Planta (1993) 191:470–481

Planta
© Springer-Verlag 1993

A novel callose synthase from pollen tubes of *Nicotiana*

Henriette Schlüpmann, Antony Bacic, Steve M. Read

Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia

Received: 31 March 1993 / Accepted: 4 May 1993

Abstract. Pollen-tube cell walls are unusual in that they are composed almost entirely of callose, a (1,3)-β-linked glucan with a few 6-linked branches. Regulation of callose synthesis in pollen tubes is under developmental control, and this contrasts with the deposition of callose in the walls of somatic plant cells which generally occurs only in response to wounding or stress. The callose synthase (uridine-diphosphate glucose: 1,3-β-D-glucan 3-β-D-glucosyl transferase, EC 2.4.1.34) activities of membrane preparations from cultured pollen tubes and suspension-cultured cells of *Nicotiana alata* Link et Otto (ornamental tobacco) exhibited different kinetic and regulatory properties. Callose synthesis by membrane preparations from pollen tubes was not stimulated by Ca^{2+} or other divalent cations, and exhibited Michaelis-Menten kinetics only between 0.25 mM and 6 mM uridine-diphosphate glucose (K_m 1.5–2.5 mM); it was activated by β-glucosides and compatible detergents. In contrast, callose synthesis by membrane preparations from suspension-cultured cells was dependent on Ca^{2+}, and in the presence of 2 mM Ca^{2+} exhibited Michaelis-Menten kinetics above 0.1 mM uridine-diphosphate glucose (K_m 0.45 mM); it also required a β-glucoside and low levels of compatible detergent for full activity, but was rapidly inactivated at higher levels of detergent. Callose synthase activity in pollen-tube membranes increased ten fold after treatment of the membranes with trypsin in the presence of detergent, with no changes in cofactor requirements. No increase in callose synthase activity, however, was observed when membranes from suspension-cultured cells were treated with trypsin. The insoluble polymeric product of the pollen-tube enzyme was characterised as a linear (1,3)-β-D-glucan with no 6-linked glucosyl branches, and the same product was synthesised irrespective of the assay conditions employed.

Key words: Callose – Callose synthase – *Nicotiana* – Pollen tube – Protease activation

Abbreviations: Ara = L-arabinose; CHAPS = 3-[(3-cholamidopropyl)dimethylammonia]-1-propane sulphonic acid; DAP = diphenylamine-aniline-phosphoric acid stain; Gal = D-galactose; Glc = D-glucose; Man = D-mannose; Mes = 2-(N-morpholino)ethane sulphonic acid; Rha = D-rhamnose; Rib = D-ribose; TFA = trifluoroacetic acid; UDPGlc = uridine-diphosphate glucose; Xyl = D-xylose

Correspondence to: S.M. Read; FAX: 61(3)3471071

Introduction

Pollen tubes are the male gametophyte generation of flowering plants. When *Nicotiana* pollen grains land on the surface of a compatible stigma, the grains germinate and pollen tubes grow extracellularly through the transmitting tissue of the style to the ovary, where fertilisation of the egg cells occurs (Heslop-Harrison 1987). Walls of pollen tubes are unusual in that they consist largely of callose, a (1,3)-β-D-glucan with some 6-linked branches; callose constitutes 75–88% of the wall polysaccharide in cultured *Nicotiana* pollen tubes, with the remainder comprising an arabinan (6–15%) and cellulose (6–10%) (Rae et al. 1985; Read et al. 1992b). During growth at the tube tip, callose is deposited subapically as an inner layer of the wall, and also forms the plugs that traverse the pollen tube at regular intervals (Cresti et al. 1977, 1985; Heslop-Harrison 1987; Steer and Steer 1989). Elsewhere in plants, callose is a major component of the phragmoplast (cell plate), the walls around plasmodesmata and sieve-plate pores, and the walls of other specialised cells such as pollen mother cells (Fincher and Stone 1981; Bacic et al. 1988). In all these cases, callose synthesis is under developmental control during cell and tissue differentiation.

In general, however, callose is not a component of cell walls, and most higher-plant cells usually synthesize callose only in response to wounding or stress. Wound-activated deposition of callose appears to correlate with cellular perturbations, which include an influx of calcium ions into the cytoplasm (Kohle et al. 1985; Kauss 1987) as well as other changes to plasma-membrane lipids

(Kauss and Jeblick 1986) and proteins (Kauss and Jeblick 1991) and possibly the redistribution of vacuolar components (Ohana et al. 1992, 1993). Membrane preparations from somatic tissues of many plant species have high callose synthase (uridine-diphosphate glucose: 1,3-β-D-glucan 3-β-D-glucosyl transferase, EC 2.4.1.34) activity in the presence of the substrate uridine-diphosphate glucose (UDPGlc), and this activity is dependent upon divalent cations (principally Ca^{2+} at micromolar levels) and β-glucosides, and is activated by amphipathic molecules (Kauss and Jeblick 1986; Girard and MacLachlan 1987; Hayashi et al. 1987; Sloan et al. 1987; Fredrikson et al. 1991; Meikle et al. 1991). These cofactor requirements reflect the conditions inside perturbed cells, and it is thus thought that cell breakage during homogenisation mimics the damage processes that activate callose synthesis in the plant.

Little attention has, however, been directed to the enzymes responsible for callose deposition where this occurs under developmental control. Pollen tubes are an ideal system for the study of non-wound-activated callose synthesis, as they can be readily grown in single cell culture (Vasil 1987) and their walls are rich in callose. Early studies found low levels of callose synthase activity in extracts of pollen tubes of two species, *Lilium longiflorum* (Southworth and Dickinson 1975) and *Petunia hybrida* (Helsper et al. 1977). Recent developments in the conditions for growth of *Nicotiana* pollen tubes in culture (Read et al. 1992a; Read et al. 1993) allow the harvest of large amounts of morphologically normal pollen tubes, with increased levels of callose in their walls and regular callose plugs (Read et al. 1992b). We show here that the callose synthase activity in extracts of cultured pollen tubes of *N. alata* differs from the typical wound-induced callose synthase activity found in extracts of suspension-cultured cells of this species, and that the pollen-tube enzyme is not activated by Ca^{2+} or other divalent cations but is activated tenfold by treatment with trypsin.

Materials and methods

Plant materials. Nicotiana alata Link et Otto (ornamental tobacco, self-incompatibility genotypes S_2S_2 and S_2S_3; seeds kindly provided by K.K. Pandey, D.S.I.R, New Zealand) were grown in a glasshouse as previously described (Anderson et al. 1986). Pollen was collected from freshly opened flowers, and was stored in liquid nitrogen. Pollen grains were germinated at 0.5 mg pollen · ml^{-1} medium and the tubes grown in the dark at 25–26° C in a shallow layer of medium (25 ml in each 14-cm-diameter plastic Petri-dish). The medium contained 5% (w/v) sucrose, 10% (w/v) poly(ethylene glycol)-6000 ('Specially Purified for Biochemistry'; BDH, Poole, Dorset, UK), 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM KCl, 1.6 mM H_3BO_3, 0.03% (w/v) Amicase (an acid-hydrolysate of casein; Sigma, St. Louis, Mo., USA), 25 mM 2-(N-morpholino)ethane sulphonic acid (Mes)-KOH (pH5.9), 10 mg · l^{-1} rifampicin and 40 μM 2-thiouracil (Read et al. 1993). After 12 h, tubes were approximately 2 mm in length.

Suspension cultures of *N. alata* cells (self-incompatibility genotypes S_2S_2, S_2S_3, S_3S_3 and S_6S_6) were initiated from cotyledons of seedlings germinated in the medium of Murashige and Skoog (1977) supplemented with 1 g · l^{-1} *myo*-inositol, 2 g · l^{-1} Mes-KOH(pH 5.7), 4% (w/v) sucrose, 0.1 mg · l^{-1} gibberellic acid and 5 mg · l^{-1} α-naphthaleneacetic acid. The cells were subcultured weekly in this medium without gibberellic acid.

Preparation of membranes. Pollen tubes (usually from 250 mg of pollen) were harvested, after overnight (12 h) growth, under gentle suction on glass-fibre filters (GFA; Whatman Far East, Singapore) containing insoluble polyvinylpyrrolidine (PVP; 0.5 g · g^{-1} pollen), and transferred with the PVP into ice-cold extraction buffer (EB) containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (Hepes)-NaOH (pH 7.8), 10 mM EDTA, 2 mM EGTA, 5 mM L-ascorbate and 10% (w/v) sucrose. Suspension-cultured cells (usually about 25 g fresh weight) were harvested on Miracloth with addition of insoluble PVP at approximately 0.1 g · g^{-1} fresh weight of cells, and transferred to cold EB. The following steps were then identical for both types of tissue. Cells were brought to 30 ml with EB and were disrupted by sonication on ice (three bursts of 20 s with a 1-cm-diameter probe at the maximal setting of a 20 Hz 250/450 Branson Sonifier; Branson, Danbury, Conn., USA). After centrifugation (7500 · *g*, 15 min, 4° C), ribonuclease (type I-A from bovine pancreas; Sigma) was added to the supernatant to 10 μg · ml^{-1}, and 10-ml aliquots were carefully added to tubes already containing two sucrose cushions (750 μl 25% (w/v) sucrose in EB over 750 μl 50% (w/v) sucrose in EB). After centrifugation at 100000 · *g* (in an SW-40.1 rotor; Beckman Instruments, Palo Alto, Calif., USA) for 1 h at 4° C, membranes at the 25%/50% sucrose interface were collected, diluted with 4 volumes of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and pelleted by centrifugation (100000 · *g*, 1 h, 4° C). The pelleted membranes were resuspended in this buffer containing 15% (v/v) glycerol, and stored at −70° C.

