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Uridine Diphosphate Glucose Metabolism and Callose Synthesis in Cultured Pollen Tubes of *Nicotiana alata* Link et Otto[1]

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Membrane preparations from cultured pollen tubes of *Nicotiana alata* Link et Otto contain a Ca^{2+}-independent (1–3)-β-D-glucan (callose) synthase activity that has a low affinity for UDP-glucose, even when activated by treatment with trypsin (H. Schlüpmann, A. Bacic, S.M. Read [1993] Planta 191: 470–481). Therefore, we investigated whether UDP-glucose was a likely substrate for callose synthesis in actively growing pollen tubes. Deposition of (1–3)-β-glucan occurred at a constant rate, 1.4 to 1.7 nmol glucose min^{-1}, in tubes from 1 mg of pollen from 3 h after germination; however, the rate of incorporation of radioactivity from exogenous [^{14}C]-sucrose into wall polymers was not constant, but increased until at least 8 h after germination, probably due to decreasing use of internal reserves. UDP-glucose was a prominent ultraviolet-absorbing metabolite in pollen-tube extracts, with 1.6 nmol present in tubes from 1 mg of pollen, giving a calculated cytoplasmic concentration of approximately 3.5 mM. Radioactivity from [^{14}C]-sucrose was rapidly incorporated into sugar monophosphates and UDP-glucose by the growing tubes, consistent with a turnover time for UDP-glucose of less than 1 min; the specific radioactivity of extracted UDP-[^{14}C]glucose was equal to that calculated from the rate of incorporation of [^{14}C]sucrose into wall glucans. Large amounts of less metabolically active neutral sugars were also present. The rate of synthesis of (1–3)-β-glucan by nontrypsin-treated pollen-tube membrane preparations incubated with 3.5 mM UDP-glucose and a β-glucoside activator was slightly greater than the rate of deposition of (1–3)-β-glucan by intact pollen tubes. These data are used to assess the physiological significance of proteolytic activation of pollen-tube callose synthase.

Pollen tubes, the male gametophyte generation of flowering plants, form when pollen grains germinate on the surface of a compatible stigma. During their growth through stylar tissue, pollen tubes carry the sperm cells to the ovary, where fertilization occurs. The processes of plant cell-wall synthesis and assembly can be readily studied in pollen tubes, because these cells maintain their differentiated state when grown in culture and have a relatively simple secondary wall (Heslop-Harrison, 1987; Vasil, 1987; Steer and Steer, 1989; Read et al., 1993a, 1993b). Callose, the major component of the pollen-tube wall, is deposited behind the tube tip as an inner wall layer and also forms the plugs that traverse older regions of the tube at regular intervals. In *Nicotiana* pollen tubes, callose is a (1–3)-β-glucan with a few (1–6)-β-linked glucosyl branches (Rae et al., 1985; Read et al., 1992).

We have recently identified a (1–3)-β-glucan synthase (UDP-Glc: 1,3-β-D-glucan 3-β-D-glucosyl transferase, EC 2.4.1.34) activity in membranes prepared from cultured pollen tubes of *Nicotiana alata* (Schlüpmann et al., 1993). This enzyme differs significantly in its kinetic and regulatory properties from the wound-activated, Ca^{2+}-dependent callose synthase characteristically detected in membrane preparations from somatic tissues (Delmer, 1987). The pollen-tube enzyme synthesizes callose at high rates independent of the concentration of Ca^{2+}, which correlates with the low level of free Ca^{2+} in the subapical region of pollen-tube tips where callose deposition occurs in vivo (Steer and Steer, 1989; Miller et al., 1992). The affinity of the pollen-tube callose synthase for its in vitro substrate UDP-Glc is low, however, with a K_m value of 1.8 to 2.5 mM compared to the K_m values of 0.25 to 0.65 mM reported for wound-activated callose synthases from other tissues (Hayashi et al., 1987; Meikle et al., 1991; Li and Brown, 1993; Schlüpmann et al., 1993). Furthermore, unlike the enzyme from somatic tissues, the activity of the pollen-tube callose synthase increased 10-fold after treatment with trypsin, whereas its cofactor requirements and low affinity for UDP-Glc remained unchanged (Schlüpmann et al., 1993).

We present here measurements of the amount and turnover of UDP-Glc and other relevant metabolites in cultured pollen tubes of *N. alata*. Rapid turnover of the large intracellular pool of UDP-Glc was detected, which attests to the metabolic specialization of pollen tubes for rapid wall synthesis. The rate of callose synthesis by pollen-tube membrane preparations in vitro was then compared with the rate of callose deposition in actively growing pollen tubes, which allowed us to put the low affinity of this enzyme for substrate and its substantial activation by proteolysis into a physiological context.

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Abbreviations: BFA, 1 M aqueous formic acid saturated with *n*-butanol; FDA, fluorescein diacetate; Glc*p*, glucopyranosyl.

MATERIALS AND METHODS

Materials

Nicotiana alata Link et Otto plants were grown in a glasshouse, and cell-suspension cultures were established and maintained, as described by Schlüpmann et al. (1993). Pollen was collected, stored in liquid nitrogen, and grown in a thin layer of liquid medium containing 5% (w/v) Suc, 10% (w/v) PEG 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), 25 mM Mes/KOH, pH 5.9, 10 mg L^{-1} rifampicin, and 40 μM 2-thiouracil (Read et al., 1993a, 1993b). The low-Suc medium for radiolabeling experiments contained a reduced Suc concentration (0.5%, w/v) plus 2.4% (w/v) mannitol as a balancing osmoticum, but otherwise was the same as the normal growth medium.

Standard nucleotides and nucleotide sugars were from Sigma, except for UDP-arabinose, which was made by incubation of mung-bean extracts with UTP, ATP, and L-arabinose and purified by high-voltage paper electrophoresis and paper chromatography (Fry and Northcote, 1983). UDP-[U-^{14}C]Glc (348 mCi $mmol^{-1}$), [U-^{14}C]Suc (350 mCi $mmol^{-1}$), and [U-^{14}C]Glc (296 mCi $mmol^{-1}$) were from Amersham.

