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Callose deposition in the primary wall of suspension cells and regenerating protoplasts, and its relationship to patterned cellulose synthesis

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Abstract: Monitoring cell-wall formation in vivo with Fluorescent Brightener 28, by fluorescence microscopy, revealed that tobacco protoplasts regeneration started within 30 min indicated by cellulose microfibril formation at distinct sites on the protoplast surface. Oriented cellulose microfibril deposition was apparent before elongation and indicated the early polarization of protoplasts. The sequence of cellulose microfibril deposition correlates with an helicoidal-like texture. Within 6 h, a texture was completed. Tobacco suspension cells, stained by decolourized aniline blue, showed radiant granular callose fluorescence in cell plates and transverse walls. During the culture cycle of suspension cells, transverse fibrillar deposits of callose gradually appeared in the lateral walls during the log-phase, and subsequently disappeared in the early stationary phase of the cell culture. Similar callose transitions were observed in regenerated elongating protoplasts. Culture cells of Morinda citrifolia L. only showed transient granular depositions in the lateral walls. The callose formations did not result from artificial wounding. The transient appearance of callose might be related to cellulose microfibril deposition.

Key words: aniline blue, Calcofluor White ST, callose, cellulose, Fluorescent Brightener 28, Morinda citrifolia, Nicotiana tabacum.

Résumé : L’observation in vivo de la formation de la paroi cellulaire à l’aide du Fluorescent Brightener 28, en microscopie en fluorescence, montre que la régénération des protoplastes de tabac commence en moins de 30 min, tel qu’indiqué par la formation des microfibrilles de cellulose à des sites distincts sur la surface du protoplaste. La déposition orientée des microfibrilles de cellulose est apparente avant l’elongation et indique une polarisation hâtive des protoplastes. La séquence de déposition des microfibrilles de cellulose est corrélée avec une texture hélicoïde. En moins de 6 h, une structure complète est formée. Des suspensions de cellules de tabac, colorées par le bleu d’aniline décoloré, montrent un callose granulaire radiante fluorescente dans les plaques cellulaires et les parois transversales. Au cours du cycle de culture de la suspension cellulaire, des dépôts fibrillaires transversaux de callose apparaissent dans les parois latérales au cours de la phase logarithmique, et disparaissent subsequemment au début de la phase stationnaire de la culture cellulaire. Les auteurs observent des dépôts de callose comparables dans les protoplastes régénérés enelongation. Les cellules cultivées du Morinda citrifolia L. ne montrent la déposition granulaire que dans les parois latérales. Les formations de callose ne proviennent pas de blessures artificielles. L’apparition transitoire de callose pourrait être reliée à la déposition des microfibrilles de cellulose.


Introduction

The texture of the cell wall arises from the spatial organization of the cellulose microfibrils (CMF) in the wall, which is achieved by oriented deposition, followed by partially passive reorientation during cell elongation. Recent reports on the various aspects of CMF deposition and reorientation are those by Emons et al. (1992), Vian et al. (1993), and Wolters-Arts et al. (1993). A relationship between cellulose deposition and similarly patterned, but transient callose deposition was reported in cotton hairs (Waterkeyn 1981). Furthermore, the deposition of callose during cell plate formation in the entire transverse initial wall (Fulcher et al. 1975), and distinctly at the site of the plasmodesmata (Longly and Waterkeyn 1977; Delmer et al. 1993), accompanies the deposition of cellulose. Although the relationship between cellulose and callose formation is unclear, it has been suggested that callose is formed by destabilized CMF synthesizing complexes in the cell membrane (e.g., Northcote 1991). This is based, for instance, upon protoplast regeneration studies (Klein et al. 1981; Galbraith 1981). Callose is generally present during plant cell development or in cells with a specific secondary function (for review, see Stone and Clarke 1992). It may appear either transiently as in micro- and mega-sporogenesis, or permanently, as in pollen tubes. How callose and cellulose deposition interrelate in all these cases is...
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dye components responsible for the typical green—yellowish
for instance, has been confirmed with immunogold labelling
over, callose may be lost, or alternatively, its formation
The specificity of binding of this particular dye, and also
specific cell walls or physiological circumstances, is still
nothing is known of the exact function of callose in plant
cells, whereas it has been established that cellulose is intrin­
sically coupled to cell growth and cell shape (e.g., Carpita
and Gibeaut 1993).