Assay of callose synthase. Incorporation of radioactivity from UDP[^{14}C]Glc into product insoluble in 66% ethanol was used to assay callose synthase activity essentially according to Hayashi et al. (1987). Assays were generally for 10 min at 30° C in 200 μl volumes containing 50 mM Hepes-KOH (pH 7.5), 20 mM cellobiose, 100 mM sucrose, 0.02% digitonin (Serva, Heidelberg, FRG), 740 Bq UDP[^{14}C]Glc (Amersham Australia, North Ryde, N.S.W., Australia) and either (for preparations from suspension-cultured cells) 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5 mM UDPGlc, or (for pollen-tube preparations) 5 mM EDTA and 1 mM UDPGlc. β-Furfuryl-β-glucoside was synthesised essentially as described by Ohana et al. (1991), but using the tetrabenzoate rather than the tetraacetate derivative of α-D-glucopyranosyl bromide; the resultant preparation contained considerable amounts of methyl benzoate, and the final concentration of β-furfuryl-β-glucoside was not determined. Amounts of protein were 5–10 μg for assay of membranes from suspension cells, approximately 40 μg for assay of untreated membranes from pollen tubes, and approximately 4 μg for assay of trypsin-treated membranes from pollen tubes. Assays were started by addition of 10 μl resuspended membranes to the otherwise complete mixture. The reactions were stopped by addition of 66% (v/v) ethanol (3 ml) and cooling on ice, and the ethanol-insoluble product was washed five times with 66% ethanol (3 ml), and once with chloroform/methanol (2/1, v/v; 2 ml), on a glass-fibre filter (GFC, Whatman). Filters were dried and immersed in 3 ml scintillation mix (PCS; Amersham), and radioactivity measured on a 1211 Minibeta Counter (LKB, Bromma, Sweden) with 80% efficiency as determined by channel ratio. Typically, 500–7000 cpm were incorporated into product from the 50000 cpm added to each assay.

Protein determination. Protein in membrane preparations was estimated by a Coomassie-blue dye-binding assay (Bio-Rad, Hercules, Calif., USA) standardised using bovine serum albumin (BSA).

Assay for trypsin activity. Amidolytic activity of trypsin was measured using N-α-benzoyl-D,L-arginine *p*-nitroanilide essentially as described by Christeller et al. (1989). Wells of 96-well microtitre plates were filled with 100-μl reaction volumes containing 10 mM Tris-HCl (pH 7.5), 0.5 mM N-α-benzoyl-D,L-arginine *p*-nitroanilide (from 10 mM stock in dimethylsulphoxide), and various concentrations of membrane protein, trypsin and trypsin-inhibitor. The plate

was incubated for 30 min at 30° C, reactions were terminated by adding 30% acetic acid (50 µl), and the absorbance of L-arginine *p*-nitroanilide measured at 405 nm with a Titertek Multiscan MC-photometer (Flow Laboratories, Irvine, Ayrshire, UK).

Trypsinisation of membrane preparations. The effect of different proteases on callose synthesis by membranes from both suspension-cultured cells and pollen tubes was tested either by the addition of protease to standard callose synthase assays (formulated as above with 0.5 mM UDPGlc, 2 mM $CaCl_2$ and 2 mM $MgCl_2$) for 5–30 min at 30° C, or by preincubating membranes with protease and stopping the reaction with inhibitor before assay of callose synthase. In the latter case, membranes from pollen tubes (105 µg protein) were preincubated for 1 h on ice with increasing concentrations of chymotrypsin (0.1–1 µg, Sigma), papain (1–5 µg, Sigma), carboxypeptidase Y (1–5 µg, Sigma) and L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (5 µg; Sigma), and proteolysis was stopped with phenylmethylsulphonylfluoride (1 mM final concentration for trypsin, chymotrypsin and papain) or allowed to continue in the case of carboxypeptidase Y. Aliquots were then assayed for callose synthase for 10 min at 30° C under the standard conditions.

Activation with trypsin was optimally performed by incubating TPCK-treated trypsin (Sigma, 1.5 mg · ml^{-1}) with pollen-tube membranes (3 mg protein · ml^{-1}) for 15 min at 30° C in 20 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonia]-1-propane sulfonic acid (CHAPS). The reaction was stopped with the addition of soybean Bowman-Birk trypsin inhibitor (Sigma; weight ratio of trypsin-inhibitor to trypsin of 1.3:1) and cooling on ice. Callose synthase activity was then assayed directly, or after diluting the reaction mixture fivefold in 20 mM Tris-HCl, 1 mM EDTA, centrifuging (100000 · *g*, 45 min, 4° C), washing the membrane pellet in this buffer by resuspension and recentrifugation, and resuspending to the initial volume.

Thin-layer chromatography of monosaccharides. Pollen-tube membranes (200 µg protein), either untreated, boiled or trypsin-treated, were reacted for 1 h at 30° C in a 600-µl volume containing 2220 Bq UDP[^{14}C]Glc and 2 mM UDPGlc under the standard EDTA-assay conditions. The reactions were stopped by adding ethanol to 66% and cooling on ice, and unincorporated UDP[^{14}C]Glc was removed by repetitive washing of the product (3 × 6 ml 66% ethanol) by centrifugation (5 min, 1000 · *g*, 20° C), discarding the supernatant. The insoluble material was lyophilised, then 1 ml 2.5 M trifluoroacetic acid (TFA) was added and the samples hydrolysed under argon at 121° C for 1 h. The TFA was removed under a stream of nitrogen, and samples were resuspended in water (120 µl). Aliquots (5 µl) of hydrolysed product and standards of galactose (Gal), glucose (Glc), mannose (Man) and arabinose (Ara) (2 µg each) were applied to an aluminium-backed silica-gel-60 TLC plate (Merck, Darmstadt, FRG), and the plate was developed either three times in ethyl acetate/pyridine/acetic acid/water (6/3/1/1, by vol.) or two times in ethyl acetate/acetic acid/water (2/1/1, by vol.). Tracks containing standards were sprayed with DAP (0.4 g diphenylamine, 0.4 ml aniline and 2 ml phosphoric acid in 20 ml acetone) and developed at 120° C for 10 min. The areas in the sample tracks that corresponded to each monosaccharide standard were cut out and placed in scintillation vials, PCS scintillant (Amersham; 3 ml) was added, and radioactivity determined by scintillation counting.

Gas-chromatography of alditol acetates. Membranes (750 µg protein), either untreated or trypsin treated, were incubated for 60 min at 30° C in 4 ml of 2 mM UDPGlc, 20 mM cellobiose, 100 mM sucrose, 0.02% digitonin and 50 mM Hepes-KOH (pH 7.5) with either 5 mM EDTA or 2 mM $CaCl_2$, 2 mM $MgCl_2$. The reactions were stopped, and insoluble material washed, lyophilised and hydrolysed with TFA as above; control samples contained no UDPGlc, or UDPGlc was added after the ethanol. *myo*-Inositol (50 µg) was added as an internal standard, and samples were resuspended in 500 µl 1 M NH_4OH, reduced with $NaBD_4$ (0.1% w/v in 500 µl 1 M NH_4OH) for 1 h at 60° C, and the reaction stopped by three sequential additions of 50 µl acetic acid. Samples were then repeatedly evaporated with 5% (v/v) acetic acid in methanol to remove boric acid (3 × 2 ml), followed by evaporation with methanol (2 × 2 ml). Acetic anhydride (2 ml) and N-methylimidazole (200 µl) were added and acetylation allowed to proceed at 20° C for 10 min (Blakeney et al. 1983). The reaction was stopped by addition of water (15 ml), and the alditol acetates were recovered by partitioning into dichloromethane (2 × 1 ml) and analysed on a high-polarity column (BPX-70; SGE, Ringwood, Victoria, Australia) with a Hewlett Packard 5890 gas chromatograph (Hewlett-Packard Australia, North Ryde, N.S.W., Australia) (Lau and Bacic 1993). The amount of each alditol acetate detected was corrected for losses using the recovery of the internal standard *myo*-inositol hexaacetate.

Methylation analysis. Product was synthesised, washed and lyophilised as above. Methylation was performed according to Ciucanu and Kerek (1984) with modifications. Samples were suspended in 250 µl dimethylsulphoxide and methylated by addition of a slurry of solid NaOH in dimethylsulphoxide (120 mg · ml^{-1}, 250 µl) followed by three sequential additions of methyl iodide (50 µl, 50 µl and 100 µl) at 15-min intervals. The reaction was stopped by addition of 1 M aqueous sodium thiosulphate (5 ml) and chloroform (2.5 ml), and methylated products were recovered in the organic phase, which was washed four times with water. Chloroform was evaporated under a stream of nitrogen, TFA (2.5 M, 400 µl) was added, the methylated products were hydrolysed at 121° C for 1 h, and the TFA evaporated under a stream of nitrogen. Reduction and acetylation of the partially methylated monosaccharides was performed as for underivatised monosaccharides above. The partially methylated alditol acetates were analysed by gas chromatography/mass spectroscopy (GC/MS) on a 1020B Finnigan MAT (Finnigan Australia, North Strathfield, N.S.W., Australia) using both a high-polarity column (BPX-70; SGE) and a low-polarity column (CP-Sil 5; Chrompack, Middelburg, The Netherlands). Chromatography conditions for BPX-70 were as in Lau et al. (1993). For CP-Sil 5, the oven was held for 2 min at 130° C, then ramped to 220° C at 3° C · min^{-1} and held at 220° C for 5 min. Compounds eluting from the GC were detected in the MS using total ion current by scanning from m/z 100 to m/z 350 in 0.3 s. Peaks were identified by comparison of their mass spectra with standard spectra, and by their retention times relative to the internal standard *myo*-inositol hexaacetate.

