Protoplasma (1993) 176: 33–42

PROTOPLASMA

A monoclonal antibody recognizes a 65 kDa higher plant membrane polypeptide which undergoes cation-dependent association with callose synthase in vitro and co-localizes with sites of high callose deposition in vivo

Deborah P. Delmer[1], M. Volokita[1], M. Solomon[1], U. Fritz[2], W. Delphendahl[2], and W. Herth[2,] *

[1] Department of Botany, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem and
[2] Zellenlehre, Universität Heidelberg, Heidelberg

Received April 5, 1993
Accepted July 7, 1993

Summary. A monoclonal antibody (MAb) capable of immobilizing detergent-solubilized UDP-glucose: (1 → 3)-β-glucan (callose) synthase activity from higher plants has been selected and characterized. On Western blots this MAb recognizes a polypeptide of about 65 kDa found in membranes isolated from a variety of plant sources. The polypeptide recognized by this MAb does not appear to bind the substrate UDP-glucose, and evidence is presented which indicates that this polypeptide associates with the enzyme complex in a cation-dependent manner under conditions where the callose synthase assumes a larger size. Indirect immunofluorescence localization with this MAb was positive with sieve plates of cucumber (*Cucumis sativus*) seedlings, and with plasmodesmata of onion (*Allium cepa*) epidermal cells, both being sites of localized, stress-induced callose deposition.

Keywords: Callose; β-1,3-Glucan synthase complex; Monoclonal antibody; 65 kDa polypeptide; Enzyme immobilization; Immunofluorescence.

Abbreviations: BSA bovine serum albumine; DMSO dimethylsulfoxide; DTT dithiothreitol; FITC fluorescein isothiocyanate; HB Hepes buffer; HBS Hepes buffer plus 0.15 M NaCl; IgG immunoglobulin G; MAb monoclonal antibody; MSB microtubule stabilizing buffer; NP 40 Nonidet P 40; PBS phosphate buffered saline; SDS-PAGE sodium dodecylsulfate polyacrylamide gel electrophoresis; UDP uridine diphosphate; UV ultraviolet.

Introduction

In higher plants, callose (β-1,3-glucan) is a polymer often synthesized in response to wounding or pathogen attack. Deposition is often highly localized at the plasma membrane-wall interface at sites of infection, and callose can also be synthesized in high amounts in plasmodesmatal regions and on phloem sieve plates, where it may play a role in blocking movement of components between cells (Eschrich 1956, Currier 1957, Schumacher 1967, Kauss 1990, Robards and Lucas 1990). Callose is also synthesized without wounding at certain stages of development; the pollen tube wall is rich in callose (Herth et al. 1974, Cornish et al. 1988), cotton fibers deposit callose at the early stages of secondary wall synthesis (Maltby et al. 1979), and the polymer is also transiently observed in the cell plate during cell division (Kakimoto and Shibaoka 1992).

Callose synthase (UDP-glucose: β-1,3-glucan synthase) is known to be localized in the plasma membrane, and is activated by micromolar amounts of Ca^{2+} in combination with a β-glucoside (Delmer 1987, Kauss 1990). Although the enzyme is stable, possesses high activity in vitro, and can be solubilized by detergents such as Chaps and digitonin, it has proven very difficult to purify to homogeneity. However, several recent reports in the literature have identified a variety of polypeptides which could be likely subunits of the enzyme. From affinity-labeling studies with the substrate UDP-glucose or its analogs, polypeptides of 57 kDa (red beet; Frost et al. 1990), 52 kDa (cotton fibers; Delmer et al. 1991), or 32 kDa (rye; Meikle et al. 1991) have been proposed as likely candidates for the catalytic subunit. Gel analyses of major polypeptides present in partially-purified preparations of callose synthase show enriched

* Correspondence and reprints: Zellenlehre, Universtät Heidelberg, Im Neuenheimer Feld 230, D-69120 Heidelberg, Federal Republic of Germany.

polypeptides in the molecular weight range of 27–34 kDa (Wasserman and Wu 1992, Fink et al. 1990, Girard et al. 1992); 65–66 kDa (Fredrikson et al. 1991), and 48–58 kDa (Meikle et al. 1991, Duggha and Ray 1991, Girard et al. 1992). Taken collectively, these studies suggest that the enzyme complex may be comprised of several non-identical subunits. This is also supported by the observations of Hayashi et al. (1987) which show that the size of the callose synthase is increased in the presence of divalent cations which could be interpreted either as aggregation or association with additional non-catalytic subunits. Recent studies with cotton fibers indicate that a 34 kDa annexin-like protein associates with the enzyme in a Ca^{2+}-dependent manner (Andrawis et al. 1993).

Callose and cellulose synthases share the common features of both being localized in the plasma membrane and utilizing the same substrate UDP-glucose (Delmer 1987). However, perturbed conditions which favor callose synthesis are not favorable for cellulose synthesis; because of these similarities combined with apparent opposing modes of regulation, Delmer (1987) suggested that the two enzymes might share common subunits which are regulated in an opposite fashion in vivo. The cellulose synthase of higher plants also has not been purified from plants, although polypeptides of 84 and 83 kDa have been identified by affinity labeling studies with cotton fibers as possibly being analogous to the catalytic and regulatory subunits of the well-characterized bacterial cellulose synthase (Amor et al. 1991). Another approach to identify specific polypeptides for proteins, the purification of which has proven difficult, is to attempt to select specific monoclonal antibodies (MAbs) which recognize specific epitopes on the native enzyme. A recent example is the selection of a MAb apparently directed against some component of the ryegrass callose synthase by use of a partially purified enzyme preparation as immunogen and subsequent selection of hybridomas which secreted MAb which could form immunoprecipitable complexes with the enzyme (Meikle et al. 1991). The precipitated complex was enriched for polypeptides of 30, 31, 54, and 58 kDa.

Results presented in this paper similarly describe the selection of a MAb which can form an immune complex and immobilize detergent-solubilized callose synthase activity. We also show that this MAb recognizes a polypeptide of about 65 kDa from several different plant sources. Immunolocalization studies show that the antigen recognized by this antibody is highly localized to sites of high callose deposition, further supporting the conclusion that the antigen is a component of the callose synthase.