Extraction of Nucleotide Sugars

Nucleotides and nucleotide sugars were extracted from suspension-cultured cells and cultured pollen tubes using an adaptation of the method of Olempska-Beer and Bautz Freese (1984). Suspension-cultured cells (duplicates of 0.8 g fresh weight from 4-d cultures) were collected by filtration through Miracloth (Calbiochem), and pollen tubes (grown in duplicate from 25 mg of pollen in 25 mL of medium) were harvested on a filter (GF/A, Whatman) under gentle suction. The Miracloth or filter was rapidly inverted and placed onto a prechilled dish containing 2 mL of BFA, sometimes supplemented with 5 mM KF, and, where appropriate, containing either UDP-[^{14}C]Glc (25 pCi, 0.07 pmol) or UDP-Glc, UMP, and UTP (100 nmol each) as internal standards. After 5 min of incubation on ice, the cells and BFA were transferred to a plastic centrifuge tube (Greiner, 12 mL) and thoroughly vortex-mixed. The filter was placed in the cut-off base of a second centrifuge tube, which was pierced and fitted onto the top of the tube holding the cells. The pair of tubes was centrifuged (3000*g*, 10 min, 4°C), the dry filter was discarded, the supernatant was removed, and the packed-cell pellet was re-extracted twice with ice-cold BFA (1 mL). Pooled supernatants were clarified by centrifugation (40,000*g*, 15 min, 4°C), lyophilized, and stored at −70°C before analysis.

Analysis of Nucleotide Sugars

The dried BFA extracts were resuspended in ice-cold water (600 μL), and aliquots (50 μL) were analyzed by anion-exchange HPLC at pH 5.0 as described below. The responses of the Yttrium-glass radioactivity detector and the UV detector were calibrated by chromatography of UDP-[^{14}C]Glc (25 pCi, 10 nmol). The recovery of UDP-Glc in each extract was calculated by adding a known amount of UDP-[^{14}C]Glc (25 pCi, 0.07 pmol) to samples during extraction with BFA, then determining the amount of radioactivity detected in UDP-Glc after chromatography. The amount of UDP-Glc present in the extract was then calculated from the amount of UDP-Glc detected by UV absorption after chromatography and the recovery value for that extract.

UV spectra of eluted fractions were recorded on a Varian DMS-90 spectrophotometer. Eluted fractions were also lyophilized and hydrolyzed in TFA (0.25 M, 100°C, 30 min), then either the hydrolyzed fractions were reanalyzed by anion-exchange HPLC at pH 5.0 or the released sugars were analyzed as their alditol acetates by GC and GC/MS as described below.

Measurement of Pollen-Tube Cytoplasmic Volume

Pollen was suspended in water at 1 mg mL^{-1} and hydrated for 30 min, and the density of grains in the suspension was determined in a hemocytometer. The frequency of pollen germination was determined 4 h after hydration of pollen in growth medium at 1 mg mL^{-1}. A grain was considered germinated if the emergent pollen tube was more than half the grain diameter. The sample size was 200 pollen grains.

Lengths of pollen tubes occupied by cytoplasm were measured in tubes mounted in growth medium on a microscope slide that had been precoated with FDA by evaporation of 2 μL of a 5 mg mL^{-1} solution of FDA in acetone. The preparation was viewed on a Zeiss IM35 microscope using BP365, FT395, and LP397 filter-sets and epifluorescence illumination and photographed, and both tube lengths and the lengths of the region staining with FDA were measured from prints. Pollen-tube diameters were measured by viewing pollen tubes under differential interference contrast optics on a Zeiss Universal microscope fitted with a Dage-MTI CCD-72 TV video camera and analyzing the images with IMAGE-1/AT software.

Labeling of Pollen Tubes with [U-^{14}C]Suc

Pollen tubes from 10 mg of pollen were grown in 10 mL of growth medium containing 5% Suc at 25 to 26°C for up to 8 h. The culture was transferred onto a glass-fiber filter (GF/A, Whatman) and the medium was passed through the filter by gentle suction. The culture dish was rinsed twice in 5 mL of low-Suc medium, and this medium was also passed through the filter. Pollen tubes on the filter were then washed with low-Suc medium (7 × 1 mL) and transferred to small culture dishes containing 1 mL of low-Suc medium with either 1 μCi of [^{14}C]Suc (for determination of incorporation into walls) or 12 μCi of [^{14}C]Suc (for determination of incorporation into soluble metabolites as well as walls). Tubes were then incubated for up to 30 min at 20°C and harvested, and nucleotides were extracted with BFA as described above. The residual pellet was transferred to a glass-fiber filter and washed sequentially with water (10 × 5 mL) and chloroform:methanol (2:1, v/v, 3 × 3 mL), and radioactivity was determined by scintillation counting.

The BFA-soluble extract was analyzed by anion-exchange HPLC at pH 5.0 as described below, loading up to 88% of the sample, and material co-eluting with radioactive peaks

was collected and lyophilized. Radioactive components that were unretarded on chromatography at pH 5.0 were re-analyzed directly by anion-exchange HPLC at pH 12.0 as described below. Radioactive components that co-eluted with Glc-P were treated with alkaline phosphatase (calf intestine, Boehringer; 1 unit) in 50 mM Tris/HCl, pH 8.5, 0.01 mM $ZnCl_2$ (100 μL) for 2 h at 30°C and the products were analyzed by anion-exchange HPLC at pH 12.0. Radioactive components that co-eluted with UDP-Glc were hydrolyzed in TFA (0.25 M, 100°C, 30 min) and lyophilized, and the products were analyzed by anion-exchange HPLC at pH 12.0.

Anion-Exchange HPLC

Nucleotides, nucleotide sugars, sugar monophosphates, and sugars were chromatographed using a Dionex BioLC Carbohydrate Analyzer HPLC system equipped with a pulsed amperometric detector (PAD, Dionex, Sunnyvale, CA), a variable-wavelength UV monitor (Dionex), and an in-line dry radioactivity counter with an Yttrium-glass solid cell (YG-150 U 4, Berthold Instruments, Wildbad, Germany).