Endo-(1,3)-β-D-glucanase digestion. Product was synthesised by untreated or trypsin-treated pollen-tube membranes in 500-µl reactions containing 50 mM Hepes-NaOH (pH 7.5), 20 mM cellobiose, 0.02% digitonin, 1 mM UDPGlc and 2220 Bq UDP[^{14}C]Glc, and either divalent cations (2 mM $CaCl_2$, 2 mM $MgCl_2$) or EDTA (5 mM). After 1 h incubation at 30° C, the reactions were stopped by addition of water (5 ml), centrifuged (3000 · *g*, 10 min, 4° C), and the supernatant discarded. The pellets were lyophilised and either hydrated in water (10 µl) or swollen in 4 M NaOH, 1% $NaBH_4$ (10 µl) for 2 h at 4° C. Sodium acetate/acetic acid buffer (pH 5.0, 50 mM, 0.5 ml) was then added, and the NaOH-treated samples were neutralised with acetic acid. Bovine serum albumin was added to 0.05% (w/v) followed by 0.1 U of an endo-(1,3)-β-D-glucanase purified from the styles of *N. alata*, and the reactions were incubated for 12 h at 30° C, when another 0.1 U of endo-(1,3)-β-D-glucanase was added and incubation continued for another 12 h at 30° C. Digestion with α-amylase (porcine pancreas; Sigma) was performed similarly but in 20 mM Hepes-KOH (pH 7.0), 20 mM NaCl, 1 mM $CaCl_2$. In both cases, ethanol was added to 66% (v/v) to stop digestion, and the samples cooled on ice, centrifuged (5 min, 3000 · *g*, 4° C), and radioactivity in supernatants and pellets determined by scintillation counting. When released material was to be analysed by HPLC, callose-synthase reactions were performed as above but in 50-µl reaction volumes containing trypsin-treated membranes, 5 mM EDTA and a higher level of radioactivity (3700 Bq UDP[^{14}C]Glc). The reaction was stopped by addition of ethanol to 66%, centrifuged (12000 · *g*, 10 min, 4° C), the

pellets washed with 1 ml of 66% ethanol, recentrifuged, and resuspended in 500 µl of 50 mM sodium acetate/acetic acid (pH 5.0). Bovine serum albumin and endo-(1,3)-β-D-glucanase were added as above and digestion carried out for two 24-h periods. Reactions were stopped with 66% ethanol, and the soluble material desalted on a 2-ml column of AG-X-801 (Bio-Rad) and analysed by anion-exchange HPLC on a Carbopac-PA1 column (Dionex, Sunnyvale, Calif., USA) equilibrated in 150 mM NaOH and run at 1 ml · min^{-1}. Oligosaccharides were eluted with a 15-min linear gradient of 0–500 mM CH_3COONa in 150 mM NaOH, starting 1 min after sample injection. Eluted carbohydrate was monitored with a pulsed amperometric detector (Dionex), and radioactivity with an in-line Yttrium-glass solid cell (YG-150 U4; Berthold Instruments, Wildbad, FRG). Acid hydrolysates of pachyman and laminarin were hydrolysed in 2.5 M TFA for 10 min at 100° C to provide standard series of (1,3)-β-D-linked oligoglucosides.

Results

Properties of the callose synthase from suspension-cultured cells. Membranes prepared from suspension-cultured cells of *Nicotiana alata* showed callose synthase activity, measured as the incorporation of radioactivity from UDP[^{14}C]Glc into product insoluble in 66% ethanol. This activity was stable at 0° C during preparation of membranes and for at least 20 min at 30° C, and could be stored at −70° C in buffer containing glycerol. Activity from leaves or seedlings, on the other hand, was unstable during preparation of membranes and could not be stored frozen, and thus suspension-cultured cells were used as a source of control activity for work on pollen-tube callose synthase.

Callose synthase activity in membranes from suspension-cultured cells was highly dependent on the presence of calcium ions (Fig. 1), and in buffers containing 2 mM

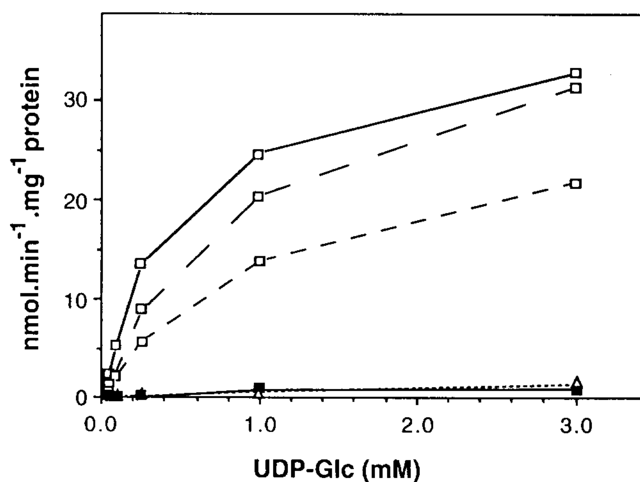

Fig. 1. Callose synthase activity of membranes from *N. alata* suspension-cultured cells. Membrane preparations (10 µg protein) were incubated for 15 min at 30° C in 200 µl of 50 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 20 mM cellobiose, 0.01% digitonin, 740 Bq UDP[^{14}C]Glc and various concentrations of UDPGlc from 0.05 mM to 3 mM. Concentrations of cations were 2 mM $CaCl_2$, 5 mM $MgCl_2$ (—□—); 0.1 mM $CaCl_2$, 5 mM $MgCl_2$ (--□--); 0.025 mM $CaCl_2$, 5 mM $MgCl_2$ (- -□- -); 2 mM EGTA, 5 mM $MgCl_2$ (...△...); and 5 mM EDTA (—■—)

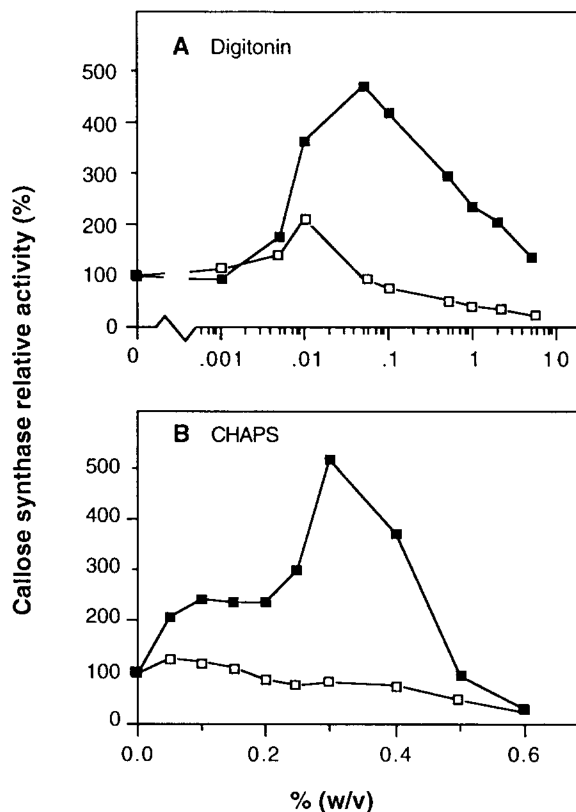

Fig. 2A, B. Effect of detergents on callose synthesis by membranes from suspension-cultured cells and pollen tubes of *N. alata*. Membranes from suspension-cultured cells (48 µg protein, —□—) and pollen tubes (48 µg protein, —■—) were incubated at 30° C under standard conditions for assay of callose synthase, with 1 mM UDPGlc, 2 mM $CaCl_2$, 2 mM $MgCl_2$ for 10 min (suspension-cultured cells) or 2 mM UDPGlc, 2 mM $CaCl_2$, 2 mM $MgCl_2$ for 15 min (pollen tubes), and various concentrations of digitonin (**A**) or CHAPS (**B**). Activity is expressed relative to the activity in the absence of any added detergent, which was 35 nmol · min^{-1} · mg^{-1} protein for membranes from suspension-cultured cells and 11 nmol · min^{-1} · mg^{-1} protein for membranes from pollen tubes

Ca^{2+} displayed Michaelis-Menten kinetics. Magnesium ions were unable to substitute for calcium ions. The pH optimum of this activity was 7.3, it required β-glucosides (data not shown), and was activated 2- to 2.5-fold by the detergent digitonin at 0.02% w/v (Fig. 2A) but only marginally by CHAPS (Fig. 2B). In the presence of 50 mM Hepes-KOH (pH 7.3), 20 mM cellobiose, 0.02% digitonin and 2 mM $CaCl_2$, the K_m for UDPGlc was 0.45 mM and the V_{max} was 40 nmol · min^{-1} · mg^{-1} protein. The kinetics of callose synthase from *N. alata* suspension-cultured cells are thus similar to those of wound-activated enzymes from somatic cells of many other species (Delmer 1987; Hayashi et al. 1987), and allow comparison of this enzyme to the *N. alata* pollen-tube callose synthase.

Properties of the callose synthase from pollen tubes. Extracts of pollen tubes grown in vitro exhibited callose synthase activity in the presence or absence of divalent cations. This activity was associated with the membrane fraction (Table 1). During sub-cellular fractionation of

Table 1. Fractionation of callose synthase activity in extracts from pollen tubes of *Nicotiana alata*. Crude extract was fractionated by centrifugation at 7500 · *g* for 15 min, and the supernatant recentrifuged on a sucrose step-gradient at 100000 · *g* for 1 h. Callose synthase was assayed under standard conditions (5 mM EDTA, 2 mM UDPGlc and 740 Bq UDP[^{14}C]Glc), adding 10 or 20 µl of each fraction to an assay mix (final volume 200 µl)

Fraction	Protein	Callose synthase activity	
	(mg.g^{-1} pollen)	(nmol.min^{-1}.g^{-1} pollen)	(nmol.min^{-1}.mg^{-1} protein)
Crude extract	420	1206	2.9
7500 · *g* pellet	82	224	2.7
7500 · *g* supernatant after centrifugation at 100000 · *g*			
supernatant	246	0	0.0
interface, 10–25% sucrose	34	166	5.0
interface, 25–50% sucrose	44	1274	29.1
pellet	3	36	11.7
Yield	409 (97%)	1700 (141%)	

the crude extract, callose synthase activity was found predominantly in the membranes at the 25%/50% sucrose interface after centrifugation at 100000 · *g*, and was enriched tenfold compared to the crude extract. Sedimentation of pollen-tube membranes onto a sucrose cushion gave consistently higher specific activities of callose synthase than did pelleting the membranes directly from cell homogenates. The amount of ethanol-insoluble product increased linearly over an assay period of 45 min at 30° C (data not shown), indicating that the activity associated with membranes from the 25%/50% sucrose interface was stable during the period of the assay. Some callose synthase activity (13% of recovered activity) was also associated with cell-wall debris in the 7500 · *g* pellet, but no activity was detected in the 100000 · *g* supernatant.