Material and methods

Isolation and partial purification of callose synthase

Membrane proteins were prepared and proteins solubilized with digitonin as described (Hayashi et al. 1987, Delmer et al. 1991). Plant tissues utilized included etiolated seedlings of mung beans (*Vigna radiata*), pumpkin (*Cucurbita pepo* var. Gelber Zentner), cucumber (*Cucumis sativus* var. Delikatess badenia), and developing cotton fibers (*Gossypium hirsutum* Acala SJ-2; see Delmer et al. 1991). Glycerol gradient centrifugation of digitonin-solubilized proteins and routine assay of callose synthase were performed as described by Delmer et al. (1991).

Hybridoma production

Balb/c female mice were injected with a mixture of *Vigna radiata* (mung bean) membrane proteins containing callose synthase activity. This mixture was obtained by solubilization of membrane proteins in digitonin and subsequent product entrapment of callose synthase essentially as described previously (Hayashi et al. 1987). The specific activity of callose synthase in this preparation was about 900 nmol glucosyl units transferred to (1 → 3)-β-glucan (callose) per min/mg protein; in terms of specific activity, this represents a minimum 20-fold purification from the crude digitonin-solubilized protein preparation. The preparation used for immunization contained about 10 clear bands detected by Coomassie Blue staining of gels from SDS-PAGE, and many more minor bands were detectable after silver staining of similar gels. The initial injections contained 90 μg protein in combination with complete Freund's adjuvant; subsequent injections contained similar amounts of protein in incomplete adjuvant. Hybridomas were prepared from one of these mice essentially as described by Galfre and Milstein (1981), and the myeloma line SP 2/O-Ag 14 described by them was used for fusion with the spleen cells.

Selection and growth of hybridomas; purification of MAbs

Screening of culture filtrates for MAbs directed against callose synthase was carried out using an immunoprecipitation procedure in which culture filtrate (50 μl) from individual hybridoma wells was incubated in 96-well plates for 2 h at 25 °C with 50 μl of crude digitonin-solubilized mung bean proteins containing about 5 μg protein and 0.15 units (1 unit = 1 nmol/min) of callose synthase activity. Anti-mouse IgG (Sigma, St. Louis, MO; 25 μl containing 17.5 μg protein in Ab buffer) was then added and incubation continued for another 1 h; following this, 25 μl of a 1% (w/v) suspension of *S. aureus* cells (Sigma) in Ab buffer was added and incubation continued for a further 30 min with occasional shaking. The plates were then centrifuged 10 min at 4 °C at 5,000 **g**. Supernatants were not routinely assayed; the pellets were resuspended in 0.1 ml 50 mM Hepes/KOH pH 7.3 containing 1mM DTT, and immunoprecipitated callose synthase was detected by assaying the resuspended pellets for 15 min at 25 °C in 150 μl final volume containing 0.13 mM UDP-[^{14}C]glucose (specific activity 93 Bq per nmol), 6.7 mM cellobiose, 3.4 mM $CaCl_2$, 0.3 mM DTT, and 50 mM Hepes/KOH pH 7.3. Reactions were terminated by addition of 75 μl of 66% (v/v) ethanol containing 0.5 N HCl. Reactions were then transferred to wells of a bio-dot apparatus (Bio-Rad, München) fitted with a piece of Type A/E glass fiber paper (Gelman, Düsseldorf). Glucan formed

was collected by filtration from the wells and washed 7 times with 66% (v/v) ethanol/0.5 N HCl. The glass fiber paper was then placed on Kodak O-Mat X-ray film overnight for radioautography of the radioactive spots containing glucan product; for quantification, the spots of glass fiber paper were cut and quantified by liquid scintillation counting.

Hybridomas which screened positive in two different tests were cloned by limiting dilution and cell lines from positive clones were frozen and stored in liquid nitrogen in 95% (v/v) fetal calf serum containing 5% (v/v) DMSO and 0.005% (w/v) gentamycin. Clones of interest were adapted to growth on Ventrex (Portland, ME) HB-1 medium containing 2% (v/v) fetal calf serum, 2 mM L-glutamine, 25 mg/l ampicillin, 100 mg/l streptomycin sulfate, and 0.1% (v/v) Fungizone (Gibco, Grand Island, NY). Culture filtrates were concentrated by pressure dialysis (Amicon; YM-10 or PM-30 filters) and MAb purified using the Affi-Gel Protein A MAPS II MAb purification system (Bio-Rad). Each MAb was subtyped with Ouchterlony immunodiffusion plates (Serotec, Blackthorn, U.K.).

Dot-blot enzyme immobilization assay

This procedure was adapted from the Bio-Rad procedure for immunoblotting using their bio-dot apparatus fitted with a sheet of nitrocellulose (Schleicher and Schüll BA 85). Various dilutions of purified MAb (50 µl) in 100 mM Hepes/KOH, pH 7.3 (HB) containing 0.15 M NaCl (HBS) were gravity filtered through wells in the apparatus. Blocking of additional protein binding sites was then performed by gravity filtration of 200 µl of 1% (w/v) BSA in HBS. This was followed by three rapid washes in vacuum with HBS, one wash with HB, and then addition and gravity filtration of 100 µl of callose synthase partially purified by glycerol gradient centrifugation (Delmer et al. 1991). This was followed by 3 washes with HB; filtration was then blocked, and 100 µl of assay mixture containing 0.2 mM UDP-[^{14}C]glucose (Amersham; 93 Bq per nmol), 20 mM cellobiose, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM DTT, and 50 mM Hepes/KOH pH 7.3 was added to the wells. After incubation for 15 min, the wells were washed 5 times with HBS; the nitrocellulose was dried, and areas underneath the wells containing [^{14}C]glucan were either subjected to radioautography or cut and product formed was quantified by liquid scintillation counting.

Other assays

Protein was assayed using the Bio-Rad protein assay kit with BSA as standard. SDS-PAGE was carried out using the procedure of Laemmli et al. (1970) as described in more detail by Delmer et al. (1991). Blotting of proteins and antibody probing of blotted proteins (Western blots) were performed as described by Elthon and McIntosh (1987) using anti-mouse IgG alkaline phosphatase conjugate (Sigma).