Nucleotides, nucleotide sugars, and sugar monophosphates were chromatographed on a PropacPA-1 column (Dionex) in 50 mM ammonium acetate adjusted to pH 5.0 with acetic acid. The flow rate was 1 mL min^{-1}. Bound components were eluted with increasing concentrations of ammonium acetate/acetic acid; 1 min after sample injection the concentration was increased linearly to 120 mM at 12 min, then linearly to 240 mM at 20 min, then linearly to 1 M at 30 min. Nucleotides were detected by monitoring A_{255}, and radioactive compounds were detected with the in-line counter.

Sugars were chromatographed on a CarbopacPA-1 column (Dionex) in 15 mM NaOH, pH 12.0 (Lee, 1990). The flow rate was 1 mL min^{-1}. Bound components were eluted with a linear concentration gradient of CH_3COONa in 15 mM NaOH, starting 1 min after sample injection and reaching 25 mM CH_3COONa, 15 mM NaOH after 20 min. Following postcolumn addition of NaOH (final concentration 150 mM), the eluant was monitored with the PAD and the in-line radioactivity counter.

Preparation of Pollen-Tube Walls

Pollen tubes from 50 mg of pollen were grown in duplicate 100-mL cultures, harvested on glass-fiber filters (GF/A, Whatman) under gentle suction, and washed with ice-cold 15 mM Mes/KOH, pH 5.9, 1 mM $CaCl_2$, 5% (w/v) Suc (3 × 20 mL). Tubes were transferred to 20 mL of ice-cold 25 mM Hepes/KOH, pH 7.0, 1 mM $CaCl_2$, 3 mM $Na_2S_2O_5$ and disrupted by sonication on ice (2 × 1 min, at setting 9 of a 20-Hz 250/450 Sonifier, [Branson, Danson, CT]). Wall fragments were pelleted by centrifugation (500*g*, 5 min, 4°C), washed once in 20 mL of this buffer, resuspended in 17 mL of this buffer, and sonicated as above (3 × 1 min). The resultant wall fragments were 10 to 50 μm in length and were washed in 25 mM Hepes/KOH, pH 7.0, 1 mM $CaCl_2$ (2 × 20 mL) by centrifugation (1000*g*, 5 min, 4°C), resuspended in this buffer (5 mL), and digested with α-amylase (porcine pancreas, Sigma; 10 units of enzyme for walls from 50 mg of pollen) for 30 h at 20°C under a drop of toluene. Wall fragments were finally washed in water (4 × 15 mL) by centrifugation (1000*g*, 5 min, 20°C), resuspended in 2.5 mL of water, and stored at −20°C.

Measurement of Total Carbohydrate

Carbohydrate determinations were performed according to Dubois et al. (1956), with Glc as a standard.

Monosaccharide Analysis

Monosaccharides were analyzed as their alditol acetates following hydrolysis in TFA (2.5 M, 1 h, 121°C) as previously described (Schlüpmann et al., 1993). Alditol acetates were separated, identified, and quantified by GC and GC-MS as described by Lau and Bacic (1993).

Linkage Analysis

Linkage analysis of wall polysaccharides was established by methylation using the NaOH method of Ciucanu and Kerek (1984) with modifications as described (Schlüpmann et al., 1993). Permethylated alditol acetates were separated, identified, and quantified by GC-MS as described by Lau and Bacic (1993).

Expression of Data

No determinations of fresh weight were made during growth of pollen-tube cultures. Therefore, amounts of metabolites, rates of carbohydrate deposition, and rates of incorporation of [^{14}C]Suc were all calculated and expressed relative to the initial fresh weight of pollen grains.

RESULTS

Separation of Nucleotide Sugars by HPLC

Anion-exchange HPLC on PropacPA-1 eluted with a gradient of ammonium acetate/acetic acid at pH 5.0 was found to be a rapid and convenient means of separating common nucleotides and nucleotide sugars. The elution profiles of UMP and standard nucleotide sugars are shown in Figure 1. The nature of the base had a greater effect on retention time at pH 5.0 than did the nature of the neutral sugar, but UDP-Ara (16.2 min), UDP-Gal (16.4 min), UDP-Glc (17.0 min), and UDP-Xyl (18.0 min) were well resolved, with UDP-Glc eluting at about 160 mM acetate. The more negatively charged UDP-GlcA (30.7 min) was well separated from the other derivatives.

UDP-Glc Content of Suspension-Cultured Cells

Extraction of suspension-cultured cells of *N. alata* in BFA, followed by analysis by anion-exchange HPLC at pH 5.0, allowed the cellular level of UDP-Glc to be determined. A known amount of UDP-[^{14}C]Glc was added to the BFA-extractant as a marker for subsequent chromatography and to permit calculation of the recovery of UDP-Glc from the cells. This showed that UDP-Glc was not stable in cell extracts in the absence of KF, with recoveries of the standard UDP-[^{14}C]Glc being less than 65%. When 5 mM KF was added to

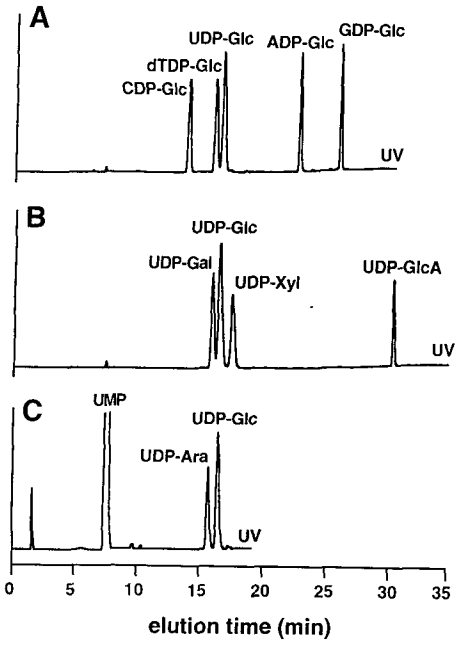

Figure 1. Separation of standard nucleotide sugars by anion-exchange HPLC. Nucleotide sugars (20 nmol each) were chromatographed on a Propac-PA1 column (Dionex), eluted with a gradient of ammonium acetate/acetic acid (pH 5.0), and detected at 255 nm as described.