Divalent cations (Ca^{2+} or Mg^{2+}) were not required for callose synthesis from pollen-tube membranes and, unlike the callose synthase from suspension-cultured cells, synthesis of insoluble product occurred in 5 mM EDTA (Fig. 3A). Saturation of the activity assayed in 5 mM EDTA was only observed at high concentrations of substrate (over 10 mM UDPGlc; data not shown), and above 2 mM UDPGlc the maximal rate of callose synthesis was reproducibly lower in the presence of Ca^{2+} and-or Mg^{2+}. Below 0.5 mM UDPGlc, however, the rate of callose synthesis was higher when either Ca^{2+} or Mg^{2+} were present, and Michaelis-Menten kinetics were not followed below 0.25 mM UDPGlc in 5 mM EDTA (Fig. 3A inset). The K_m values determined after either Lineweaver-Burke or Cornish-Bowden and Eisenthal (1978) were 1.8 mM (with 2 mM $CaCl_2$, 2 mM $MgCl_2$) and 2.5 mM (with 5 mM $MgCl_2$, 2 mM EGTA), but could not be accurately measured in the presence of 5 mM EDTA due to the lack of saturation of the enzyme. The V_{max} ranged from 55 to 80 nmol · min^{-1} · mg^{-1} protein in the presence of divalent cations.

In the absence of divalent cations (5 mM EDTA), the pH optimum for callose synthase activity was 7.5 (Fig. 4A); this value was unchanged when the enzyme was assayed in 2 mM $CaCl_2$, 2 mM $MgCl_2$. β-Glucosides such as cellobiose or laminaribiose were required for

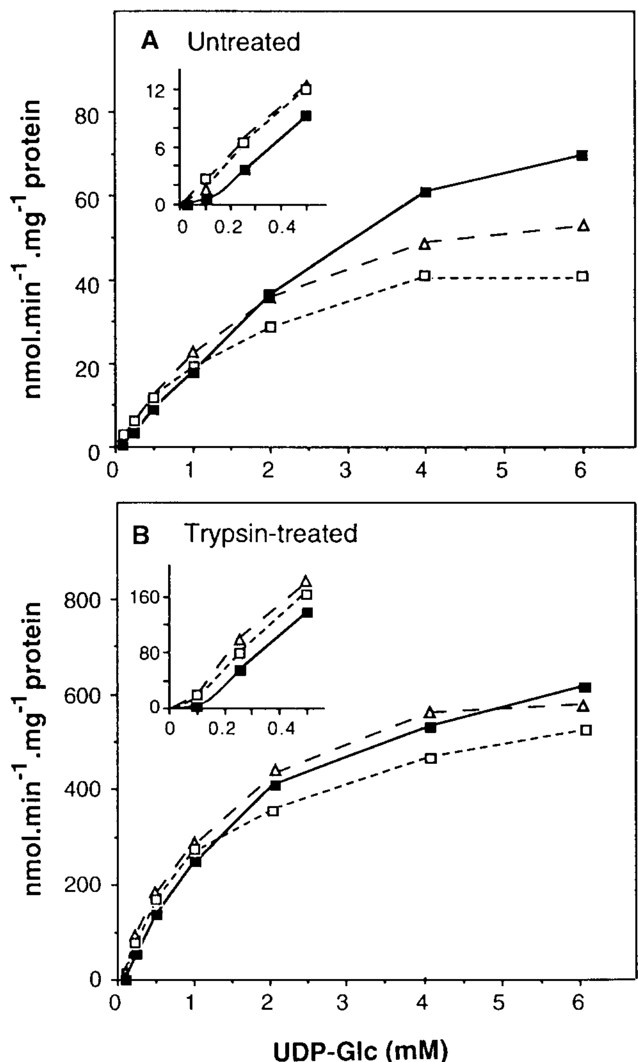

Fig. 3A, B. Callose synthase activity of membranes from *N. alata* pollen tubes. Pollen-tube membranes, either untreated (**A**) or trypsin-treated (**B**), were assayed under standard conditions for assay of callose synthase in the presence of 5 mM EDTA (—■—); 5 mM $MgCl_2$ 2 mM EGTA (---△---); and 2 mM $MgCl_2$, and 2 mM $CaCl_2$ (—□—)

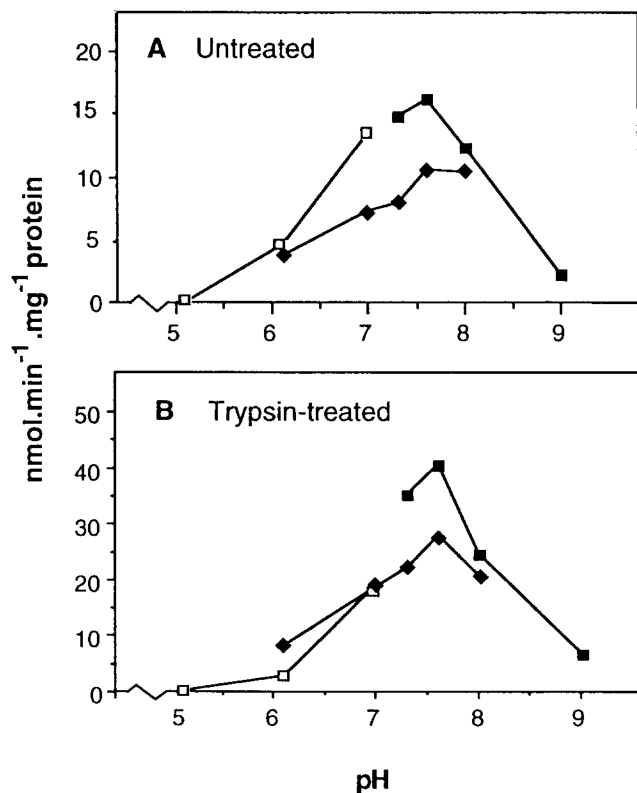

Fig. 4A, B. Dependence of pollen-tube callose synthase on pH. Pollen-tube membranes, either untreated (**A**) or trypsin-treated (**B**), were assayed under standard conditions for assay of callose synthase (5 mM EDTA, 1 mM UDPGlc) in 50 mM of the following buffers: Mes-KOH at pH 5.2, 6.2 and 7.0 (—□—); Hepes-KOH at pH 6.2, 7.0, 7.3, 7.6 and 8.0 (—◆—); and Tris-HCl at pH 7.3, 7.6, 8.0 and 9.0 (—■—)

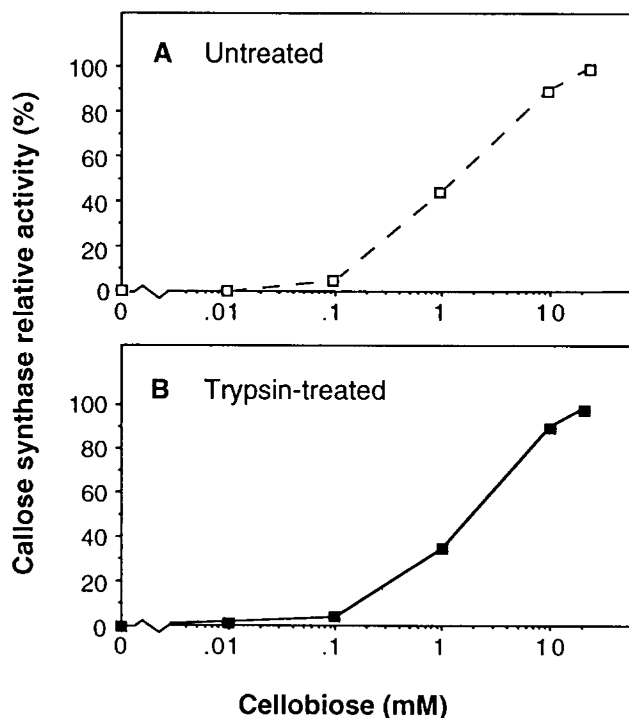

Fig. 5A, B. Effect of cellobiose on pollen-tube callose synthase. Pollen-tube membranes (200 µl, 3 mg protein · ml^{-1}), either untreated (**A**, —□—) or trypsin-treated (**B**, —■—) were diluted in 10 ml of 20 mM Hepes-KOH (pH 7.5), 1 mM EDTA, centrifuged at 100000 · *g* for 60 min, 4° C, resuspended in 10 ml of this buffer, and recentrifuged as before to remove glycerol and sucrose. The pellets were resuspended to 3 mg protein · ml^{-1} (untreated membranes) or 0.6 mg protein · ml^{-1} (trypsin-treated membranes). Callose synthase assays (10 µl of membrane) were performed under standard conditions (5 mM EDTA, 1 mM UDPGlc) with various concentrations of cellobiose (0.01–20 mM); activity is expressed as the percentage of the activity obtained with 20 mM cellobiose, which was 14 nmol · min^{-1} · mg^{-1} protein for untreated membranes and 250 nmol · min^{-1} · mg^{-1} protein for trypsin-treated membranes

callose synthase activity when both glycerol and sucrose were absent (Fig. 5A), and 20 mM cellobiose was therefore included in the assay. An impure preparation of β-furfuryl-β-glucoside also activated the pollen-tube enzyme, giving 75% of the activity obtained with 20 mM cellobiose. Inclusion of the detergents digitonin and CHAPS in the assay led to a fivefold increase in activity, with maximal activity at 0.01–0.1% digitonin or 0.3–0.4% CHAPS, and the pollen-tube enzyme retained more activity at higher levels of detergent and was activated to a greater extent than was the enzyme from suspension-cultured cells (Fig. 2). Above its critical micellar concentration (0.6%, w/v), CHAPS completely inhibited callose synthesis by both types of membrane, whereas significant levels of pollen-tube activity were still detected in the presence of 5% (w/v) digitonin (critical micellar concentration of 0.005%, w/v).