Cell wall texture analysis is relatively easy using whole
mounts or cleared preparations, which show the CMF after
rigorous chemical extraction (Emons 1988; Amstel and
Derkson 1993). The formation of a new cell wall can also be
studied using freeze-etching (e.g., Willison and Cocking
1975) or scanning electron microscopy (e.g., Katsiradakis
and Roubelakis-Angelikis 1992 and references therein).

Rapid in vivo determination of plant cell wall reconstitution to date
is commonly achieved through fluorescence microscopy using
Calcofluor White as a probe (e.g., Kranz et al. 1995). This method was first introduced by Nagata and Takebe (1970).
The specificity of binding of this particular dye, and also
related dyes to CMF, has been thoroughly studied. In general,
the consensus is that Calcofluor White can be applied to
detect fibrillar cellulose patterns (e.g., Sengull 1986), although
it does bind to other β-(1→3) glucans linked backbones like
xylanoglu can as well. Moreover, it is clear that fluorescent
amorphous material does not represent fibrillar cellulose
(e.g., Hahne et al. 1983), that cellulose may be “contami­
nated” with β-(1→3) glucans (Herth and Meyer 1977), and
that it stains pachyman (fungal callose) and chitin as well
(e.g., Maeda and Ishida 1967). Nevertheless, Calcofluor
White staining is still considered suitable today for studying
nascent CMF (e.g., Sauter et al. 1993).

It is much more difficult to study the deposition of callose
because of its possible transient character and variable stain­
ing behaviour in electron microscopic preparations. More­
ever, callose may be lost, or alternatively, its formation
might be initiated during experimentation (Stone and Clarke
1992). In standard electron microscopy, callose appears elec­
tron lucent and its location in the tobacco pollen tube wall,
for instance, has been confirmed with immunogold labelling
(e.g., Meikle et al. 1991). Determination of callose deposits
in vivo is preferentially carried out with phosphate-buffered
solutions of aniline blue using fluorescence microscopy. The
dye components responsible for the typical green—yellowish
fluorescence after binding to callose do not strictly bind to
pure β-(1→3) glucans (see Evans et al. 1984). For this reason,
it is always considered essential to refer to the specific source
of the callose (see Stone and Clarke 1992 for discussion).

Whether the occurrence of callose is a general feature in
the formation of plant cell walls, or is only restricted to the
specific cell walls or physiological circumstances, is still
uncertain. This is elaborately discussed in the review by

To address the occurrence of callose and its possible func­
tion and relationship with cellulose synthesis in primary
walls, we chose to study de novo synthesis of callose and
 cellulose in vivo, using decolourized aniline blue (Linskens
1957) and Calcofluor White (Herth and Schnepf 1980) respec­
tively as probes. We specifically chose to study cells and
protoplasts from suspension cultures, which are generally
considered extremely suitable for various types of plant
research (see Willison 1986), but specifically suitable for
wall regeneration and cell elongation studies. This biological
detection in vivo, without the risk of inducing callose synthesis
unavoidable during isolation or preparation from whole tissues.
Moreover, protoplasts are of particular use for studying
 cellose biosynthesis in primary walls (Herth and Meyer
1977). We used the BY-2 G0 cell line from Nicotiana tabacum
(Nagata et al. 1992) and suspended calli from Morinda citrifolia
(Zenk et al. 1975), as they exhibit controllable
 elongation during protoplast regeneration and in suspension.