the BFA extractant, however, recoveries of added UDP-[^{14}C]-Glc increased to about 90%. Even in the presence of KF, UDP-Glc was not a major UV-absorbing component in suspension-cultured cell extracts (Fig. 2A). The UDP-Glc content of the cells was determined from the A_{255} of the eluted UDP-Glc peak and the recovery of UDP-[^{14}C]Glc measured for each extract. In this way, suspension-cultured cells of *N. alata* were calculated to contain 30 nmol UDP-Glc g^{-1} fresh weight during exponential growth.

UDP-Glc Content of Cultured Pollen Tubes

Extraction of cultured pollen tubes with BFA, followed by anion-exchange HPLC at pH 5.0, showed that these cells contained a major component that absorbed at 255 nm and co-eluted with authentic UDP-[^{14}C]Glc (Fig. 2B). The UV *A* maximum for this component, measured after elution in 160 mM ammonium acetate/acetic acid, pH 5.0, was 263 nm, identical with that of authentic UDP-Glc under these conditions, but different from that of other nucleotide sugars (λ_{max} values measured for CDP-Glc, 274 nm; dTDP-Glc, 266 nm; ADP-Glc, 259 nm; GDP-Glc, 249 nm). Mild acid hydrolysis of the eluted component, followed by rechromatography on the same system, resulted in disappearance of the peak migrating with UDP-Glc and the appearance of a new UV-absorbing peak that co-eluted with UMP (Fig. 2C). Monosaccharides in the acid hydrolysate were analyzed by GC (Fig. 2D) and GC/MS after reduction and acetylation, and Glc was the only sugar detected; other sugars, if present, would have constituted less than 1% of the total sugar. Taken together, these analyses confirm that the material in pollen-tube extracts that co-eluted with authentic UDP-Glc consisted solely of UDP-Glc. Therefore, UDP-Glc constitutes a major part of the UV-absorbing metabolites extracted from pollen tubes.

Recoveries of UDP-Glc from pollen tubes, determined by addition of UDP-[^{14}C]Glc to the BFA extractant, were consistently around 90%, and were not improved by addition of 5 mM KF. The UDP-Glc contents of pollen tubes at various

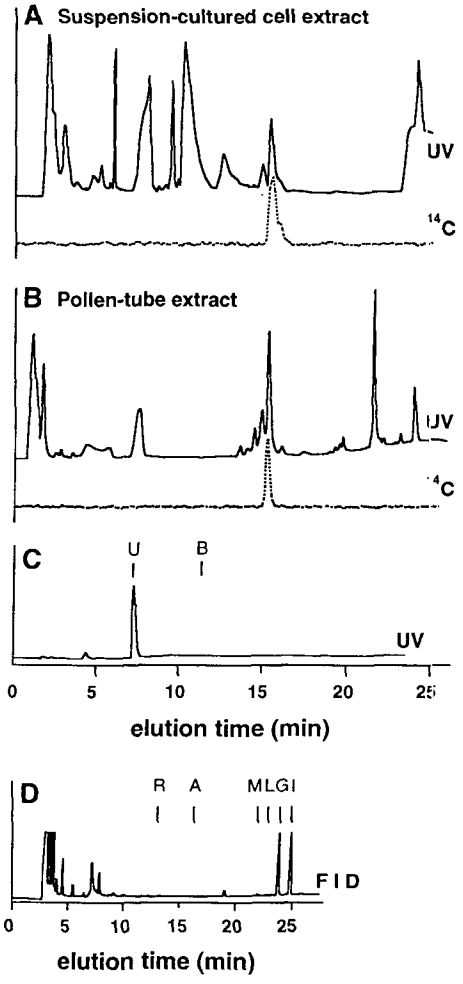

Figure 2. Analysis of extracts of suspension-cultured cells and cultured pollen tubes of *N. alata*. Suspension-cultured cells (A) and cultured pollen tubes (B, 4 h after hydration of pollen) were harvested and extracted in BFA containing 5 mM KF and UDP-[^{14}C]Glc (25 pCi, 0.07 pmol) as described in "Materials and Methods." Extracts were lyophilized and analyzed by anion-exchange HPLC at pH 5.0, monitoring the eluant at 255 nm (UV) and with an in-line radioactivity counter (^{14}C). Material in pollen-tube extracts co-eluting with the standard UDP-Glc was collected and hydrolyzed in 0.25 M TFA for 30 min at 100°C. Part of this material was re-analyzed by anion-exchange HPLC at pH 5.0 (C, elution of standard nucleotides marked for UMP [U] and AMP [B]; GMP eluted at 25.5 min), and after addition of inositol as standard the other part was reduced, acetylated, and analyzed by GC with a flame-ionization detector (FID) (D, elution of standard penta- and hexa-acetates marked for Rha [R], Ara [A], Man [M], Gal [L], Glc [G], and inositol [I]).

times after germination were calculated using the recovery value for each extract, as for suspension-cultured cells. The UDP-Glc content increased over the first 3 to 4 h after pollen hydration and then remained constant at about 1.6 nmol in tubes grown from 1 mg of pollen (Fig. 3).

Calculation of UDP-Glc Concentrations

The bulk of the UDP-Glc content of suspension-cultured cells has been presumed to be located in the cytoplasm (Wagner and Backer, 1992), but the cytoplasmic volume of these cells cannot be easily determined. Assuming that the cytoplasm accounts for 10% of the packed cell volume (Carpita and Delmer, 1981), we calculate an approximate cytoplasmic concentration of 0.3 mM UDP-Glc in *N. alata* suspension-cultured cells in their exponential growth phase.