Trypsin activation of the callose synthase from pollen tubes. Addition of TPCK-treated trypsin (7.5 µg) to a standard callose synthase assay (10 min at 30° C, 5 mM EDTA) containing 12 µg pollen-tube membrane protein led to a more than tenfold increase of activity; the rate of glucan synthesis dropped rapidly, however, with continued exposure to trypsin. In a similar experiment, Lys-C endoproteinase (12.5 µg) also stimulated activity (4-fold over the first 10 min) whereas Arg-C endoproteinase (25 µg) was less effective (1.5-fold over the first 10 min). Preincubation of pollen-tube membranes for 60 min at 4° C with a much lower ratio of trypsin (5 µg trypsin to 105 µg membrane protein), followed by subsequent assay of aliquots for callose synthase activity, gave five fold activation of the enzyme, but even under these conditions similar weight-ratios of chymotrypsin, papain or carboxypeptidase Y did not stimulate the enzyme and at higher levels caused inactivation.

The most effective and reproducible conditions for protease activation occurred when trypsin (1.5 mg · ml^{-1}) and membrane protein (3.0 mg · ml^{-1}) were preincubated for 20 min at 30° C in the presence of 0.17% CHAPS, giving a tenfold increase in subsequently assayed activity (Fig. 6); if detergent was omitted, activation was only fivefold (Fig. 6). Bowman-Birk trypsin-inhibitor from soybean at an equal amount by weight to trypsin was found to be an effective inhibitor of trypsin activity either alone or in the presence of an equal weight

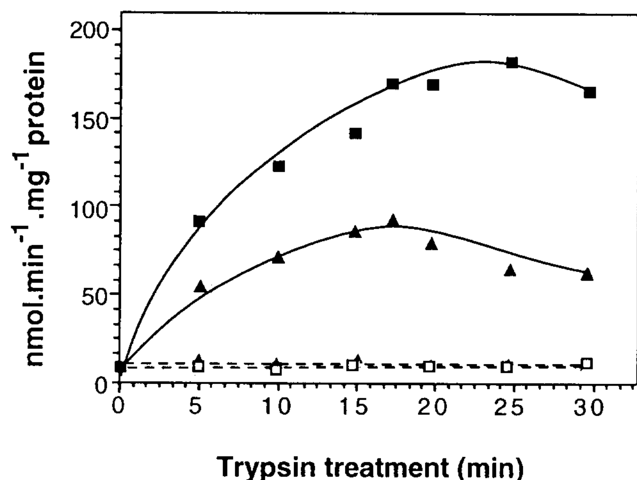

Fig. 6. Activation of callose synthase by trypsin treatment of pollen-tube membranes. Membranes (3 mg · ml^{-1}) were mixed with TPCK-treated trypsin (1.5 mg · ml^{-1}), and proteolysis either immediately stopped by addition of soybean Bowman-Birk trypsin inhibitor (2.25 mg · ml^{-1}) and cooling on ice, or allowed to continue at 30° C for the time shown before addition of trypsin-inhibitor. Trypsin treatment was in the presence (—■—) or in the absence (—▲—) of 0.17% CHAPS. Control incubations without trypsin but with trypsin inhibitor added at the time shown were also performed in the presence (--□--) or in the absence (--△--) of 0.17% CHAPS. Callose synthase activity was then assayed under standard conditions (5 mM EDTA, 1 mM UDPGlc)

of pollen-tube membrane protein, and so this amount of trypsin inhibitor was added to stop proteolysis before proceeding to the standard callose synthase assay. Preincubation and assay of membranes in the presence of phenylmethylsulphonylfluoride (1 mM), leupeptin (0.1 mg · ml^{-1}) or trypsin inhibitor (0.1 mg · ml^{-1}) did not reduce the basal level of callose synthase activity, and no activation occurred when the trypsin inhibitor was added immediately after the addition of trypsin to the membranes (Fig. 6).

The callose synthase activity remained associated with trypsin-treated membranes after these were pelleted by ultracentrifugation and washed to remove trypsin, trypsin inhibitor and any soluble proteolytic products. This was also the case when the trypsin-treated membranes were brought to 0.4% CHAPS before ultracentrifugation (pollen-tube callose synthase was stable after addition of 0.4% CHAPS to both untreated and trypsin-treated membranes; see Fig. 2). Thus, activation of callose synthase was not reversible or mediated by a soluble factor generated by proteolysis, and trypsin treatment did not solubilise the enzyme.

Activation with trypsin in the presence of CHAPS did not change the relative cation independence of the pollen-tube callose synthase (Fig. 3B), and high levels of activity were still observed in assay buffers containing 5 mM EDTA. The V_{max} was increased to 670 nmol · min^{-1} · mg^{-1} in line with the tenfold activation of the enzyme, and the affinity for UDPGlc was also increased somewhat: K_m values decreased to 1.5 mM (with 2 mM $CaCl_2$, 2 mM $MgCl_2$), 1.5 mM (with 5 mM $MgCl_2$, 2 mM EGTA) and 2.5 mM (in the presence of 5 mM EDTA). The graph of activity in 5 mM EDTA remained sigmoidal at low levels of UDPGlc (Fig. 3B inset), however, and still showed a slow increase in activity even above 6 mM UDPGlc. The pH optimum of trypsin-treated callose synthase was unchanged at pH 7.5 (Fig. 4B), and the absolute dependence on β-glucosides for activity remained (Fig. 5B). The trypsin-activated enzyme was similar to the untreated enzyme in retaining activity at high levels of CHAPS (up to 0.4%) or digitonin (up to 5%).

Treatment of membranes prepared from suspension-cultured cells with low levels of trypsin also stimulated callose synthase activity, but that stimulation was only 1.5-fold and only occurred in the absence of CHAPS (callose synthase from suspension-cultured cells was not stable in the presence of 0.17% w/v CHAPS). Trypsin treatment did not alter the calcium dependence of the suspension-culture enzyme. Extraction of suspension-cultured cells with buffer containing protease inhibitors (10 mM EDTA, 1 mg · ml^{-1} trypsin inhibitor, 5 mg · ml^{-1} bovine serum albumin) did not significantly alter the yield of callose synthase activity, and the resultant callose synthase activity was not more susceptible to activation by trypsin.

Analysis of the product from pollen-tube membranes. The product synthesized from UDPGlc by pollen-tube membranes was insoluble in aqueous buffers but dispersed in 4 M NaOH containing 1% $NaBH_4$; on neutralisation of the alkali, however, the material reprecipitated. Product synthesised by trypsin-treated membranes appeared typically as fine crystalline needles, whereas product synthesised at lower rates by untreated membranes appeared as a white cloudy suspension.

Thin-layer chromatography of an acid-hydrolysate of the 66%-ethanol-insoluble ^{14}C-product from pollen-tube membranes showed that most of the radioactivity comigrated with glucose (Fig. 7), and the material was therefore a glucan. Recovery of radioactivity applied to the TLC plate was 102% and 91% for material synthesized by untreated and trypsin-treated membranes respectively, and 87% and 92% of this was glucose; all the residual label remained at the origin and presumably represented unhydrolysed glucan. Only background levels of radioactivity comigrated with other monosaccharides. Similar results were obtained using two different solvent systems (data not shown).

The 66%-ethanol-insoluble product from pollen-tube membranes was also analysed by gas-chromatography following acid hydrolysis, reduction and acetylation. Membranes (750 μg protein) not reacted with UDPGlc contained 90 μg ethanol-insoluble carbohydrate consisting of Ara (65% w/w), Gal (13%), Glc (9%), Xyl (3%), Man (3%), Rha (3%) and Rib (5%, presumably derived from membrane-bound ribosomal RNA). After reaction of membranes for 60 min with 2 mM UDPGlc in standard EDTA assay conditions and extensive washing, both untreated and trypsin-treated membranes had produced considerably more carbohydrate than they contained protein, and this consisted of very high levels of Glc (95% and 97% by weight of carbohydrate, respec-

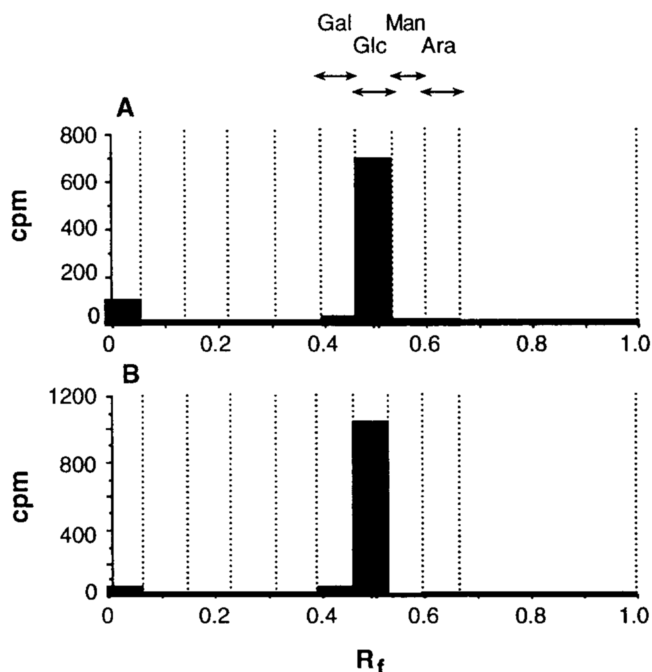

Fig. 7A, B. Thin-layer chromatography of acid-hydrolysed product synthesised from UDP[^{14}C]Glc by pollen-tube membranes. Radioactive product was synthesized, washed, hydrolysed in TFA and redissolved in water as described in *Materials and methods*. Aliquots of 5 µl containing 767 cpm (**A**) from the product from untreated membranes. 1216 cpm (**B**) from the product from trypsin-treated membranes and 16 cpm (not shown) from a control incubation with boiled membranes, were loaded in parallel with monosaccharide standards and chromatographed on silica-gel in ethyl acetate/pyridine/acetic acid/water (6/3/1/1, by vol., three ascents). Locations of the standard monosaccharides, determined by the DAP stain, are shown at the top. The R_f values of the standards were 0.41 (Gal), 0.50 (Glc), 0.55 (Man), 0.64 (Ara). The tracks containing radioactivity were cut as shown by the *dotted lines*, and radioactivity was determined by scintillation counting as described

tively); absolute amounts of other neutral monosaccharides were similar to those found in membranes not reacted with UDPGlc.