In situ localization of antigen

The antigen recognized by MAb 2-1 was localized using an indirect immunofluorescence procedure essentially as described by Quader et al. (1987) for the detection of microtubules. Longitudinal razor blade sections of the root region of cucumber (*Cucumis sativus* var. Delikatess badenia) seedlings, of mung bean (*Vigna radiata*) seedlings, and freshly prepared adaxial epidermal cells of onion bulbs were used as source of tissue. Pollen tubes of *Lilium longiflorum* were cultivated in vitro as described (Herth et al. 1974). Following fixation, reduction, cell wall digestion, and extraction with 5% DMSO and 5% NP-40, all as described (Quader et al. 1987), the objects were incubated for 1 h at 37 °C with MAb 2-1 (18 µg/ml) in PBS. Specimens were either directly proceeded for immunolocalization and staining procedures as described above, or dehydrated in an ethanol series, embedded in resin LR White, sectioned with glass knifes on a Reichert OMU 2 ultramicrotome to yield 3 µm sections. After 3 washes with PBS, and further wash with freshly dissolved milk powder (5% milk powder in PBS; Moore 1989) to block nonspecific absorption, they were then reacted with MAb 2-1 (18 µg/ml) in PBS. After 3 washes in PBS the objects or sections then reacted for 1 h at 37 °C with anti-mouse IgG-FITC conjugate (Sigma; 1 : 15 dilution in PBS), and then were rinsed 3 times with PBS. Specimens were mounted for microscopy using Diazobicyclooctan (Sigma; 25–100 mg/ml in 10% PBS/90% glycerol, pH 9.0). Aniline blue staining and Calcofluor white staining methods were as described (Eschrich and Currier 1964, Herth 1980). Fluorescence microscopy was performed with a Zeiss IM 35 microscope equipped with epifluorescence illumination, using either UV illumination or an FITC-specific filter combination; objectives used were Plan Neofluar 40, 0.9; NPL Fluotar 40, 1.3 (Leitz, Wetzlar) and Neofluar 100, 1.3. Photographs were taken with an Olympus OM 2 camera body with Ilford XP 1 film.

Results

Initial screening and characterization of MAbs

Tests of the polyclonal serum taken from the immunized mouse several days before removing spleen cells for fusion indicated that the serum contained antibodies which both inhibited and allowed immunoprecipitation of enzyme activity. Since our hybridoma screen followed only activity in the pellet fraction, we would have selected against clones which produced MAb which inhibited enzyme activity. On Western blots of mung bean membrane proteins, the immune serum at a 1 : 500 dilution allowed clear detection of a number of protein bands (not shown).

The fusion yielded more than 600 hybridomas; of these about 400 were screened. Of these, culture fluid from 37 screened positive; in a second test 24 repeated as positive, although many of these only gave a weakly positive response; 10 of them most promising were cloned by limiting dilution. In more rigorous testing of affinity purified antibody from these clones using the dot-blot assay (see below), only 3 clones were considered worthy of further study; these clones were designated 2-1, 3-3, and 9-1. All of these clones were found to produce the IgG_1 class of antibody. None inhibited callose synthase activity even at high concentrations of antibody.

Testing of purified MAbs using the dot-blot enzyme immobilization assay

Figure 1 shows the reaction of the MAb of the clone of most interest, 2-1, with digitonin-solubilized mung bean or cotton fiber callose synthase partially purified

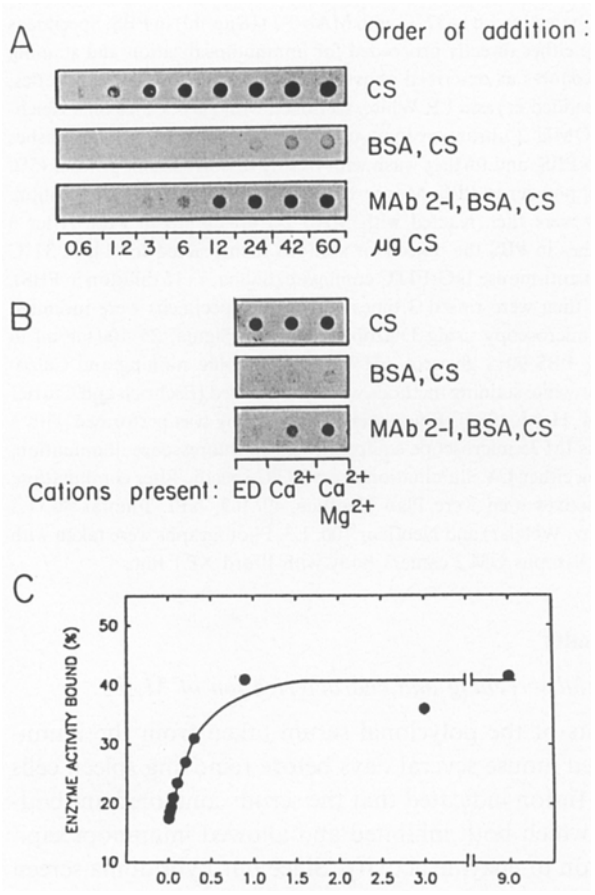


Fig. 1. Immobilization of callose synthase activity using affinity-purified MAb 2-1. **A** Radioautograms of dot blots showing the production of [^{14}C]glucan produced by varying amounts of immobilized cotton fiber callose synthase. *CS* Gradient-purified callose synthase; produced 330 cpm (0.07 nmol) of product per μg protein when applied directly to the matrix. *BSA* A blocking step with bovine serum albumin; *MAb 2-1* 0.6 μg of affinity-purified MAb 2-1. In all cases, CS was applied and subsequently washed in HBS containing 1 mM $CaCl_2$ and 5 mM $MgCl_2$. **B** Experiment similar to **A** using 20 μg of CS and 0.6 μg of MAb 2-1. In this experiment, the CS was applied and washed in HBS which contained either 10 mM EDTA (ED), 1 mM $CaCl_2$, or 1 mM $CaCl_2$ plus 5 mM $MgCl_2$ as indicated. **C** Varying amounts of MAb 2-1, as indicated, were applied to wells, blocked with BSA, and followed by application of 7.2 μg of mung bean callose synthase, partially purified by glycerol gradient centrifugation. The applied 7.2 μg were set as 100% of activity and were capable of producing 15,500 cpm (3.1 nmol) of product when immobilized directly on the matrix. Following assay of bound enzyme, the wells were washed and regions of bound activity were cut and quantified by liquid scintillation counting