Methods

Plant material

Cells of Nicotiana tabacum ‘‘Bright Yellow’’ 2 G0 L. were suspension
cultured in 100 mL modified Linsmaier-Bednar/Skoog (LS)
medium, supplemented with 3% sucrose, 255 mg/L KH2PO4 and
0.2 mg/L 2,4-dichlorophenoxy-acetic-acid (2,4-D), pH 5.6,
in 300 mL Erlenmeyer flasks on a rotary shaker (130 rpm) at
25°C in the dark. The cells were subcultured every week (Nagata et al.

Cells of Morinda citrifolia L. (Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH, Germany) were grown
as calli on 0.7% agar solidified DAX medium (modified Gamborg
medium; Zenk et al. 1975), supplemented with 2% sucrose, 2%,
2% 3-ammoni A (Sigma Chemical Co., St. Louis, Mo.), and 2.0 mg/L
2,4-D, pH 5.5, at 25°C in the dark. Callus was transferred to liquid
medium to obtain cells in suspension. After approximately 4 weeks,
cell clusters were of appropriate size for further experiments.

Preparation of protoplasts

Protoplasts were prepared from early log-phase tobacco suspension
cells in 1% (w/v) cellulase “Onozuka” RS (Yukult Pharmaceutical
Ind. Co., Ltd., Tokyo, Japan) and 0.1% pectolyase Y-23 (Seishi
Pharmaceutical Co., Ltd., Tokyo, Japan) in 0.4 M mannitol for 1 h
at 28°C on a rotary shaker (30 rpm). Afterwards, protoplasts were
spun down at 800 rpm, 125 × g for 2.5 min, and rinsed three
times with Fukuoda-Murashige-Skoog (FMS) medium (modified (MS
medium plus 1% sucrose, 0.4 M mannitol, 1 mg/L benzyladenine
BA) and 0.1 mg/L α-naphthale-acetic-acid (NAA)). Vital and
wall-less protoplasts were purified and collected by centrifugation
in 300 rpm, 125 × g on 0.4 M sucrose. Subsequent transfer to
FMS medium stimulates the elongation of regenerating protoplasts
(Hasezawa and Sono 1983).

Staining procedures

The formation of CMF was studied during the first 6 h of protoplast
regeneration. Freshly isolated tobacco protoplasts were mixed with
a stock solution of Fluorescent Brightener 28 (the active component
of Calcofluor White ST; Sigma Chemical Co.) in a final dye con­
centration of 0.0005% (w/v; see Meadows 1984). Preliminary
investigations showed no differences in the use of this batch com­
pared with Fluostain-I (Dojindo, Japan; see Mizuta et al. 1991).

The formation of CMF was studied during the culture cycle of
tobacco suspension cells, in regenerated elongating protoplasts, and
in small cell clusters of the Morinda culture. Wound callose was
induced in regenerating protoplasts and suspension cells by vigour­
ously pipetting the cells. To study changes in callose deposition,
another form of membrane perturbation and wounding was applied
to suspension cells by allowing them to slowly dry on glass slides.

Cell aliquots of 25 μL were mixed with 5 μL of a decolourized
aniline blue (DAB) solution prepared as described by Linskens
(1957). Cells were immobilized on Vectabond-treated glass slides
(Vector Laboratory, Burlingame, Calif.). The DAB solution
was tested on various reference material including pollen tubes