Linkage analysis of ethanol-insoluble material from pollen-tube membranes before and after reaction with UDPGlc is summarised in Table 2. Ethanol-insoluble carbohydrate in membranes not reacted with UDPGlc, or in membranes boiled before incubation with UDPGlc, had an identical linkage composition, containing mostly 5-linked and terminal Ara*f*, terminal Ara*p*, and 6-linked and 3,6-linked Gal residues; 4-linked Glc was present at 2–4 mol% and 3-linked Glc at 1–2 mol%. After reaction with UDPGlc, however, the ethanol-insoluble carbohydrate of untreated and trypsin-treated membranes contained primarily 3-linked Glc (94 and 97 mol%, respectively), confirming the bulk synthesis of the 3-linked glucan backbone of callose.

The ratio of terminal Glc to 3-linked Glc in the ethanol-insoluble material after reaction of membranes with UDPGlc was less than one to 1200, and the level of 3,6-linked Glc was similarly low, indicating that a very long, linear 3-linked glucan with no branches was made. Further, terminal Glc and 3,6-linked Glc were present at

Table 2. Linkage analysis of carbohydrates in preparations of pollen-tube membranes before and after incubation with UDPGlc. Reactions with UDPGlc were performed for 30 min at 30° C in 600 µl of standard callose synthase assay mix containing 5 mM EDTA, 1 mM UDPGlc and 200 µg membrane protein. Samples were then precipitated in 66% ethanol at −20° C for 2 h, and washed twice by centrifugation and resuspension in 66% ethanol and once in chloroform-methanol (2/1, v/v), before processing for linkage analysis by methylation. tr, trace (<0.1 mol%); –, not detected

Deduced glycosidic linkage (mol%)	−UDPGlc	+UDPGlc		
		Boiled	Untreated	Trypsin-treated
Ara*f*				
terminal	13.2	12.2	0.9	0.6
terminal*p*	10.0	9.3	tr	tr
5–	46.6	44.1	4.4	2.3
Glc*p*				
terminal	tr	tr	tr	tr
3–	1.2	2.2	94.4	97.2
4–	2.1	4.0	–	–
3,6	tr	tr	tr	tr
Gal*p*				
terminal	1.0	1.0	–	–
6–	11.5	12.3	0.3	tr
3,6–	9.8	10.3	tr	tr

a similar ratio to the control residue 5-linked Ara*f* both before and after reaction of the membranes with UDPGlc, and thus no detectable levels of chain initiation or branching occurred in this system. Similarly, no increase in 4-linked Glc was detected under any conditions of synthesis, implying that no cellulose was made by the pollen-tube membrane preparations.

The radiolabelled product synthesised by untreated and trypsin-treated pollen-tube membranes was susceptible to digestion by an endo-(1,3)-β-D-glucanase purified from *N. alata* styles. The [^{14}C]glucan polymer synthesised in vitro was digested much more slowly than was laminarin (which is soluble in water), but typically 40–60% of the radioactivity was released to 66%-ethanol-soluble material whether the product was washed with water or ethanol before digestion. Glucan made in the presence or absence of cations was digested to a similar extent, and pre-swelling of the glucan with NaOH did not increase the extent of digestion. Only background levels of radioactivity were released in the absence of the endo-(1,3)-β-D-glucanase, or on treatment of the glucan with α-amylase. When the radiolabelled product was treated with endo-(1,3)-β-D-glucanase and the material released was analysed by anion-exchange HPLC in 150 mM NaOH, radioactivity coeluted with a series (1,3)-β-D-oligoglucosides generated by partial acid hydrolysis of laminarin or pachyman (Fig. 8), as well as with glucose and an unknown degradation product. This confirmed that the material synthesised in vitro was a (1,3)-β-D-glucan.

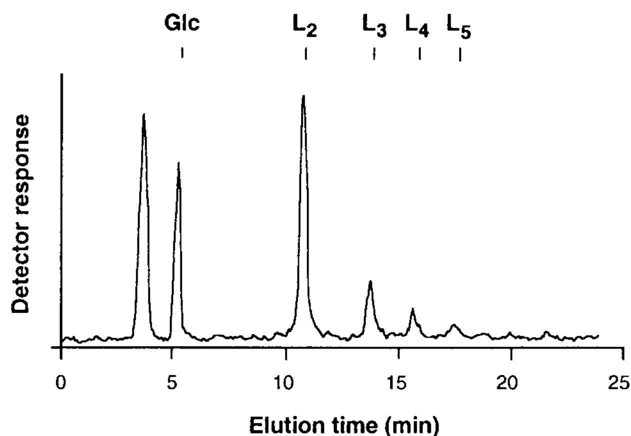

Fig. 8. Anion-exchange chromatography of material released by endo-(1,3)-β-D-glucanase treatment of the product synthesised from UDPGlc by trypsin-treated pollen-tube membranes. Radioactive product was synthesised in the presence of 5 mM EDTA, washed, digested with endo-(1,3)-β-D-glucanase, and the 66%-ethanol-soluble products desalted as described in *Materials and methods*. An aliquot containing 41000 cpm of released material was loaded on a Carbopac-PA1 column equilibrated in 150 mM NaOH and eluted with a gradient of 0–500 mM CH_3COONa in 150 mM NaOH. Radioactivity in the eluant was detected with an in-line dry counter

Discussion

This paper describes the properties of a developmentally regulated callose synthase from pollen tubes of *Nicotiana alata*. The enzyme has a high activity in vitro, and displays unique properties that distinguish it from the wound-activated callose synthase previously described from somatic tissues of plants and analysed here from suspension-cultured cells of *N. alata*. The properties of the callose synthase activities from pollen tubes and other tissues are compared in Table 3.

Callose synthesis by untreated or trypsin-treated membranes from *N. alata* pollen tubes was independent of Ca^{2+}, except at very low substrate levels (less than 0.25 mM UDPGlc) when some stimulation by divalent cations (Mg^{2+} or Ca^{2+}) was observed. This contrasted strongly with the Ca^{2+} dependence of callose synthesis by membranes from *N. alata* suspension-cultured cells, which is characteristic of the classical, wound-induced activity that is activated by the influx of Ca^{2+} ions occurring on cell perturbation (Kauss 1987). In growing pollen tubes, the wall at the tip contains cellulose and neutral and acidic pectins, while callose is deposited as an inner layer of the wall in the subapical zone, starting 10 μm back from the tip (Cresti et al. 1977, 1985; Rae et al. 1985; Heslop-Harrison 1987). Fluorescence ratiometric imaging has given evidence for a gradient of free Ca^{2+} within pollen-tube cytoplasm, with an increased concentration of Ca^{2+} at the tube tip, and using Fura-2 dextran Miller et al. (1992) showed that this gradient was restricted to the tip-most 10–20 μm of *Lilium* pollen tubes; the basal level of free Ca^{2+} in the remainder of the pollen-tube cytoplasm was about 0.17 μM. The Ca^{2+} concentration is therefore not elevated in the region where callose is deposited, which correlates with the observed Ca^{2+} independence of the pollen-tube callose synthase.

The sensitivity of activity to either Mg^{2+} or Ca^{2+} at low substrate concentrations (below 0.25 mM UDPGlc) suggests that Mg^{2+} (normally present at millimolar levels in plant cytoplasm) may play a non-regulatory role in catalysis. Since under these conditions stimulation by Ca^{2+} was not observed in the presence of Mg^{2+}, there was no evidence for any classical, Ca^{2+}-dependent callose synthase in the pollen-tube extracts, although a low level of wound-activated callose synthase may not have been observed against the background of the Ca^{2+}-independent pollen-tube enzyme. This contrasts with observations on isolated phragmoplasts (cell plates, the sites of callose deposition during cell division), which showed only a callose synthase activity with properties of the wound-activated enzyme, with no distinct, developmentally related activity being detected (Kakimoto and Shibaoka 1992). Callose is deposited at pollen-tube tips in incompatible pollinations, but this may simply be due to the disorganisation that occurs with loss of the Ca^{2+} gradient on cessation of growth (Miller et al. 1992).