by gradient centrifugation. Using affinity-purified MAb from this clone, we consistently observed immobilization of callose synthase activity. Figure 1 A and B shows this qualitatively using callose synthase from cotton fibers. These radioautograms of dot blots show the [^{14}C]glucan produced by various amounts of enzyme immobilized on the blot. The upper-most track shows the immobilization of various amounts of the enzyme alone (CS), and indicates that activity can be preserved when enzyme is immobilized directly on blots, although activity recovered is somewhat inhibited compared to comparable assays in solution. If all binding sites on the matrix are first blocked by a treatment with bovine serum albumin, then very little CS activity can subsequently be bound (BSA, CS). However, addition of 0.6 μg of affinity-purified MAb 2-1 to the blot prior to blocking results in the immobilization of CS activity (MAb 2-1, BSA, CS). Figure 1 B shows that immobilization is more efficient when divalent cations are present in the buffer when enzyme is applied. Figure 1 C shows a quantitative study performed using a similar enzyme preparation from mung beans. Saturation of binding of enzyme occurred with about 1 μg of affinity-purified MAb 2-1. In all these studies, the maximum amount of enzyme which could be bound at saturation was never more than about 40% of that applied, a result which might relate to the affinity between antibody and antigen, and/or to the difficulty in immobilizing a large enzyme with an antibody recognizing only a single epitope. Negative controls using similar amounts of purified MAb from five other random clones showed no ability whatsoever to bind the enzyme (not shown). MAb 3-3 also was able to consistently immobilize enzyme activity, but at saturation, only about 20% of total enzyme applied could be immobilized. MAb 9-1 on occasion also gave positive results, but reactions were less consistent than with MAb 2-1.

Reaction of affinity-purified MAb 2-1 with blotted membrane proteins

Figure 2 shows the reaction of purified MAb 2-1 on Western blots of membrane proteins from mung bean, two varieties of cucumber, and cotton fibers. For all preparations, a reaction with a polypeptide of about 65 kDa is observed which, in cotton fibers, sometimes resolves into a doublet of proteins of very similar molecular weight. We have also shown similar reactions using membrane preparations derived from pea, corn and onion (not shown). Thus, this MAb appears to recognize a determinant on a polypeptide which is fairly conserved in the plant kingdom. When the other potentially-positive clones were tested, both 9-1 and 3-3 showed reactions with the same 65 kDa polypeptide, whereas several MAbs which were selected as controls

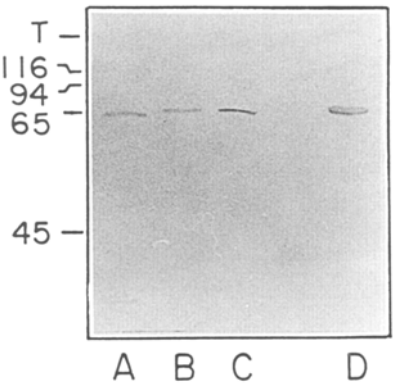


Fig. 2. Western blots of membrane proteins following reaction with MAb 2-1. Approximately 5 µg of membrane protein was loaded per lane onto a 10% acrylamide gel; following SDS-PAGE, the proteins were blotted onto nitrocellulose and reacted with 1 µg/ml of affinity-purified MAb 2-1. *A* Mung bean; *B* pumpkin var. Gelber Zentner; *C* cucumber var. Delikatess badenia; *D* cotton fibers. Numbers to the left of figure indicate the position of migration of molecular weight markers; *T* top of separating gel

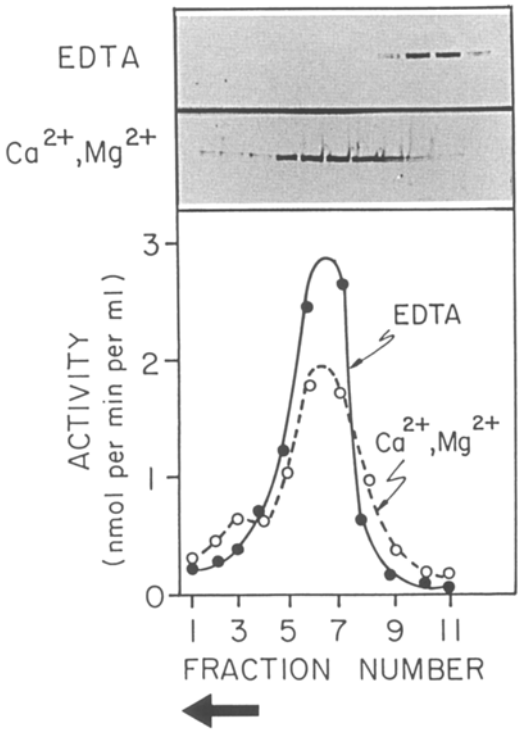


Fig. 3. Pattern of migration of callose synthase activity and 65 kDa polypeptide during glycerol gradient centrifugation of digitonin-solubilized cotton fiber membrane proteins in the presence or absence of divalent cations. *EDTA* Gradient contained 5 mM EDTA and was centrifuged 20 h at 100,000 g. *Ca^{2+}, Mg^{2+}* Gradient contained endogenous levels of Ca^{2+} and 5 mM $MgCl_2$ and was centrifuged 10 h at 100,000 g. Affinity-purified MAb 2-1 at 0.6 µg/ml (EDTA) or 1.8 µg/ml (Ca^{2+}, Mg^{2+}) was used as probe in Western blotting. Arrow indicates the direction of centrifugation

showed reaction with different polypeptides (data not shown). Thus, a good correlation exists between ability to immobilize callose synthase activity and reaction with this 65 kDa polypeptide. Since MAb 2-1 was the most effective of the three clones, it was used for further studies.