Concentrations of UDP-Glc in pollen tubes were calculated after direct measurement of cell volume and of the proportion of this volume occupied by cytoplasm. Growing tubes were stained with the vital stain FDA (Fig. 4). From 3 h after pollen hydration, vacuoles formed in the grain and the distal portion of the growing tube, and the cytoplasmic volume remained approximately constant thereafter. At 4 h after hydration, pollen tubes had an average diameter of 9 μm and length of 340 μm, of which an average 180 μm behind the tip contained cytoplasm. From these data, the cytoplasmic volume of each pollen tube could be calculated as 11.5 pL. The number of grains in 1 mg of pollen was determined as 51,000 by counting pollen suspensions in a hemocytometer, and the pollen germination frequency in growth medium was measured as 78%. Therefore, pollen tubes cultured for 4 h from 1 mg of pollen contained 0.46 μL of cytoplasm, and the cytoplasmic UDP-Glc concentration in these pollen tubes was calculated as 3.5 mM. UDP-Glc is thus present at a considerably higher concentration in pollen-tube cytoplasm than in the cytoplasm of suspension-cultured cells.

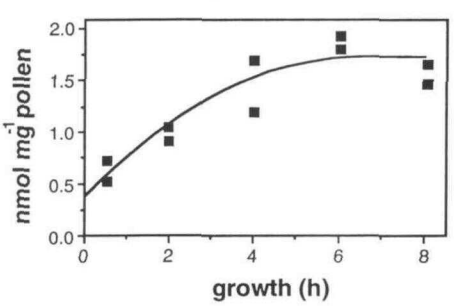

Figure 3. UDP-Glc content of cultured pollen tubes. Pollen tubes were grown for the times shown in duplicate cultures of 25 mg of pollen in 25 mL of medium. Pollen tubes were harvested and metabolites were extracted with BFA containing UDP-[^{14}C]Glc (25 pCi, 0.07 pmol); extracts were lyophilized and analyzed by anion-exchange HPLC at pH 5.0 as described. The recovery of UDP-[^{14}C]Glc was determined for each individual sample, and the content of UDP-Glc in each extract was calculated as described. Results are expressed relative to the initial fresh weight of pollen grains used.

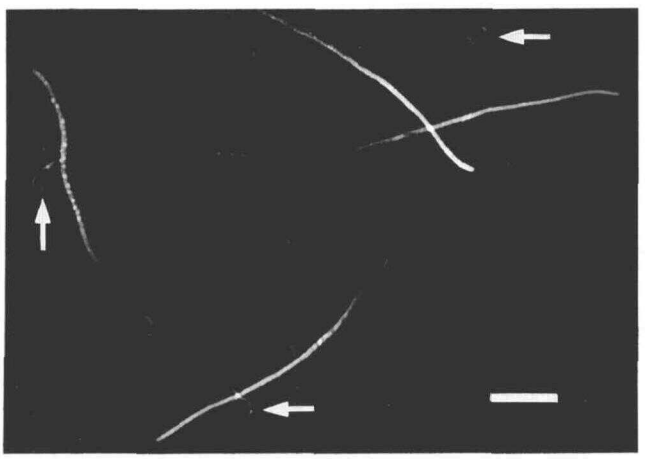

Figure 4. Cytoplasmic content of *N. alata* pollen tubes after 4 h of growth. Pollen was cultured for 4 h at 1 mg pollen mL^{-1} medium, then transferred to a microscope slide precoated with FDA (2 μL, 5 mg mL^{-1} in acetone) and examined by fluorescence microscopy. Bar, 50 μm. Arrows point to pollen grains.

Change in the Carbohydrate Content of Pollen-Tube Walls

The rates of UDP-Glc metabolism and glucan deposition were compared to determine whether UDP-Glc has the properties of a precursor for pollen-tube wall glucans.

Pollen grains were hydrated and germinated in growth medium and walls were prepared from the resultant pollen tubes. After a 3-h lag, the content of water-insoluble carbohydrate in these walls increased linearly at about 26 μg carbohydrate h^{-1} mg^{-1} pollen (Fig. 5, inset), which is equivalent to 2.4 nmol hexose min^{-1} mg^{-1} pollen. The neutral sugar composition of these walls was measured by GC of alditol acetates (Fig. 5). Wall material isolated from pollen grains after 0.5 h in culture, by which time grains had hydrated but had not begun to germinate, was rich in Ara (27% w/w), Gal (27%), Glc (17%), and Xyl (11%), with some Rha (9%), Man (6%), and Fuc (3%). In contrast, walls from pollen tubes grown for 8 h, by which time tubes were about 1 mm long, contained only two major neutral sugars, Glc (68%) and Ara (19%). Analysis over time showed that the amounts of Glc and Ara increased steadily during pollen-tube growth (Fig. 5), whereas the other sugar residues remained essentially constant in amount and were presumably confined to the pollen grain or the tube tip. Between 4 and 8 h after hydration, Glc was incorporated into pollen-tube walls at a rate of 18 μg h^{-1} mg^{-1} pollen according to monosaccharide analysis (Fig. 5), equivalent to 1.7 nmol Glc min^{-1} mg^{-1} pollen.