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Figs. 1—6. Cellulose microfibril detection in vivo on the surface of regenerating tobacco protoplasts during the first 6 h after isolation. Fig. 1. Bright-field image of a freshly isolated protoplast with several cytoplasmic strands and an off-centre nucleus. Figs. 2—6. Fluorescence of CMF stained with 0.005% Fluorescent Brightener 28. Fig. 2. Cellulose microfibril development in the first 30 min showing relatively short bundles of microfibrils initiated at different sites. Fig. 3. Continuation of CMF deposition in the next 1.5 h. Cellulose microfibrils showed some alignment, and were unevenly distributed on the protoplast surface. Fig. 4. The subsequent period of about 2 h showed the presumable onset of wall layering and parallel swirling of CMF. Fig. 5. During approximately the 5th h, a dense framework of CMF developed with a definite parallelism of CMF. Some cells started to elongate. Cell axis parallel to the vertical. Notice the transverse orientated CMF in the central cell region and a more dispersed and less dense presence at one of the cell’s poles. Scale bars (Figs. 1—5) = 12 μm. Fig. 6. At the end of 6 h, a cell clearly elongated or elongating. The CMF were transversely deposited. The cell has collapsed, showing folds in the wall. Notice the difference in density between the upper and lower side of the cell. Scale bar = 25 μm. Figs. 7—12. Callose detection in vivo in regenerating protoplasts and elongating cells tobacco using decolourized aniline blue. Fig. 7. Protoplasts showing thread-like and partially parallel aligned callose deposits within 30 min. Fig. 8. As in Fig. 7, staining patch callose deposit. Fig. 9. As in Figs. 7 and 8, after induced wounding, radiance fluorescence of spot-like callose appeared at the presumed edges of the wound. Scale bars (Figs. 7—9) = 30 μm. Fig. 10. Bright-field image of a 7-day-old elongated cell with numerous cytoplasmic strands and a just off-centre nucleus. Fig. 11. A cell similar to that in Fig. 10 showing fine thread-like transverse oriented callose, interspersed with fine granular spots. Fig. 12. Elongated cell after induced wounding showing intense radiance callose deposits. Scale bars (Figs. 10—12) = 55 μm.

from different species (callous wall), onion epidermal cell strips (plasmodiacta), root tips from various sources (cell plates, pit fields), stylar tissue (phloem bundles, plasmodiacta), chiony epidermal cell strips (developing guard cells), tobacco micro- and mega-sporogenic tissue, and glandular cells of Drosera. Wounding of these specimens was achieved by vigorous pipetting or shearing strips along glass edges. Staining patterns were identical to general descriptions (see Stone and Clarke 1992).

To compare any specific effect of experimentation, special perfusion chambers, which allow optimal environmental control, were used next to normal glass slides and cover slips. No differences were observed in callose and cellulose deposition with either set-up.

Cell viability was constantly monitored using 0.005% fluorescein diacetate (Sigma Chemical Co.).

Epifluorescence of stained cells was observed in an Ortholux/Orthomont microscope combination (Leitz Wetzlar, Stuttgart, Germany) with the appropriate filters (BG 3 250-450 exciter and S405 barrier).

Photographs were taken using 100 ISO KONICA positive film, which was reprinted on Agfapan 25 ASA negative film or photo- graphed directly on Agfapan 100 ASA negative film. Additionally, micrographs were taken using a Nikon inverted microscope Diaphot with the appropriate filters (BG 3 250-450 exciter and S405 barrier). Photographs were taken using 100 ISO KONICA positive film, Orthomat microscope combination (Leitz Wetzlar, Stuttgart, Germany) equipped with a MRC-600 Confocal Imaging System (Bio-Rad, Cambridge, Mass.). An argon laser provided the spot source for illumination. Optical section thickness varied between 0.2 and 0.5 μm using 0.1—0.5 μm increments. Photographs were taken from a high resolution monochrome monitor on Agfapan 100 ASA negative film.