MAb 2-1 does not appear to be recognizing sugar determinants on the protein; treatment of the proteins prior to SDS-PAGE with anhydrous HF, under conditions which destroy glycosidic, but not peptide, linkages (Sanger and Lamport 1983), does not change the pattern of migration of the doublet nor its ability to be detected on Western blots (not shown). The protein appears to be an integral plasma membrane protein since it co-purifies with plasma membranes of cotton fibers during two-phase plasma membrane purification procedures (Larsson et al. 1987), and requires either Chaps or digitonin for effective solubilization.

Previous results of ours with cotton fibers indicated that a polypeptide of 52 kDa could be affinity-labeled with the substrate UDP-glucose in a Ca^{2+}-dependent manner, that this polypeptide co-localized with callose synthase activity on gradients, and was suggested to be the catalytic subunit (Delmer et al. 1991). Since, in those studies, no labeling was observed for a polypeptide in the range of 65 kDa, this indicates that the polypeptide recognized by MAb 2-1 is most likely not the catalytic subunit of the enzyme. In order to study further the relationship of the 65 kDa polypeptide to callose synthase, we tested whether there was a coincidence between the pattern of migration of this polypeptide with callose synthase activity during glycerol gradient centrifugation using digitonin-solubilized proteins from cotton fibers. During centrifugation in the presence of the divalent cations Mg^{2+} and Ca^{2+}, callose synthase activity migrates as a defined peak of activity, and Western blotting of these fractions shows a perfect correlation of localization of enzyme activity and of the antigen recognized by MAb 2-1 (Fig. 3). However, as observed previously by Hayashi et al. (1987), if gradients are run instead in buffer containing EDTA in place of divalent cations, the rate of migration of callose synthase is about 2 times slower, while the migration behavior of the bulk of solubilized proteins remains unchanged. When gradients were run in the presence of EDTA for twice the time used for the gradient containing cations, we reproducibly recovered more callose synthase activity, and the enzyme migrates to a similar position in the gradient; however, the 65 kDa polypeptide detected by Western blots with MAb 2-1 no

longer migrates coincidently with callose synthase activity (Fig. 3). An identical shift was also observed in a comparable experiment using digitonin-solubilized proteins from mung beans (not shown). Such a result supports the idea that the increase in size of the callose synthase is due to a cation-dependent association with other subunit(s), and that the 65 kDa polypeptide is most likely one such subunit.

In situ localization of the antigen recognized by MAb 2-1

Attempts at localization with mung bean seedlings or pollen tubes of *Lilium* were hampered by the fact that a high nonspecific reaction with the second antibody could not be eliminated. However, a positive reaction with sieve plates was observed with MAb 2-1 when hand sections of the phloem region of cucumber seedlings were tested (Fig. 4 b, c). The cucumber root sieve plates stained also with aniline blue indicating the presence of callose in situ (Fig. 4 a). Controls with the second antibody alone showed only a weak diffuse general staining (not shown). Because of difficulties in fixation of fresh material, cotton fibers were not examined.

Most localization experiments in this study were performed with adaxial epidermal cells of onion bulbs. This material was chosen because of being a classical object for callose deposition (Eschrich 1956); the cells show a strong wound reaction with intensely localized callose deposition in the plasmodesmatal regions; these regions can be localized by aniline blue fluorescence of the callose (Fig. 5 a). Figure 5 c and d shows that indirect immunofluorescence reaction, using MAb 2-1 as first Ab, is restricted to the plasma membrane/cell wall region and is most intensely localized in the plasmodesmatal regions where callose synthesis is most intense. A control using only FITC-conjugated second antibody gave no reaction; in addition, use of another mouse MAb against brain alpha-tubulin as first Ab, showed specific reaction only with microtubules and not in the areas specifically recognized by MAb 2-1 (Fig. 5 b). When tested on the 3 µm resin sections of embedded onion epidermal cells, Calcofluor white staining was restricted to cell walls except plasmodes-