Linkage analyses of polysaccharides in pollen-tube walls over the first 8 h of culture are shown in Table I. The predominant glycosyl linkages in the walls of hydrated but ungerminated pollen grains (0.5 h) were 5-linked arabinofuranosyl and 4-linked and 4,6-linked Glc*p*. The major neutral polysaccharides can be deduced to be arabinan, xyloglucan, and cellulose, with arabinogalactans and pectins present at lower levels. The wall deposited during pollen-tube growth was different and was dominated by 3-linked Glc*p* from

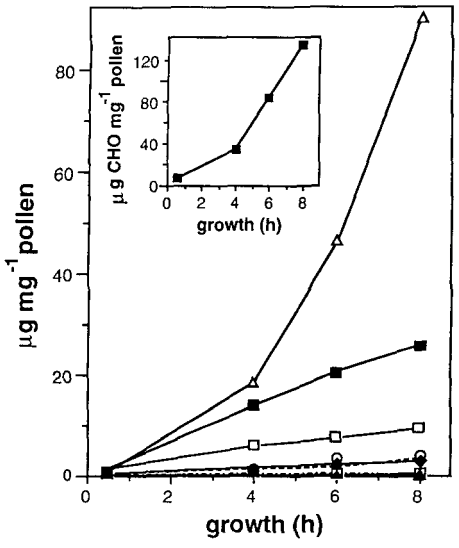

Figure 5. Total carbohydrate content and monosaccharide composition of walls of cultured pollen tubes. Pollen tubes were cultured from 50 mg of pollen in 100 mL of medium for various periods, harvested, and broken by sonication in buffer. Walls were extensively washed, treated with α-amylase and acid hydrolyzed (2.5 M TFA, 1 h, 121°C), and the released monosaccharides were analyzed as their alditol acetates by GC (—Δ—, Glc; —■—, Ara; —□—, Gal; —♦—, Rha; - - -O- - -, Xyl; —▲—, Man; ····□····, Fuc). In addition, total carbohydrate content of the α-amylase-treated walls (inset) was determined after Dubois et al. (1956). Results are expressed relative to the initial fresh weight of pollen grains used.

callose; terminal Glc*p* and 3,6-linked Glc*p*, indicative of branching of the callose backbone, were also present. Combining data from linkage analysis (Table I) and total carbohydrate determination (Fig. 5, inset) showed that, although the proportions of 4-Glc*p* and 5-arabinofuranosyl decreased over time, their absolute amounts increased steadily. The tube wall deposited between 4 and 8 h after germination was thus calculated to contain callose (81 mol% of neutral polysaccharide), cellulose (10 mol%), and arabinan (9 mol%); no accumulation of xyloglucan or arabinogalactan was detected. Residues of the callose backbone, 3-Glc*p* plus 3,6-Glc*p*, accounted for 75% of the carbohydrate synthesized between 4 and 8 h, and were deposited in pollen-tube walls at a rate of 1.7 nmol min^{-1} mg^{-1} pollen (we performed calculations for these residues to permit comparison with the in vitro callose synthase activity, which synthesizes linear 3-linked glucan). Alternatively, combining data from linkage analysis (Table I) and monosaccharide determination (Fig. 5) showed that these residues accounted for 81% of the glucan synthesized between 4 and 8 h, and that they were deposited at a rate of 1.4 nmol min^{-1} mg^{-1} pollen.

The rate of synthesis of the 3-linked Glc residues of the callose backbone was thus determined to be 1.4 to 1.7 nmol min^{-1} in actively growing tubes from 1 mg of pollen. This implies that the intracellular UDP-Glc pool, shown above to be 1.6 nmol in tubes from 1 mg of pollen, would turn over completely every 0.9 to 1.2 min if UDP-Glc were the precursor only for the callose backbone; the actual turnover time will be shorter, because UDP-Glc is also used for other biosynthetic purposes.

Labeling Walls and Wall Precursors with [^{14}C]Suc

Pollen tubes were normally cultured at 25°C to 26°C in medium containing 5% Suc, but, for studying the incorporation of [^{14}C]Suc into the cell-wall fraction (taken as material insoluble in BFA, water, and chloroform/methanol), tubes were grown in medium containing 5% Suc, then transferred into a small volume of low-Suc medium containing 0.5% [^{14}C]Suc (1 µCi, 137 cpm nmol^{-1}) and incubated at 20°C.

The incorporation of exogenous [^{14}C]Suc into walls during a 15-min incubation increased with pollen-tube age for at least 8 h after pollen hydration (Fig. 6A), even though the rate of deposition of wall carbohydrate had reached a constant value by 3 to 4 h after hydration (Fig. 5). Pollen tubes grown for 6 h and then incubated with [^{14}C]Suc accumulated radioactivity in their walls at a linear rate, and no lag in incorporation was observed when incubations were as short as 1 min (Fig. 6B). The measured rate of incorporation, 18,000 cpm in 30 min by tubes grown for 6 h from 10 mg of pollen, is equivalent to 0.88 nmol hexose min^{-1} mg^{-1} pollen, which

Table I. *Glycosyl-linkage composition of walls of cultured pollen tubes*

Duplicate samples of wall material were methylated, hydrolyzed, and then reduced and acetylated for analysis as partially methylated alditol acetates by GC/MS as described. Values are mol% (+, trace; –, not detected). *p* after a sugar abbreviation indicates pyranosyl. Ara*f*, Arabinofuranosyl.

Deduced Glycosyl Linkage		Pollen Tube Growth			
		0.5 h	4 h	6 h	8 h
Rha*p*	Terminal	2	–	–	–
	2–	+	–	–	–
	2,4–	3	1	–	–
Fuc*p*	Terminal	2	+	+	–
Ara*f*	Terminal	4	3	2	+
	Terminal*p*	2	1	+	–
	2–	2	+	–	–
	3–	1	+	+	+
	5–	28	20	15	11
	2,5–	1	2	2	1
	3,5–	–	3	2	2
Xyl*p*	Terminal	5	1	1	–
	2–	3	2	+	+
	4–	1	–	–	–
Man*p*	4–	1	–	–	–
Gal*p*	Terminal	3	+	+	+
	2–	2	–	–	–
	3–	1	1	+	+
	4–	1	–	–	–
	6–	2	2	1	1
Glc*p*	Terminal	2	5	6	6
	3–	1	35	49	61
	4–	23	17	13	12
	2,3–	–	1	1	1
	3,6–	–	3	5	4
	4,6–	11	3	2	1