Results

Protoplasts

Freshly isolated protoplasts of tobacco (Fig. 1) showed no fluorescence after Fluorescent Brightener 28 staining (not shown). Figures 2 to 6 show the progressive formation of presumed bundles of CMF within the first 6 h after removal of the original wall. Initial formations appeared randomly distributed at the cell surface (Fig. 2). Gradually, the number of CMF increased showing an unevenly, parallel distribution (Fig. 3). Further deposits in different orientations, under similar sites, indicated the beginning of wall layering (Fig. 4). Unfortunately, this was only visible in direct view while focussing at different levels. Swirls of parallel CMF were also visible. Protoplasts in the early stages of elongation showed transversely oriented CMF in the middle part and less abundant and more dispersed formations at the polar regions (Fig. 5). After 6 h, some cells were clearly elongated and showed transverse CMF (Fig. 6). In due course, over several weeks, these elongated cells showed predominantly transversely oriented CMF. The intense fluorescence of these abundant transversely oriented CMF makes it impossible to identify possible differently oriented CMF. Changing dye concentration or using extensive rinsing after applying various concentrations of the dye did not improve visibility of such differently oriented CMF.

Freshly isolated protoplasts were devoid of callose deposits (not shown), but they may show thread-like (Fig. 7) and (or) patch-like (Fig. 8) deposits within the first hour after isolation. These were clearly distinguishable from wounding-induced callose (Fig. 9). In contrast with CMF deposition, no significant increase in the amount of callose deposits nor a pattern development could be observed at later stages. During the elongation phase of wall-regenerated cells (Fig. 10), fine thread-like callose, interspersed with fine granular deposits, were transiently present (Fig. 11) and showed a transverse or near-transverse orientation. The presence of callose in these elongating cells was highly variable with no detectable order (i.e., correlation between, for instance, cell length). Induced wound callose in these cells was easily recognizable as intense radiance spots surrounding an unstained area (Fig. 12). Wounding of reference material resulted in similar patterns (not shown).

Culture cells

Cellulose microfibrils were mainly transversely oriented according to Fluorescent Brightener 28 induced fluorescence (not shown). As in elongated cells, other orientations of CMF were difficult to recognize, as the brightness of transversely oriented CMF may have hidden differently oriented CMF (see above).

The lateral (longitudinal) walls of tobacco log-phase cells (Fig. 13) showed fibrillar or thread-like formations of callose with near-transverse orientation (Fig. 14). The thread-like deposits gave the impression of being composed of aligned spots, especially during focussing. These threads distinctly showed beginnings and endings.

Callose was persistently present at the cell plates and
The pattern of callose in the lateral walls varied during growth of the cell culture. Callose was present in spots (Fig. 16), or short thread-like deposits (Fig. 17) after 24 h of subculturing. Over the next 2 days, the number of fibrillar deposits increased dramatically (Fig. 18). Three to 4 days after subculturing, when growth declined, callose deposits rapidly disappeared. Occasionally, similar callose deposits were found in stationary cultures (not shown).

Cells that were made to collapse by drying retained their pattern of fluorescence, and this procedure allowed the simultaneous observation of callose deposits in several cells (Fig. 19). No deposits could be detected beyond the callose formed initially, and redistribution seemed not to occur. Daughter cells always showed similar callose patterns. Within the total population, the amount of callose formation between cells varied significantly. Optical sections made with the confocal laser scanning microscope (CLSM) revealed similarly oriented, thread-like deposits of callose compared with conventional fluorescence microscopy. At the site of the transverse wall, fibrillar callose showed a higher density (Fig. 20, left). Moving inward through the cell, deposits were only peripherally visible, whereas the transverse deposits were still observed where the connection between daughter cells constricted at this particular site. Fig. 21. As in Fig. 20, the projection of four optical sections through a cell at the end of a ribbon-like cluster of cells. The cell shows transverse folds in the upper half (no fluorescence). On the right: projection of four successive optical sections. Bleeding of (auto) fluorescence of the nucleus (centre) into these sections seems apparent. Notice that in the upper half, the fluorescence of the folds from the left figure appears. Fig. 22. Bright-field image of a cluster of globular cells of Morinda citrifolia. Fig. 23. Complementary fluorescence images of the same cells from Fig. 22 showing spot-like deposits of callose. Scale bar = 32 µm.