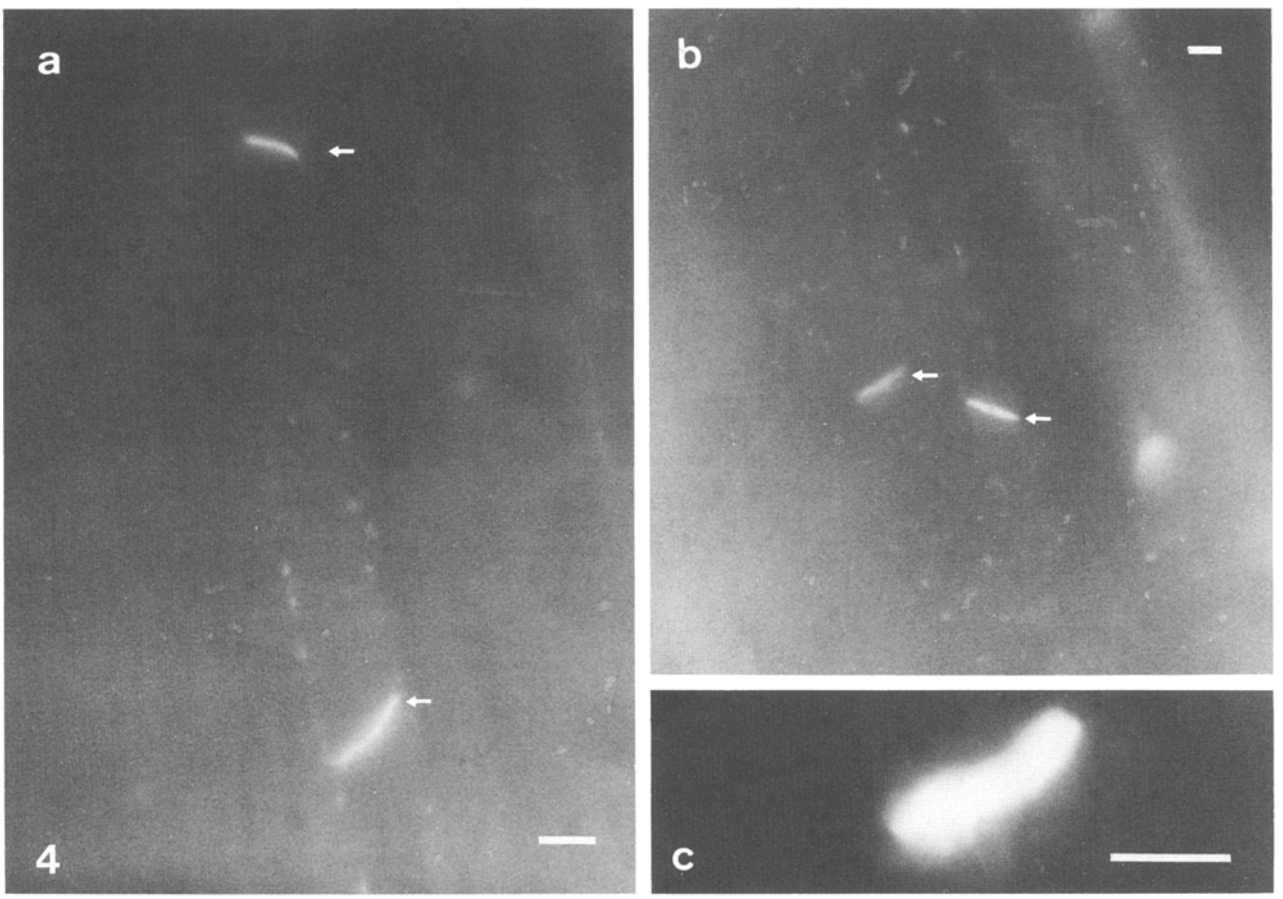


Fig. 4. Cucumber seedlings, longitudinal hand section of the phloem region of the root. **a** Two sieve plates of a phloem element stained with aniline blue (arrows). **b** and **c** Positive reaction of sieve plates with MAb 2-1; **b** survey (arrows), **c** high magnification. Bars: 10 µm

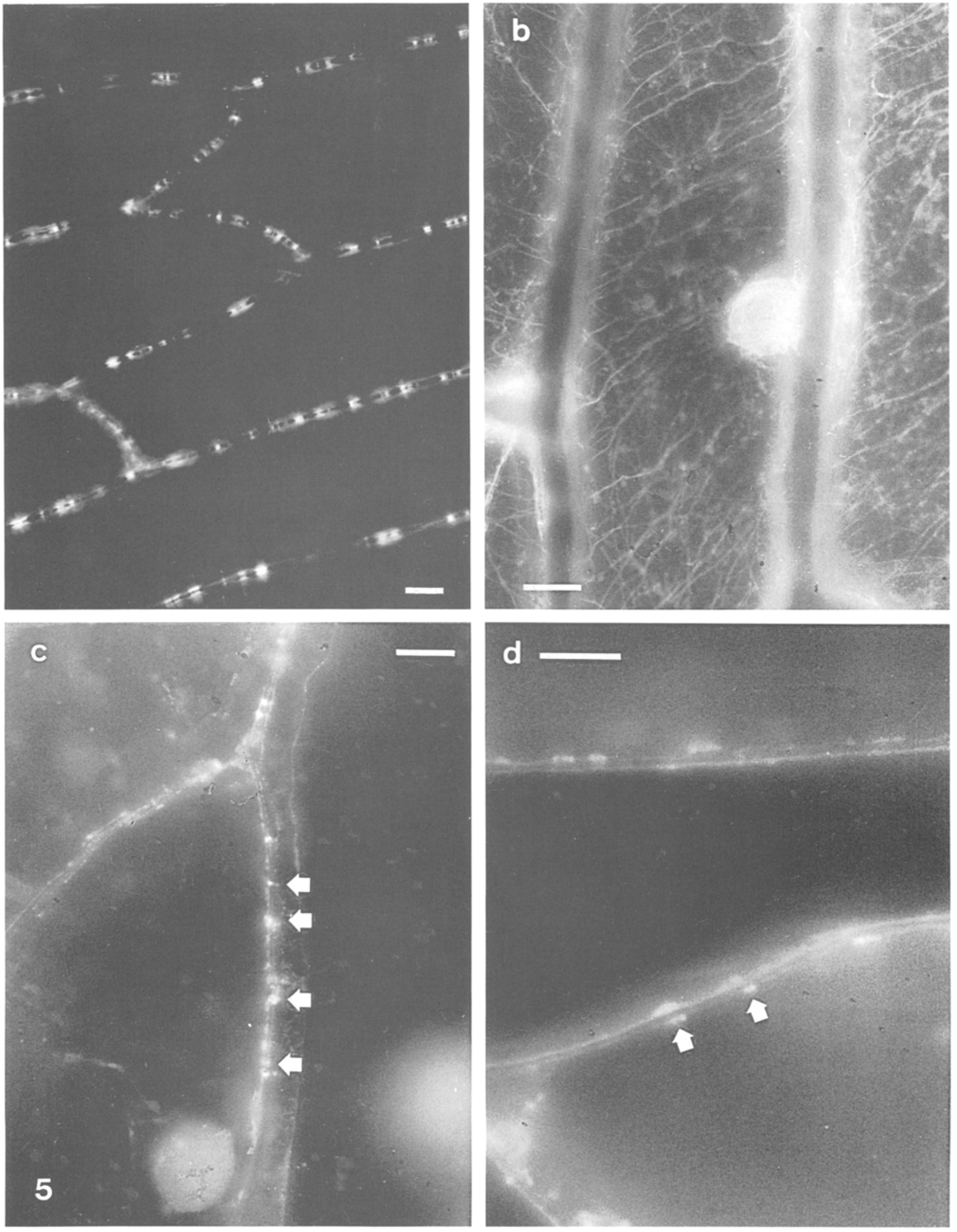
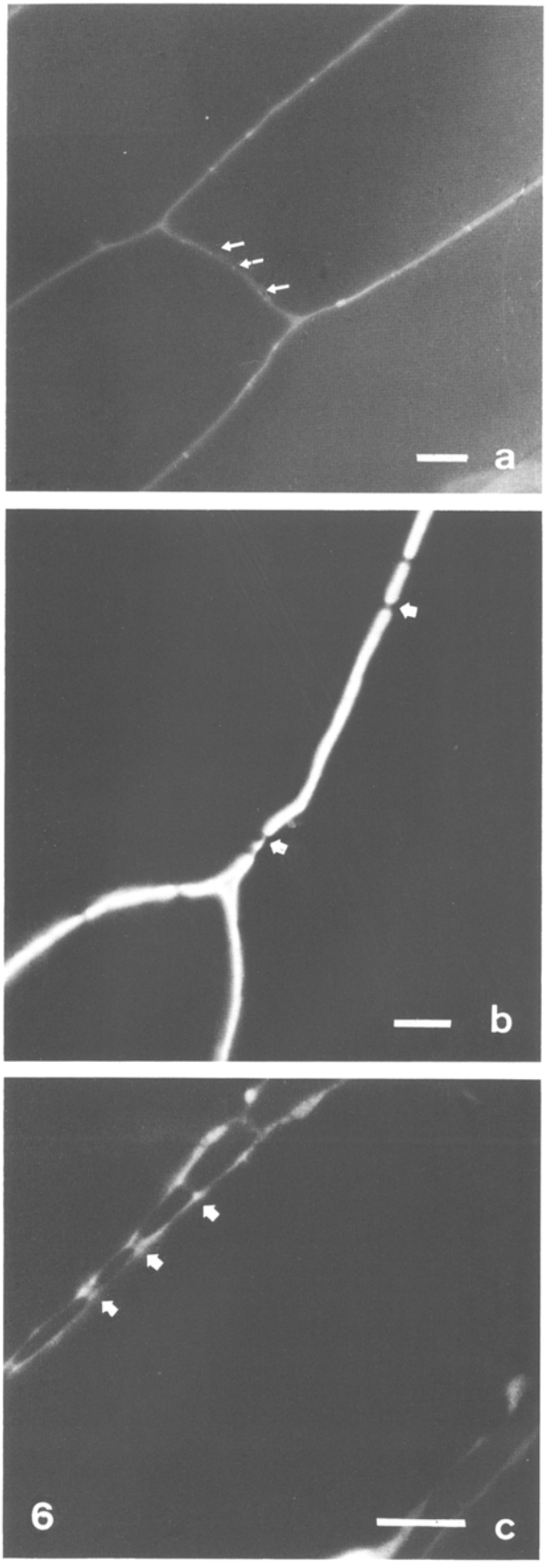