The pollen-tube callose synthase has a much lower affinity for its substrate UDPGlc (K_m of 1.5–2.5 mM) than does the wound-activated enzyme from suspension-cultured cells (K_m of 0.45 mM). Plant cells in culture have

Table 3. Properties of callose synthase activities from pollen tubes of *N. alata* and somatic tissues. The data for pollen-tube callose synthase come from the present study, and the data for callose synthases from somatic tissues come from the present study and the work of Kauss and Jeblick (1986), Girard and MacLachlan (1987), Hayashi et al. (1987), Sloan et al. (1987), Fredrikson et al. (1991) and Meikle et al. (1991)

Properties	Pollen tubes	Somatic tissues
Cellular level of control	Induced at germination	Activated on cell damage
Ca^{2+} requirement	No	Yes
K_m for UDPGlc	1.5–2.5 mM	0.25–0.6 mM
Activation by digitonin and CHAPS	Over a wide range of detergent concentrations	Over a narrow range of detergent concentrations
Effect of trypsin treatment	Tenfold activation	No significant activation
Product	Linear (1,3)-β-D-glucan	Linear (1,3)-β-D-glucan
pH Optimum	7.5	7.0–7.5
β-Glucoside requirement	Yes	Yes

up to 100 nmol UDPGlc · g^{-1} fresh weight (Meyer and Wagner 1985), corresponding to cytoplasmic concentrations of up to approximately 1 mM, but we have found much higher levels of UDPGlc in extracts of rapidly growing pollen tubes. The lower affinity of the pollen-tube enzyme for UDPGlc may thus reflect the high availability of this substrate in pollen tubes and not reduce rates of callose deposition in vivo. Callose synthesis has also been reported for membranes prepared from pollen tubes of *Lilium longiflorum* (Southworth and Dickinson 1975) and *Petunia hybrida* (Helsper et al. 1977), but in both these cases a very low level of synthase activity was observed and no kinetic analysis was possible: callose synthase activity in extracts of *Lilium* pollen tubes was 0.6 nmol · min^{-1} · mg^{-1} protein in the presence of 0.6 mM UDPGlc, while the activity reported for *Petunia* pollen tubes was assayed at nanomolar levels of UDPGlc and the rates obtained were in the order of 7 pmol · min^{-1} · mg^{-1} protein.

Callose synthase from pollen tubes was activated tenfold by treatment of membranes with trypsin. There was no indication that the basal level of untrypsinised activity in the pollen-tube extracts was due to proteolytic cleavage during extraction. Activation by trypsin and Lys-C endoproteinase implies cleavage at sequences containing lysine, which could be the positively charged peptide sequences usually esposed at the surface of the lipid bilayer where membrane proteins interact with the negatively charged heads of phospholipids. Optimal activation was only achieved in the presence of detergent, which suggests that permeabilisation of membranes is needed for trypsin to reach those callose synthase molecules that are located inside sealed membrane vesicles. Fredrikson and Larsson (1989) used measurements of enzyme latency in vesicles of defined orientation to show that the callose synthase from cauliflower (*Brassica oleracea*) inflorescences is vectorially oriented in the membrane, and our results suggest that a similar, vectorially oriented transmembrane glucosyl-transferase also exists in pollen tubes.

Understanding the mechanism of this activation by trypsin will be important in revealing the regulation of callose synthesis in intact pollen tubes. Activation of callose synthesis in extracts could be due to proteolytic removal of an inhibitory domain on the callose synthase enzyme, to proteolysis of a separate membrane-associated inhibitor, or to proteolytic activation of a previously inactive synthase zymogen; retention of activation after washing of the trypsin-treated membranes with detergents excludes the generation by proteolysis of a soluble activator of callose synthase. The proton-pumping ATPase of plant plasma membranes is activated by treatment with trypsin or chymotrypsin through removal of a C-terminal autoinhibitory peptide (Palmgren et al. 1990, 1991); this activation can be mimicked by addition of lysophospholipids, while plasma-membrane vesicles isolated from cells pretreated with fusicoccin contain an activated but full-size ATPase (Johansson et al. 1993). Fusicoccin pretreatment presumably induces stable displacement of the C-terminal peptide by a mechanism such as a phosphorylation or dephosphorylation, while lysophospholipids induce this displacement directly. Similarly, the level of active, and presumably plasma-membrane-bound, callose synthase within pollen tubes could be controlled either by removal of an inhibitory domain by an endogenous protease or by displacement of such a domain through some other, non-proteolytic mechanism; either mechanism could function to activate callose synthase as this is transferred from the endomembrane system to the plasma membrane. We show elsewhere that the level of callose synthase activity in pollen-tube extracts not treated with trypsin is sufficient to make callose at the rate that this polymer is deposited in the intact pollen tube, which implies that trypsin treatment reveals enzyme that is held in an inactive form in vivo. Protease treatment also activates enzymes of fungal wall polysaccharide synthesis, including the (1,3)-β-glucan synthase of *Saprolegnia* (Fèvre et al. 1988) and chitin synthases I and II in yeasts (Cabib et al. 1988); in neither of these cases, however, has the relevance of protease activation in extracts been related to the control synthase activity in intact cells.

We were unable to find significant activation by trypsin of callose synthase from *N. alata* suspension-cultured cells: results were variable and activation was always less than 1.5-fold. Callose synthase of suspension-cultured soybean cells, assayed in the presence of Ca^{2+}, was not activated by incubation of unfractionated homogenates with trypsin or an endogenous protease, but the dependence of activity on Ca^{2+} was abolished leading to apparent activation when the enzyme was assayed in EGTA (Kauss et al. 1983); and Girard and McLachlan (1987) proposed that pea hypocotyl extracts contained an inhibitor capable of protecting β-glucan synthase from inactivation, or a zymogen from activation, by added or endogenous protease activity. The variable and irreproducible nature of trypsin activation of callose synthase from cells other than pollen-tubes could be due to the lower and more variable rate of plasma-membrane turnover in non-tip-growing cells, whereas the plasma membrane and its components in pollen-tubes undergo rapid turnover (Steer 1988).

The pollen-tube callose synthase showed a remarkable degree of stimulation by a wide range of concentrations of digitonin and CHAPS. Activation by detergents could be due either to permeabilisation of sealed membrane vesicles to substrate UDPGlc or to increasing fluidity of the lipid bilayer. The maximal increase in activity was a balance between activation and denaturation, and depended on the detergent used; for the enzyme from suspension-cultured cells, this balance was reached at low concentrations of detergent, whereas for the pollen-tube enzyme this balance shifted to higher concentrations of detergents and a higher level of activation was attained. The tolerance of high detergent levels implies that the pollen-tube enzyme is active in a very fluid membrane bilayer.

The requirement for a β-glucoside activator for pollen-tube callose synthase is unexplained, but the same is found for the wound-induced callose synthase. It has been suggested that endogenous β-glucosides, such as

β-furfuryl-β-glucoside, are synthesised in the cytoplasm of all cell types, transported into the vacuole, and upon cell perturbation are released into the cytoplasm where they activate callose synthesis (Ohana et al. 1992, 1993). The pollen-tube enzyme, however, is active in the intact cell, and the physiological roles of β-glucosides in the pollen-tube cytoplasm remain to be explained. Alternatively, β-glucosides may originate in the cell wall and be transported into the cytoplasm or even act directly at the extracytoplasmic face of the callose synthase complex: for example, an enzyme from *Candida* cell walls that introduces 6-linked branches into linear (1,3)-β-glucan chains has been shown to release laminaribiose (Hartland et al. 1991).

Characterisation of products showed that the pollen-tube enzyme synthesised a linear (1,3)-β-D-glucan with a degree of polymerisation considerably greater than 1200 under all conditions tested (untreated or trypsin-treated membranes, and in the presence of EDTA or divalent cations). No 6-linked branch points were detected in the product synthesised in vitro, although these are found in the walls of pollen tubes (Rae et al. 1985; Read et al. 1992b). Similarly, cotton-fibre cell walls contain a sparingly branched 3-glucan while in vitro the cotton-fibre enzyme only makes linear 3-linked chains of a high degree of polymerisation (Hayashi et al. 1987). The branching enzyme could remain bound to the wall on breakage of plant cells, as is the case for the (1,3)-β-glucan branching enzyme from *Candida* (Hartland et al. 1991). The pollen-tube membranes did not synthesise detectable levels of 4-linked glucan, implying that the pollen-tube cellulose synthase was inactivated on extraction, as in the case of cellulose synthase from all other plant cells (Delmer 1987).

The subcellular location of the pollen-tube callose synthase has not been conclusively determined (Jensen and Fisher 1970; Cresti and van Went 1976; Cresti et al. 1977, 1985), whereas the wound-activated enzyme of other cell types is known to be localised in the plasma-membrane (Delmer 1987). Membrane vesicles prepared from pollen tubes contained only minimal levels of 3-linked Glc, which suggests that the callose synthase was not located in the endomembrane system; the membranes did contain substantial amounts of 5-linked Ara*f* corresponding to the linear arabinan of the pollen-tube wall, a polymer known to be synthesised within membrane vesicles (Rae et al. 1985; Read et al. 1992b; Schlüpmann et al. 1992), and terminal Ara*f* and Ara*p* residues and 3-linked and 3,6-linked Gal residues presumably corresponding to membrane-bound or secreted arabinogalactan proteins (Fincher et al. 1983). Partitioning of the pollen-tube callose synthase activity in an aqueous polyethylene glycol-dextran phase system (prepared as described in Larsson et al. 1988) was consistent with localisation of some of the enzyme in plasma-membrane-derived vesicles (data not shown).

In summary, the callose synthase activity of isolated pollen-tube membranes has kinetic and regulatory properties that can be related to the physiology of callose deposition in growing pollen tubes, including the lack of Ca^{2+} dependence and the novel activation by trypsin. Elucidation of the individual components of the solubilised and purified pollen-tube callose synthase will now allow a molecular analysis of the developmental control of callose assembly in these differentiated plant cells.

This research was supported by funds from a Special Research Centre of the Australian Research Council. H.S. was funded by a Melbourne University Postgraduate Scholarship and an Overseas Postgraduate Research Studentship; S.M.R. was supported by a Queen Elizabeth II Research Fellowship. We thank Bruce McGinness and Susan Mau for greenhouse assistance, and Deborah Delmer and Adrienne Clarke for advice and encouragement throughout this project.