Discussion

This paper presents the first description of the in vivo formation of CMF into an organized texture at the surface of transverse walls (Fig. 15). The intense fluorescence in transverse walls might imply the presence of massive amounts of callose. However, in face view the fluorescence seemed to be restricted to the edges, whereas the centre stained in a patchy manner. Towards the stationary phase of the culture, this intense fluorescence of transverse walls markedly decreased.

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regenerating tobacco protoplasts. In numerous studies, Fluorescent Brightener 28 or related dyes have been used to follow general wall reconstitution. However, demonstration of CMF in other higher plant protoplasts and even in tobacco, particularly in the detail as presented here, has not been shown before. To some lesser extent, detailed observations on the alga Boodlea are comparable (Mizuta et al. 1994).

Cellulose formation starts almost immediately after protoplast isolation at distinct sites throughout the protoplast surface. Such scattered patterns seem to be common, as determined by more invasive techniques (Willison 1986; Burgess 1983; Katsirdakis and Roubelakis-Angelikis 1992). Wall formation at a restricted surface area, as frequently reported (e.g., Hahne et al. 1992), is often interpreted as indicative of diminished cell integrity. In preliminary observations, we found that in such cases the nuclei of protoplasts were located at the periphery of the cell. Such cell types also possess large central vacuoles with few cytoplasmic strands. The cytoplasm is often concentrated at one side of the cell. We found that such cells degenerate easily. Moreover, Rutten and co-workers (1991) have earlier described the aberrant cell wall regeneration of tobacco subprotoplasts, showing the typical unequal cytoplasmic distribution. Although such cells supply valuable information on cytoskeletal reorganization and cell polarization, they cannot be used to study wall reconstruction. Performing cytological examination of individual protoplasts before and at resuspension in regeneration media proves to be essential for a valid interpretation of wall renewal. The central position of the nuclei in freshly isolated protoplasts is a direct result of enzymatic wall degradation and not of mitotic activity (e.g., Lloyd et al. 1980; Katsuta and Shibaoka 1988). This is important with respect to speculations on cell dedifferentiation and polarization.

The time course of wall renewal is rather variable (e.g., Poinar et al. 1967; Nagata and Takebe 1970; Williamson et al. 1977). Complete chemical reconstitution of the wall may even take several weeks (e.g., Mock et al. 1990). Tobacco protoplasts in our study developed an organized texture within 6 h, which was much faster than earlier reports on BY2-G0 cells (24 h, see Hasezawa et al. 1989) or other tobacco varieties (Burgess et al. 1978). The variation in wall renewal time depends on differences in the protoplast isolation procedure (see Burgess 1983), even apart from the particular species. An extreme example is shown by subprotoplasts. Cell wall formation can be delayed for up to 16 h after initial wall removal, but will start at any time within 10 min as soon as the protoplasts are simply rinsed again in fresh regeneration medium (Rutten and Derksen 1990). Wall reformation can also be influenced by changes in the cell line because of sub-culturing over time (Cassels and Temma 1987). The present study confirms this, as our culture of this tobacco variety clearly differed in morphology from the original cell line, and we also adopted the protoplast isolation procedure, although to a lesser extent.

The organized pattern of cellulose deposition on protoplasts that are still spherical demonstrates a polarization of these cells before actual elongation. To some extent, this polarization is also reflected by the cytoskeletal organization (Kengen and Derksen 1991). The organization of the cortical cytoskeleton does not change because of the protoplast isolation procedure, and it shows distinct domains with ordered patterns showing extensive co-localization of microtubules and actin filaments (Kengen and De Graaf 1991). It was concluded that the so-called overall "random" organization must be considered a superficial view. We presume that the rapid wall reconstitution in our experiments might be related to the highly conserved cytoskeleton considering its presumed role in wall formation (Seagull 1991; Emmons et al. 1992).