Fig. 5. Onion epidermal cells. **a** Aniline blue staining of plasmodesmata; **b** tubulin localization with an antibody to alpha-tubulin; **c** and **d** localizations of sites reacting with MAb 2-1 (arrows). Bars: 10 μm



mata (Fig. 6 b), aniline blue weakly but selectively stained the plasmodesmatal regions (Fig. 6 a), and localization with MAb 2-1 (Fig. 6 c) was positive for plasma membrane and plasmodesmatal regions.

Discussion

The findings that MAb 2-1 can immobilize callose synthase activity and that the antigen recognized is concentrated in situ in an area of high callose deposition (plasmodesmata and sieve plates) argue strongly that this MAb is directed against a polypeptide component of the enzyme. The initial difficulties encountered with experiments to immunolocalize the MAb 2-1 reactive sites, like nonspecific absorption as in mung bean and pollen tubes of *Lilium*, are frequently encountered in such studies. They may be due to nonappropriate preparation and fixation conditions which might be overcome with further variations of the experimental protocol. First attempts with immunogold localization of MAb 2-1 reactive sites were also not yet successful due to nonspecific absorption. The positive immunolocalizations of MAb 2-1 reactive antigens with cucumber sieve plates and onion plasmodesmata show the main reaction at the same sites which also react strongly with aniline blue. In onion, however, the positive sites are not so well defined as the callose plaques deposited. Further studies with electron microscopic localizations are needed to resolve in detail whether the antigen is restricted to the plasma membrane or also trapped in the callose being deposited.

The immunolocalizations with MAb 2-1 in no case so far showed reactions with sites of high cellulose deposition, like root hair tips or developing xylem elements (same study, not shown). Thus it seems that the MAb 2-1 positive polypeptide does not interact with the cellulose synthase complex. This does not exclude the possibility that other components are shared in β-1,3- and β-1,4-glucan synthases (see Introduction).

Western blot analyses indicate that this MAb 2-1 positive antigenic component is a polypeptide of about 65 kDa. Affinity-labeling studies (Delmer et al. 1991) do not indicate that this polypeptide interacts with the

Fig. 6. Onion epidermal cells, LR White embedded, 3 µm sections. **a** Aniline blue staining, relatively weak reaction in plasmodesmatal regions (e.g., at arrows), cell wall with weak autofluorescence; **b** Calcofluor white staining, strong reaction of cell wall except plasmodesmata (e.g., at arrows); **c** localization of plasmodesmata positive to MAb 2-1 (arrows). Bars: 10 µm

enzyme substrate UDP-glucose; rather our studies indicate that this polypeptide interacts with the enzyme in a cation-dependent manner. We note that a fairly highly-purified callose synthase from cauliflower also contained a doublet of polypeptides in the range of 65–66 kDa (Fredrikson et al. 1991), and it may be that these polypeptides are related to the one recognized by our MAb 2-1. Because of differences in running conditions during SDS-PAGE, we also cannot exclude the possibility that one of the polypeptides in the range of 55–60 kDa identified in other systems as being enriched in partially-purified callose synthase preparations (Meikle et al. 1991, Dhugga and Ray 1991) could as well be homologs of the 65 kDa polypeptide.

The specific function of this polypeptide is unknown, and these studies raise new questions about the structure and mechanism of such glucan synthases. The most intriguing result concerns the nature of the larger enzyme complex formed in the presence of divalent cations. This complex always has reduced activity compared to the smaller complex in EDTA. Other recent studies of ours with the cotton fiber enzyme have identified another 34 kDa annexin-like protein which also apparently associates with the enzyme in a calcium-dependent manner (Andrawis et al. 1993). In those studies, the association of the annexin protein with the enzyme has been shown to correlate with reduction of the activity of the enzyme. Further studies will be necessary to determine the significance of these associations with respect to the regulation of callose synthesis in vivo. We have recently observed that the polypeptide recognized by MAb 2-1 undergoes a calcium-dependent phosphorylation in vitro (unpubl. obs.). Such a finding may indicate that regulation of phosphorylation is one factor which regulates enzyme activity, size, and/or localization in vivo. Possible functions for these non-catalytic subunits might be to play a role in priming the glucan synthase reaction, in regulation of activity, or in anchoring the enzyme to the cytoskeleton. This last possibility is particularly attractive, as callose synthesis is often highly localized in vivo.