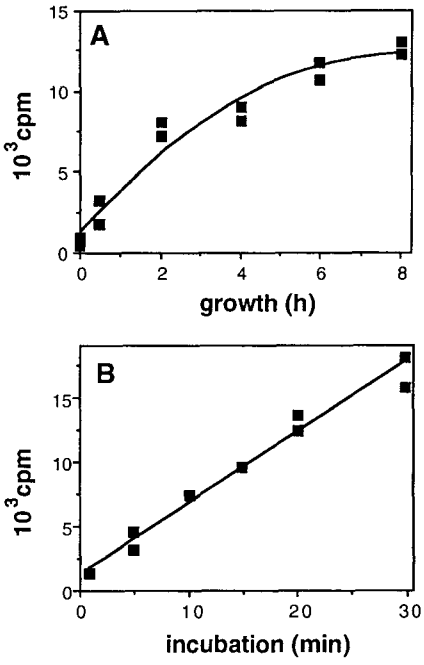

Figure 6. Incorporation of radioactivity from [^{14}C]Suc into pollen-tube walls. A, Pollen tubes were cultured from 10 mg of pollen in 10 mL of growth medium containing 5% (w/v) Suc for various times, in duplicate, then collected on a glass filter, washed, and incubated for 15 min in 1 mL of low-Suc medium containing 0.5% (w/v) Suc, 2.4% (w/v) mannitol, and 1 μCi of [^{14}C]Suc. Labeling was stopped by adding 5 mL of ice-cold BFA containing 5 mM KF, and pollen tubes were washed with BFA, then water, then chloroform/methanol, and radioactivity in the resultant insoluble cell-wall fraction was measured by scintillation counting. B, Pollen tubes were grown, incubated with [^{14}C]Suc, and radioactivity in the wall fractions was determined as in A, except that tubes were cultured in growth medium for 6 h before incubation for various times in low-Suc medium containing [^{14}C]Suc.

is 52% of the value of the rate of total Glc deposition over 4 to 8 h, 1.7 nmol min^{-1} mg^{-1} pollen, determined above. These measurements thus show an increasing use of exogenous Suc for wall synthesis over at least the first 8 h of pollen-tube growth; this is consistent with the initial use for wall synthesis of nonradioactive carbon sources, probably reserves stored in the pollen grain, and with a decreasing contribution of these internal reserves over time.

The kinetics of incorporation of radioactivity from [^{14}C]Suc into metabolites soluble in BFA was examined using pollen tubes grown for 6 h, because tubes cultured beyond this time were too long and tangled to handle with the speed required for short labeling periods. Tubes were thus cultured at 25°C to 26°C for 6 h in medium containing 5% Suc, then transferred to a small volume of low-Suc medium containing [^{14}C]Suc (12 μCi, 1640 cpm nmol^{-1}) and incubated at 20°C. Metabolites soluble in BFA were then analyzed by anion-exchange HPLC at pH 5.0 (Fig. 7). The amount of UDP-Glc in the pollen-tube extracts, determined from the A_{255} of the eluted UDP-Glc peak, was constant over the 20 min of incubation (Fig. 8A), and assuming a recovery of 90% the mean value was calculated as 1.5 nmol mg^{-1} pollen. After 1 min of incubation with [^{14}C]Suc, the earliest feasible time point, three radioactive components were detected: peak 1 (2.1 min) contained neutral or cationic material not bound to the column; peak 2 (5.4 min) co-eluted with sugar monophosphates (not shown); and peak 3 (16.9 min) co-eluted with UDP-Glc. After 20 min of incubation with [^{14}C]Suc, another prominent radioactive component eluting after UDP-Glc was detected (peak 4, 18.9 min).

Material contained in peak 3 was collected and acid-hydrolyzed and the products were analyzed by anion-exchange HPLC at pH 12.0. The only radioactive component detected was [^{14}C]Glc (not shown), which confirmed that peak 3 corresponded to UDP-Glc. The level of radioactivity in UDP-Glc reached a maximal value of 590 to 670 cpm mg^{-1} pollen within 1 min of incubation in [^{14}C]Suc and remained constant thereafter (Fig. 8A). The specific radioactivity of extracted

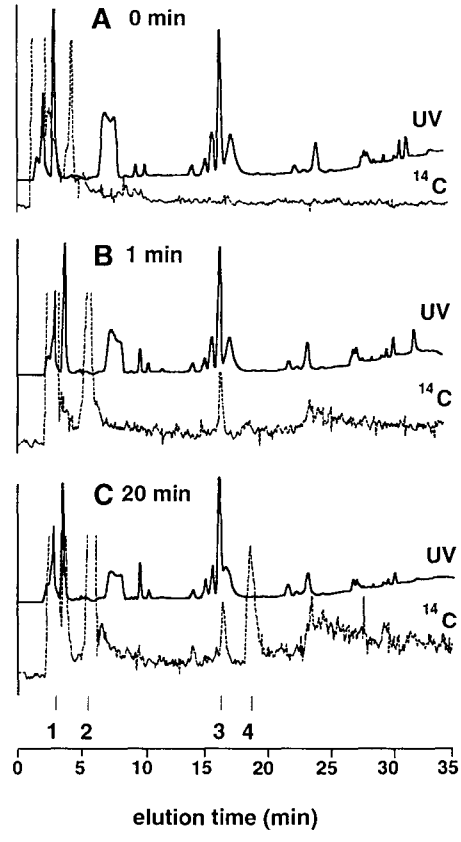

Figure 7. Anion-exchange HPLC of components extracted from pollen tubes incubated with [^{14}C]Suc. Pollen tubes were cultured from 10 mg of pollen in 10 mL of growth medium for 6 h, washed on a glass filter, incubated for various times in 1 mL of low-Suc medium containing 12 μCi of [^{14}C]Suc (A, 0 min; B, 1 min; C, 20 min), and then collected on a glass filter and transferred to 2 mL of ice-cold BFA containing 5 mM KF. For the 0-min incubation, the washed pollen tubes were transferred directly into 2 mL of ice-cold BFA that contained 12 μCi of [^{14}C]Suc and 5 mM KF. Preparation of extracts and anion-exchange HPLC at pH 5.0 were as described. Individual radioactive peaks (marked 1–4) were collected for analysis.

