incubation (8 h or more) wall formation occurred mainly in the centre of the surface under which the cytoplasm was concentrated. Finally, wall formation was completely limited to this site and long fibres were made (Fig. 3a). These fibres were up to 800 μm long and 1–8 μm wide, but their diameter ranged predominantly between 3 and 4 μm. They stained with AB indicating the presence of callose (Fig. 3b, c). When studied under DIC the fibres seemed to be built up of several strands (Fig. 3d). After extraction with H_2O_2/HAc the fibres showed a fibrillar component with a helical or longitudinal orientation (Fig. 4). However, cellulose microfibrils were not observed. Bi-refringence

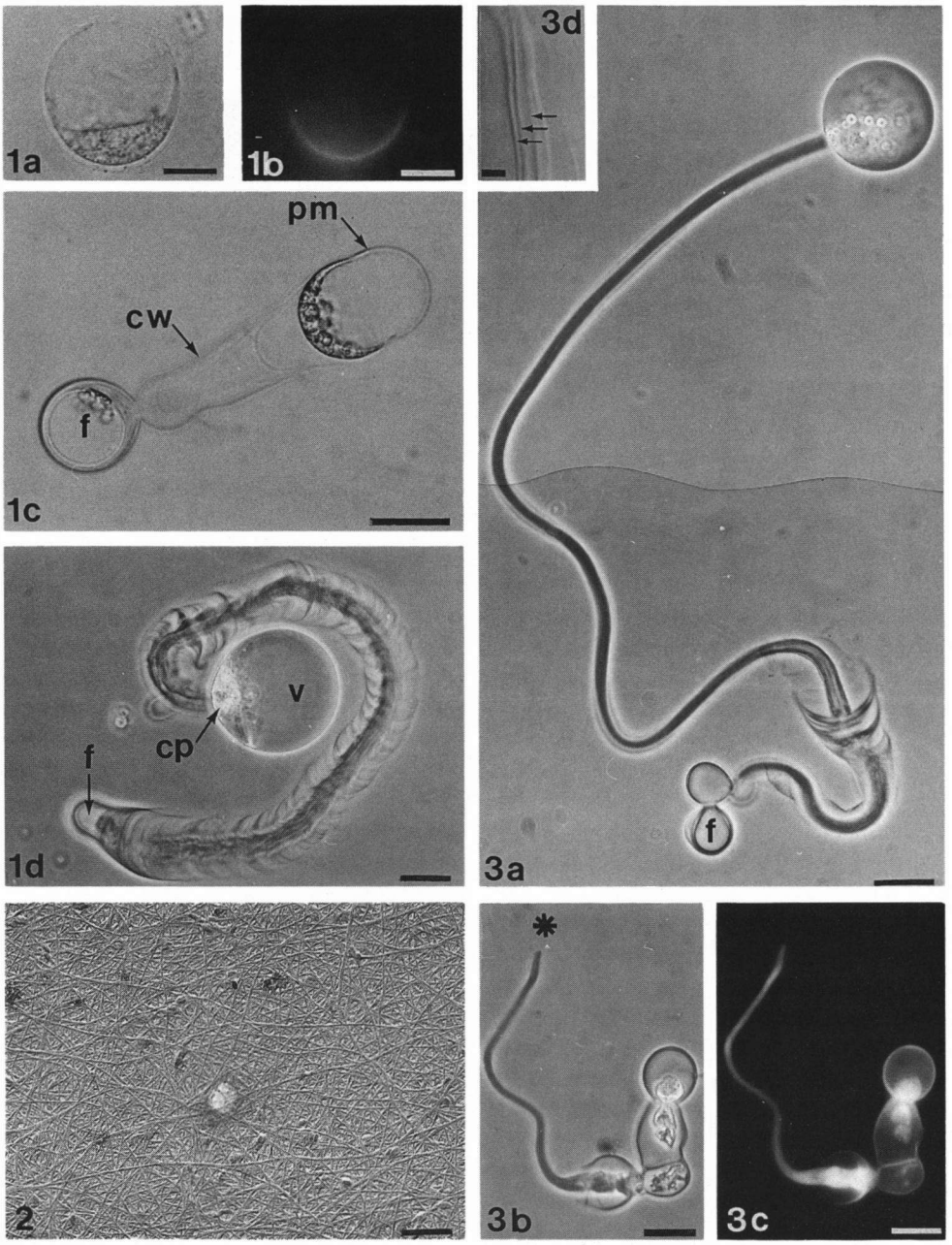

Fig. 1(a–d). Growth of pollen-tube SPPs. (a,b) Calcofluor-white staining of a vacuolated SPP shows wall material to be present at the site of the cytoplasm but almost absent at the site of the vacuole; (a) without, (b) with calcofluor white. (c) 4-h of incubation: the plasmamembrane (pm) has detached from the cell wall (cw); note that the cytoplasm is concentrated into a single area. (d) 8-h of incubation: wall formation continues after the SPP has left the tube-like cell-wall. Repeated withdrawals of the plasmamembrane have resulted in a pile of closely packed cup-shaped walls; f = former SPP. × 700 (a,b,d), × 1100 (c), bars = 10 μm.