The early tobacco cell wall shows layers with parallel swirls in changing orientations, similar to those reported in spherical cells of the alga Boergesenia (Legge and Brown 1988). In the alga Boodlea, the texture formation was interpreted as a change from random to helicoidal (Mizuta et al. 1991, 1994). The designation "random" was based on absence of birefringence. However, this approach is inconclusive, as, for example, the highly ordered helicoidal and cross-lamellate walls do not show birefringence either (Emmons 1988). In first view, such organization of CMF is initially interpreted as random in electron microscope preparations. However, it was recently demonstrated by Wolters-Arts and co-workers (1993) that primary walls are organized in a helicoidal-like manner as well.

From the very moment of cell elongation, predominantly transversely oriented CMF become clearly visible (see also Hasezawa et al. 1989). Transverse orientation, however, is a simplification of actual orientation. It was shown previously that "transverse" CMF are in fact part of typical S and Z helical alternation (see Wolters-Arts et al. 1993). Orientation, other than the abundant "transverse", is difficult to distinguish in Fluorescent Brightener 28 staining because of the brightness of fluorescence. Moreover, only nascent CMF are initially presumed to be stained (Sauter et al. 1993). Recently, Verbeelen and Stickens (1994) introduced polarization CLSM using Congo red to study wall formation in another tobacco variety. They observed a predominant transverse CMF orientation in elongating cells. Although this technique is elegant in addressing CMF orientation non-invasively and uses the advantages of an "ordinary" CLSM set-up, it is still restricted in that it will only detect parallel-ordered CMF.

We have reconstructed a time sequence of wall reconstitution from a number of wall-regenerating protoplasts. Although Calcofluor White is absorbed to the crystalline core of CMF (Herth and Schnepf 1980; Burgess 1983), it also interferes with cellulose crystallization (Haigler et al. 1980). Therefore, interpreting presumed CMF patterns at the surface of regenerating protoplasts is only correct where cells have been exposed to the dye for just a brief period. Abnormalities in development and growth, even at a relatively low dye concentration, are unavoidable (Itoh et al. 1984; Belliveau et al. 1990).

We have already addressed the question whether Calcofluor White is specific enough for crystalline cellulose. It may detect single or small bundles of CMF (Hasezawa et al. 1989), but its sensitivity depends on the extent of cellulose crystallization (e.g., Blaschek et al. 1982). The discriminating qualities of the dye for various polysaccharides, as discussed, are only effected by varying the concentration (Wood and Fulcher 1983; Meadows 1984). It may bind to fibrillar pectins (Colvin and Leppard 1973), but when used at low concentration, as applied in this study, binding to pectins can be ignored. During wall regeneration of proto-
plasts, components like pectins have a tendency to dissolve into their surroundings, unlike CMF (Hanke and Northcote 1974; Takeuchi and Komamine 1978). Similar observations were also reported for in vitro tip growth of tobacco, lily, and Petunia pollen tubes (Kappler 1988; Derksen et al. 1995). We exclude the possibility that Fluorescent Brightener 28 simultaneously stains the marked patterns of callose deposits, as staining for callose results in completely different patterns during the development of regenerated protoplasts as well as suspension cells.

Demonstration of callose deposits in vivo was accomplished using a decolourised aniline blue solution. Purification of dissolved water-soluble aniline blue, by simple paper chromatography, suppresses background staining almost completely and results in high-performance stainings of callose-containing reference material. The purified fluoresceinchrome Sirofluor (Evans et al. 1984) does not present any advantage to this purified aniline blue solution with regard to specific staining. The sensitivity of both dyes depends largely on how the β(1→3)-glucan chains form complexes with other wall components, as well as the type of addition to side chains (see Evans et al. 1984; Stone and Clarke 1992). The aniline blue solution that we have used has no deleterious effects on living cells. It may even increase tobacco pollen tube growth (see Kappler 1988). With respect to the ability of the aniline blue solution to show actual detailed patterns of callose deposits, and not just location, we have previously tested a monoclonal antibody against callose on tobacco pollen tube walls. Both techniques demonstrated that callose in the secondary wall is deposited in a fine reticular manner (see Amstel and Kengen 1992).