In conclusion, the results presented once again point out the complexity of glucan synthesis in plants, but at the same time, offer further approaches for isolating and characterizing specific polypeptides involved in glucan synthesis, and of studying factors which control the assembly and activity of the native complexes.

Acknowledgments

We thank T. Elthon and R. Nickels for advice and help with MAb techniques and J. Walton and H. Kende for providing laboratory facilities for the MAb selection which was carried out at the MSU-DOE Plant Research Laboratory at Michigan State University. This work was supported by a grant No. I-34-046.9/87 from the German-Israeli Foundation for Scientific Research and Development (GIF) and also by contract DE-AC02-76ERA-1338 (to DPD) from the U.S. Department of Energy.

References

Amor Y, Mayer R, Benziman M, Delmer DP (1991) Evidence for a cyclic diguanylic acid-dependent cellulose synthase in plants. Plant Cell 3: 989–995

Andrawis A, Solomon M. Delmer DP (1993) Cotton fiber annexins: a potential role in the regulation of callose synthase. Plant J (in press)

Cornish EC, Anderson MA, Clarke AE (1988) Molecular aspects of fertilization in flowering plants. Annu Rev Cell Biol 4: 209–228

Currier HB (1957) Callose substances in plant cells. Amer J Bot 44: 478–488

Delmer DP (1987) Cellulose biosynthesis. Annu Rev Plant Physiol 38: 259–290

Delmer DP, Solomon M, Read SM (1991) Direct photolabeling with [^{32}P]UDP-glucose for identification of a subunit of cotton fiber callose synthase. Plant Physiol 95: 556–563

Dhugga KS, Ray PM (1991) Isoelectric focussing of plant plasma membrane proteins. Plant Physiol 95: 1302–1305

Elthon TE, McIntosh L (1987) Identification of the alternative terminal oxidase of higher plant mitochondria. Proc Natl Acad Sci USA 84: 8399–8403

Eschrich W (1956) Kallose. Protoplasma 47: 487–530

– Currier HB (1964) Identification of callose by its diachrome and fluorochrome reactions. Stain Technol 39: 303–307

Fink J, Jeblick W, Kauss H (1990) Partial purification and immunological characterization of 1,3-β-glucan synthase from suspension cells of *Glycine max*. Planta 181: 343–348

Fredrikson K, Kjellbom P, Larsson C (1991) Isolation and polypeptide composition of 1,3-β-glucan synthase from plasma membranes of *Brassica oleracea*. Physiol Plant 81: 289–294

Frost DJ, Read SM, Drake RR, Haley BE, Wasserman BP (1990) Identification of the UDPG-binding polypeptide of (1,3)-β-glucan synthase from a higher plant by photoaffinity labeling with 5-azido UDP-glucose. J Biol Chem 265: 2162–2167

Galfre G, Milstein C (1981) Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol 73: 3–46

Girard V, Bulone V, Fevre M (1992) Separation and partial peptide characterization of β-1,3-glucan synthase from *Saprolegnia*. Plant Sci 82: 145–153

Hayashi T, Read SM, Bussell J, Thelen M, Lin FC, Brown RM Jr, Delmer DP (1987) UDP-glucose: (1,3)-β-glucan synthase from mung bean and cotton. Differential effects of Ca^{2+} and Mg^{2+} on enzyme properties and on macromolecular structure of the glucan product. Plant Physiol 83: 1054–1062

Herth W (1980) Calcofluor white and congo red inhibit chitin microfibril assembly of *Poterioochromonas*: evidence for a gap between polymerization and microfibril formation. J Cell Biol 87: 442–450

– Franke WW, Bittiger H, Kuppel A, Keilich G (1974) Alkali-resistant fibrils of β-1,3- and β-1,4-glucans: structural polysaccharides in the pollen tube wall of *Lilium longiflorum*. Cytobiologie 9: 344–357

Kakimoto T, Shibaoka H (1992) Synthesis of polysaccharides in phragmoplasts isolated from tobacco BY-2 cells. Plant Cell Physiol 33: 353–361

Kauss H (1990) Role of the plasma membrane in host-pathogen interactions. In: Larsson C, Moller IM (eds) The plant plasma membrane. Springer, Berlin Heidelberg New York Tokyo, pp 320–350

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Larsson C, Widell S, Kjellbom P (1987) Preparation of high purity plasma membranes. Methods Enzymol 148: 558–568

Maltby D, Carpita NC, Montezinos D, Kulow C, Delmer DP (1979) β-1,3-glucan in developing cotton fibers. Plant Physiol 63: 1148–1164

Meikle PJ, Ng KF, Hohnson E, Hoogenraad NJ, Stone BA (1991) The β-glucan synthase from *Lolium multiflorum*. Detergent solubilization, purification using monoclonal antibodies, and photoaffinity labeling with a novel photoreactive pyrimidine analogue of uridine 5′-diphosphoglucose. J Biol Chem 266: 22569–22581

Quader H, Herth W, Ryser U, Schnepf E (1987) Cytoskeletal elements in cotton seed hair development in vitro: their possible regulatory role in cell wall organization. Protoplasma 137: 56–62

Robards AW, Lucas WJ (1990) Plasmodesmata. Annu Rev Plant Physiol Plant Mol Biol 41: 369–419

Sanger MP, Lamport DTA (1983) A microapparatus for liquid hydrogen fluoride solvolysis: sugar and amino sugar composition of *Erysiphe graminis* and *Triticum aestivum* cell walls. Anal Biochem 128: 66–70

Schumacher W (1967) Die Fernleitung der Stoffe im Pflanzenkörper. In: Ruhland W, Schumacher W (eds) Encyclopedia of plant physiology, vol 13. Springer, Berlin Heidelberg New York, pp 61–77

Wasserman BP, Wu A (1992) Determination of the molecular architecture of the (1,3)-β-glucan (callose) synthase complex by limited proteolysis and polypeptide depletion. In: Proceedings of the Sixth Cell Wall Meeting, Nijmegen, The Netherlands, p 126