Fig. 2. Regenerated cell wall extracted with H_2O_2/HAc and shadowed with platinum shows a network of cellulose microfibrils with a random orientation. × 21,000, bar = 0·2 μm.

Fig. 3(a–d). Fibre production by pollen-tube SPPs after 16 h of incubation. (a) wall deposition is restricted to a single, small area on the plasmamembrane, resulting in production of a long fibre; (b–c) both fibre and cell wall are stained with aniline blue; note that the SPP itself is no longer present (asterisk). (b) without, (c) with Aniline Blue; (d) DIC image of a fibre: the fibre seems to be composed of several strands (arrows); × 800 (a), × 340 (b,c), × 1500 (d); bars = 10 μm.

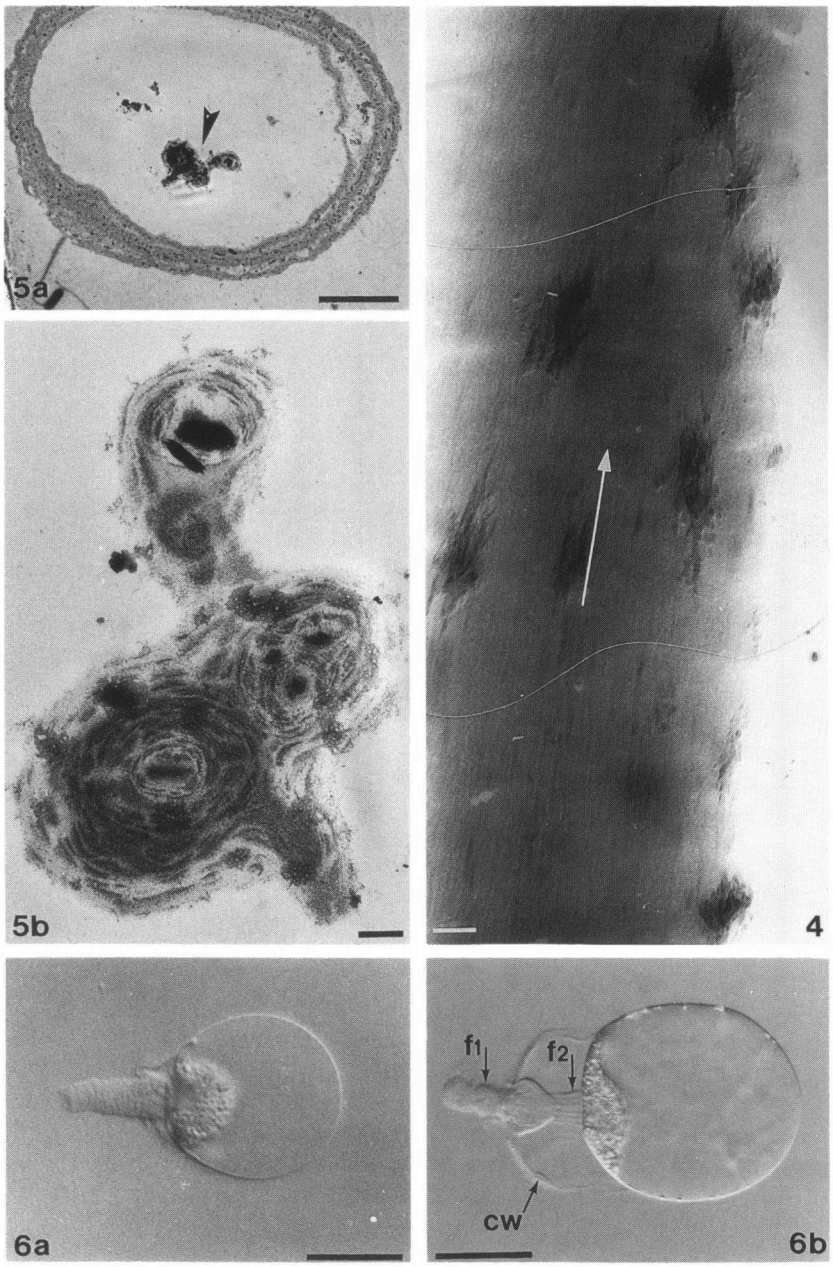

Fig. 4. The fibre extracted with H_2O_2/HAc shows the presence of fibrillar material with a longitudinal/net-helical orientation (arrow); microfibrils seem to be absent. × 31,000 (a); bar = 0·2 μm.

Fig. 5(a–b). Electron micrograph of a transected fibre. (a) The fibre (arrowhead) is composed of several strands. Transected walls of closely packed cup-shaped wall structures surround the fibre. (b) The individual strands are built up of a central core with distinct particles of wall material deposited in a spiral or concentrical organization. × 2500 (a), × 13,800 (b); bar = 5 μm (a), 0·5 μm (b).