References

Anderson, M.A., Cornish, E.C., Mau, S.L., Williams, E.G., Hoggart, R., Atkinson, A., Bönig, I., Grego, B., Simpson, R., Roche, P.J., Haley, J.D., Niall, H.D., Tregear, G.W., Coghlan, J.P., Crawford, R.J., Clarke, A.E. (1986) Cloning of a cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. Nature **321,** 38–44

Bacic, A., Harris, P.J., Stone, B.A. (1988) Structure and function of plant cell walls. In: The biochemistry of plants, vol. 14: Carbohydrates, pp 297–371, Preiss J., ed. Academic Press

Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A. (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carb. Res. **113,** 291–299

Cabib, E., Silverman, S.J., Sburlati, A., Slater, M.L. (1988) Chitin synthesis in yeast *(Saccharomyces cerevisiae)*. In: Biochemistry of cell walls and membranes in fungi, pp 31–40, Kuhn , P.J. ed. Springer

Christeller, J.T., Shaw, B.D., Gardiner, S.E., Dymock, J. (1989). Partial purification and characterisation of the major midgut proteases of grassgrub larvae (*Costelytra zealandica*. Coleoptera: Scarabaeideae). Insect Biochem. **19,** 221–231

Ciucanu, J., Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates. Carb. Res. **131,** 209–217

Cornish-Bowden, A., Eisenthal, R. (1978) Estimation of Michaelis constant and maximum velocity from the direct linear plot. Biochim. Biophys. Acta **523,** 268–272

Cresti, M., van Went, J.L. (1976) Callose deposition and plug formation in *Petunia* pollen tubes in situ. Planta **133,** 35–40

Cresti, M., Pacini, E., Ciampolini, F., Sarfatti, G. (1977) Germination and early tube development in vitro of *Lycopersicon peruvianum* pollen: ultrastructural features. Planta **136,** 239–247

Cresti, M., Ciampolini, F., Mulcahy, D.L.M., Mulcahy, G. (1985) Ultrastructure of *Nicotiana alata* pollen, its germination and early tube formation. Am. J. Bot. **72,** 719–727

Delmer, D.P. (1987) Cellulose biosynthesis. Annu. Rev. Plant Physiol. **38,** 259–290

Fèvre, M., Girard, V., Nodet, P. (1988) Cellulose and β-glucan synthesis in *Saprolegnia*. In: Biochemistry of cell walls and membranes in fungi, pp. 97–107, Kuhn, P.J., ed. Springer

Fincher, G.B., Stone, B.A. (1981) Metabolism of non-cellulosic polysaccharides. In: Encyclopedia of plant physiology, N.S., vol. 13B: Plant carbohydrates II, Extracellular carbohydrates, pp. 68–132, Tanner, W., Loewus, F.A., eds. Springer

Fincher, G.B., Stone, B.A., Clarke, A.E. (1983) Arabinogalactan-proteins: structure, biosynthesis and function. Annu. Rev. Plant Physiol. **34,** 47–70

Fredrikson, K., Larsson, C. (1989) Activation of 1,3-β-glucan synthase by Ca^{2+}, spermine and cellobiose. Localization of activator sites using inside-out plasma membrane vesicles. Physiol. Plant. **77,** 196–201

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

Girard, V., MacLachlan, G. (1987) Modulation of pea membrane β-glucan synthase activity by calcium, polycation, endogenous protease, and protease inhibitor. Plant Physiol. **85**, 131–136

Hartland, R.P., Emerson, G.W., Sullivan, P.A. (1991) A secreted β-glucan-branching enzyme from *Candida albicans*. Proc. R. Soc. Lond. B **246**, 155–160

Hayashi, T., Read, S.M., Bussell, J., Thelen, M., Lin, F.-C., Brown Jr, R.M., Delmer, D.P. (1987) UDP-Glucose: (1,3)-β-glucan synthases 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

Helsper, J.P.F.G., Veerkamp, J.H., Sassen, M.M.A. (1977) β-Glucan synthetase activity in Golgi vesicles of *Petunia hybrida*. Planta **133**, 303–308

Heslop-Harrison, J. (1987) Pollen germination and pollen-tube growth. Int. Rev. Cytol. **107**, 1–78

Jensen, W.A., Fisher, D.B. (1970) Cotton embryogenesis: the pollen tube in the stigma and style. Protoplasma **69**, 215–235

Johansson, F., Sommarin, M., Larsson, C. (1993) Fusicoccin activates the plasma membrane H^+-ATPase by a mechanism involving the C-terminal inhibitory domain. Plant Cell **5**, 321–327

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

Kauss, H. (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. **38**, 47–72

Kauss, H., Jeblick, W. (1986) Influence of free fatty acids, lysophosphatidylcholine, platelet-activating factor, acylcarnitine, and echinocandin B on 1,3-β-D-glucan synthase and callose synthesis. Plant Physiol. **80**, 7–13

Kauss, H., Jeblick, W. (1991) Induced Ca^{2+} uptake and callose synthesis in suspension-cultured cells of *Catharanthus roseus* are decreased by the protein phosphatase inhibitor okadaic acid. Physiol. Plant. **81**, 309–312

Kauss, H., Kohle, H., Jeblick, W. (1983) Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from soybean cells. FEBS Lett. **158**, 84–88

Kohle, H., Jeblick, W., Poten, F., Blaschek, W., Kauss, H. (1985) Chitosan-elicited callose synthesis in soybean cells is a Ca^{2+}-dependent process. Plant Physiol. **77**, 544–551

Larsson, C., Widell, S., Sommarin, M. (1988) Inside-out plant plasma membrane vesicles of high purity obtained by aqueous two-phase partitioning. FEBS Lett. **229**, 289–292

Lau, E., Bacic, A. (1993) Capillary gas chromatography of partially methylated alditol acetates on a high polarity, cross-linked, fused silica BPX70 column. J. Chrom. **637**, 100–103

Meikle, P.J., Ng, K.F., Johnson, E., Hoogenraad, N.J., Stone, B.A. (1991) The β-glucan synthase from *Lolium multiflorum*. J. Biol. Chem. **266**, 22569–22581

Meyer, R., Wagner, K.G. (1985) Determination of nucleotide pools in plant tissue by high-performance liquid chromatography. Anal. Biochem. **148**, 269–276

Miller, D., Callahan, D., Cresti, M., Gross, D., Hepler, P. (1992) Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. J. Cell Sci. **101**, 7–12

Murashige, T., Skoog, F. (1977) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. **15**, 473–497

Ohana, P., Delmer, D.P., Steffens, J.C., Matthews, D.E., Mayer R., Benziman, M. (1991) β-Furfuryl-β-glucoside: and endogenous activator of higher plant UDP-glucose: (1-3)-β-glucan synthase. J. Biol. Chem. **266**, 13742–13745

Ohana, P., Delmer, D.P., Volman, G., Steffens, J.C., Matthews, D.E., Benziman, M. (1992) β-Furfuryl-β-glucoside: an endogenous activator of higher plant UDP-glucose:(1-3)-β-glucan synthase. Biological activity, distribution and in vitro synthesis. Plant Physiol. **98**, 708–715

Ohana, P., Benziman, M., Delmer, D.P. (1993) Stimulation of callose synthesis in vivo correlates with changes in intracellular distribution of the callose synthase activator β-furfuryl-β-glucoside. Plant Physiol. **101**, 187–191

Palmgren, M.G., Larsson, C., Sommarin, M. (1990) Proteolytic activation of the plant plasma membrane H^+-ATPase by removal of a terminal segment. J. Biol. Chem. **265**, 13423–13426

Palmgren, M.G., Sommarin, M., Serrano, R., Larsson, C. (1991) Identification of an autoinhibitory domain in the C-terminal region of the plant plasma membrane H^+-ATPase. J. Biol. Chem. **266**, 20470–20475

Rae, A.E., Harris, P.J., Bacic, A., Clarke, A.E. (1985) Composition of the cell walls of *Nicotiana alata* Link et Otto pollen tubes. Planta **166**, 128–133

Read, S.M., Bacic, A., Clarke, A.E. (1992a) Control of morphology and generative nucleus division in cultured pollen tubes of *Nicotiana*. In: Angiosperm pollen & ovules, pp. 162–167, Mulcahy, D., Ottaviano, E., eds. Springer-Verlag

Read, S.M., Schlüpmann, H., Whitten, E., Clarke, A.E., Bacic, A. (1992b) The composition, structure and synthesis of cell walls of tobacco pollen tubes grown in different culture conditions. In: Sixth Cell Wall Meeting, p. 112, Sassen, M.M.A., Derksen, J.W.M., Emons, A.M.C., Wolters-Arts, A.M.C., eds. University Press Nijmegen

Read, S.M., Clarke, A.E., Bacic, A. (1993) Requirements for division of the generative nucleus in cultured pollen tubes of *Nicotiana*. Protoplasma, in press

Schlüpmann, H., Read, S.M., Bacic, A. (1992) A novel callose synthase activity from tobacco pollen tubes. In: Sixth Cell Wall Meeting, p. 116, Sassen, M.M.A., Derksen, J.W.M., Emons, A.M.C., Wolters-Arts, A.M.C., eds. University Press Nijmegen

Sloan, M.E., Rodis, P., Wasserman, B.P. (1987) CHAPS solubilisation and functional reconstitution of β-glucan synthase from red beet (*Beta vulgaris* L.) storage tissue. Plant Physiol. **85**, 516–522

Southworth, D., Dickinson, D.B. (1975) β-1,3-Glucan synthase from *Lilium longiflorum* pollen. Plant Physiol. **56**, 83–87

Steer, M.W. (1988) Plasma membrane turnover in plant cells. J. Exp. Bot. **39**, 987–996

Steer, M.W., Steer, J.M. (1989) Pollen tube tip growth. New Phytol. **111**, 323–358

Vasil, I.K. (1987) Physiology and culture of pollen. Int. Rev. Cytol. **107**, 127–174