Freshly produced protoplasts do not show any fluorescence if stained with aniline blue. This supports our conclusion that the applied isolation procedure is relatively mild and conservative, compared with most others (e.g., Klein et al. 1981) in which the formation of callose is believed to directly result from the protoplast state. As for CMF formation, callose deposits are formed over the entire protoplast surface. Fibrillar and patch-like structures were observed, but during the observation period, no patterns similar to CMF deposits developed. The amount of callose seemed to be rather constant and wound callose could only be induced by vigorous pipetting, giving rise to completely different staining patterns. Callose synthesis in these cells seems to confirm that it is not just a matter of nonspecific membrane perturbation, as suggested by Kauss and Jeblick (1992).

Examining the development of callose deposits during the culture cycle of suspension cells first showed familiar staining patterns. Cell plates and the transverse walls are characterized by the presence of callose (see also Fulcher et al. 1975). The patches of callose in the central region are probably associated with the plasmodesmata (Longly and Waterkeyn 1977). The difference in intensity of fluorescence between marginal and central regions was attributed to differences in molecular weight or to conformational changes of the callose (Kakimoto and Shibaoka 1992). These callose deposits form a closed ring at the edge that, according to CLSM, consist of fibrillar strands. It is suggested that callose may be transiently involved in connecting the initial wall of the cell plate with the primary, lateral wall as fluorescence decreases towards the stationary phase.

Tobacco BY2-G0 cells show an increase in spot-like and fibrillar, mainly transversely oriented callose deposits in the lateral walls during growth of the culture and a decrease when growth declines. They gradually encircle cells completely, and, as far as can be determined, are located near the membrane. Such deposits occur also during elongation of regenerated protoplasts. To date, only developing cotton hairs have shown similar structures (Waterkeyn 1981). In these cells, callose anticipates similarly oriented CMF. The dimensions and general staining features of tobacco callose deposits seem to rule out that they might be mistaken for cellulose. Yet, their exact chemical nature has to be resolved first. Microfibrillar callose synthesis in vitro has recently been achieved using a higher plant synthase (Bulone et al. 1995), indicating that fibrillar callose production can be a constructive phenomenon. The tobacco deposits do not result from cell death, wounding, dehydra­tion, or staining. Therefore, as proposed for cotton hairs, they probably are directly related to wall formation. Contemplating a particular function for callose in these specific cells is difficult considering all the other listed observations (see also Stone and Clarke (1992) for discussion). Callose must be related to changes in growth, as it appears only transiently. This might also be concluded from observing daughter cells, which always exhibit similar patterns. Considering the stability of regenerating protoplasts, we believe it unlikely that the patterns evolve from destablized cellulose-producing units (Northcote 1991), at least not as a function of wounding processes. Growth of cells implies elongation and oriented CMF deposition to a defined degree. As callose orients similarly, i.e., transversely and perpendicular to the axis of elongation, it might be involved as a temporal stabilizer of the newly incorporated plasma membrane. Thus, perhaps the function is in consolidating the new directionality of CMF deposition. Moreover, callose deposits in walled cells may stabilize or connect the plasmamembrane to the cell wall at the sites of the plasmodesmata (Longly and Waterkeyn 1977), ectodesmata (Amstel 1996), or to non-plasmodesmal binding sites for the Hechtian strands (Pont-Lezica et al. 1993; Oparka et al. 1994). Although speculative in part, such a versatile function may explain different reaction patterns, as shown for Morinda, but for other cells and regenerating protoplasts in other studies as well (Klein et al. 1981; Mock et al. 1990; Schaeffer and Walton 1990).

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References