Fig. 6(a–b). Pollen-tube SPPs incubated without shaking. (a) 2-h incubation: fibre production without the prior regeneration of a normal cell-wall; (b) 2-h incubation without shaking followed by 6-h incubation on a shaker. Initial fibre-production (f_1) was follwed by the deposition of a normal cell-wall (cw) finally, fibre production (f_2) was started; × 1500 (a,b); bars = 10 μm.

was absent in both extracted and non-extracted cells, indicating that the observed longitudinally-oriented fibrils do not have a crystalline nature.

The fibres were composed of several (up to three) electron dense strands (Fig. 5a, b). They consisted of a central core with distinct spots of cell-wall material grouped in a concentrical or helical organization (Fig. 5b). The connection between the fibres and the plasma membrane could not be studied due to its fragility; all treatments resulted in detachment of the fibres from the SPPs.

Formation of outgrowths, wall, and fibres occurred independent of the presence of a nucleus and/or generative cell. Varying the calcium concentration from 1 mM up to 125 mM did not affect growth and wall formation of the SPPs. The treatments with colchicine (10^{-3} M) and cytochalasin D (10^{-5} M) resulted in a complete breakdown of microtubules and actin filaments respectively, but did not affect outgrowth and wall formation. However, fibre formation seemed to be affected by mechanical forces. Fibre formation without the prior regeneration of a cell wall was observed in non-shaken cultures (Fig. 6a). Like in the 8 h old shaken cultures, frequence of fibre formation in the non-shaken cultures was about 8%. When shaking was started 2 h after isolation, fibre production was stopped and a cell wall was regenerated. Only after prolonged incubation on a shaker (4 h or more) fibres appeared again (Fig. 6b). In contrast, fibre formation in cultures incubated for 8 h or more on a shaker was an irreversible process.

DISCUSSION

The walls of regenerating SPPs seem to bear similarities to the walls of pollen tubes (Kroh & Knuiman 1982), as they contain a large amount of callose, indicated by the strong aniline blue staining, and randomly organized cellulose microfibrils (see also Kroh & Knuiman 1988). However, as similar walls are also found in other types of protoplasts (Willison & Klein 1982, Willison 1985, Hough *et al.* 1985), it is impossible to decide whether any specific property of the pollen-tube wall is preserved in the SPP. Outgrowths similar to those in SPPs have been recorded for a number of protoplasts (for discussion see Kroh & Knuiman 1988). Also the formation of long fibres has been shown for other protoplasts (Moore 1973, Fannin & Shaw 1983, Hahne *et al.* 1983), but not for pollen-tube SPPs. Though the fibres do not show cellulose microfibrils or bi-refringence, cellulose may yet be present. It might occur either in a non-cristalline form, as shown for microspore protoplasts from *Lilium* (Miki-Hirosige *et al.* 1988), or microfibrils and their bi-refringence might be obscured by a non-extractable material. As the non-extractable material strongly stains with uranyl acetate and lead citrate, it is probably not cellulose (Emons, 1988). In our hands, the only substances, except cellulose, that could withstand H_2O_2/HAc extraction are esterified fatty-acids such as cutin or sporopollinin, but these substances are not known to form stable fibre-like structures.

Fannin & Shaw (1983) have suggested that a high calcium concentration in the medium favours fibre formation. This is obviously not the case in the SPPs studied here. In contrast to the observations on *Zea mays* SPPs by Lörz *et al.* (1981), who reported cell-wall formation to be absent from cytoplasts, the absence of a nucleus had no influence on wall formation in pollen-tube SPPs. Also microtubules and actin filaments did not exert any apparent control on wall or fibre formation in the SPPs.

The transition from wall deposition to fibre formation seems to result from a clustering of definite sites where wall materials are deposited; each strand in the fibre representing such a site. As indicated by the reversible fibre-formation on young SPPs at rest,

clustering may occur spontaneously but is prevented by mechanical forces, i.e. shaking. The irreversibility in older SPPs may result from redistributions of organelles in the cytoplasm or of proteins in the plasma membrane of the SPPs. The clustering of wall-depositing sites might also affect the cellulose synthesizing complexes in the membrane, leading to the synthesis of β 1–3 linked glucans, i.e. callose, instead of β 1–4 linked cellulose (Emons 1991). This might explain the cessation of cellulose-microfibril deposition after the start of fibre formation. Possible redistributions however, could not be traced as the site of fibre formation could not be identified electron microscopically, due to the fragility of the connection between SPP and fibre.

Cell wall, microtubules, and actin filaments, the main morphogenetic elements in a plant cell (Derksen *et al.* 1990), are still present in the SPP. The loss of co-ordination between these systems may be the main cause for the inability of the SPP to resume tip growth as it occurs in pollen tubes.

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