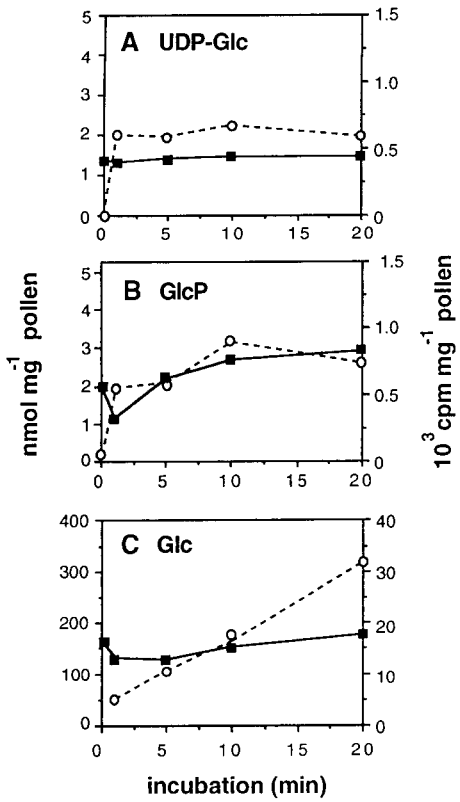

Figure 8. Incorporation of radioactivity from [^{14}C]Suc into pollen-tube metabolites. BFA extracts from pollen tubes grown for 6 h then incubated with [^{14}C]Suc for various times were analyzed as in Figure 7, and radioactive material from peaks 1 to 3 was collected. A, The amount of UDP-Glc (peak 3) and the level of radioactivity incorporated into UDP-Glc were calculated after calibration of the UV detector and radioactivity counter with standard amounts of UDP-[^{14}C]Glc. B, Material in peak 2 was treated with alkaline phosphatase and the products were re-analyzed by anion-exchange HPLC at pH 12.0. The amount of Glc and the level of radioactivity incorporated into Glc were calculated after calibration of the PAD and radioactivity counter with standard [^{14}C]Glc. C, Radioactive material eluting in the unbound fraction (peak 1) was directly re-analyzed by anion-exchange HPLC at pH 12.0; the amount of Glc and level of radioactivity incorporated into Glc, calculated as above, are shown. (—■—, amount; - - -O- - -, radioactivity). Results are expressed relative to the initial fresh weight of pollen grains used.

UDP-[^{14}C]Glc was determined from its A_{255} and radioactive content as 410 cpm nmol^{-1}, and the specific radioactivity of [^{14}C]Glc released by acid hydrolysis of the extracted UDP-[^{14}C]Glc was determined from the PAD response and its radioactive content as 450 cpm nmol^{-1}. These values are 50 to 55% of the specific radioactivity of the hexoses present in the exogenous [^{14}C]Suc (1640 cpm nmol^{-1} Suc, which is 820 cpm nmol^{-1} hexose), and therefore there was a significant nonradioactive contribution to the UDP-Glc pool, presumably from internal reserves. This ratio of specific radioactivities is similar to, and can explain, the discrepancy between the rate of incorporation of radioactivity from [^{14}C]Suc into the wall by pollen tubes grown for 6 h and the measured rate of total glucan synthesis at this time.

Material contained in peak 2 was collected and treated with alkaline phosphatase and the products were analyzed by anion-exchange HPLC at pH 12.0. Components detected were Glc (85 mol%), Fru (12 mol%), and Suc (4 mol%) (not shown), and the calculated amount of Glc-P contained in this material was in the range of 2.2 to 2.7 nmol mg^{-1} pollen over most of the 20-min incubation (Fig. 8B); the range of these values was larger than for UDP-Glc because recoveries of sugars were not measured during phosphatase treatment and subsequent chromatography. The level of radioactivity in the Glc released by alkaline phosphatase was 550 to 870 cpm mg^{-1} pollen over this period (Fig. 8B).

Material contained in peak 1 was collected and re-analyzed directly by anion-exchange HPLC at pH 12.0. The neutral sugars detected were Glc (130–154 nmol mg^{-1} pollen, Fig. 8C), Fru (135–148 nmol mg^{-1} pollen), and Suc (55–65 nmol mg^{-1} pollen), and these pool sizes did not vary significantly over the 20-min incubation. Radioactivity in both Glc (Fig. 8C) and Fru was initially low but increased steadily over this period. The specific radioactivity of the extracted Suc (1700–1850 cpm nmol^{-1}) remained constant, however, and since this was similar to the specific radioactivity of the exogenously supplied [^{14}C]Suc (1640 cpm nmol^{-1}) it probably represented [^{14}C]Suc in the culture medium that remained with the labeled tubes.

DISCUSSION

Pollen tubes develop synchronously in culture and rapidly deposit a wall that is relatively simple in composition and architecture; thus, they are an excellent system for investigating the pathways leading to synthesis of wall carbohydrates. Previously, Loewus and co-workers investigated *myo*-inositol metabolism by growing pollen tubes of *Lilium* on an osmotically balanced medium containing no metabolizable carbohydrate (Chen and Loewus, 1977). Pollen of *Nicotiana* needs a source of Suc for long-term morphologically normal growth, however, and the studies presented here were possible only because of the high rate of wall synthesis in tubes growing in an optimized medium (Read et al., 1993a, 1993b). The major wall component synthesized during growth of *Nicotiana* pollen tubes is the (1–3)-β-glucan callose, and we have recently demonstrated that membranes prepared from cultured pollen tubes of *N. alata* contain a callose synthase activity that is distinct from the wound-induced callose synthase of somatic cells (Schlüpmann et al., 1993). Callose synthase uses UDP-Glc as substrate in vitro, and here we present measurements comparing the rates of callose deposition and UDP-Glc metabolism in cultured pollen tubes. These data relate the properties of callose synthase in isolated membranes to the physiology of callose deposition in vivo.

Polysaccharide Deposition in Pollen-Tube Walls

The pectocellulosic nature of the inner wall of pollen grains (the intine) was originally deduced from cytological observations (Knox, 1984; Heslop-Harrison, 1987), and Nakamura and Suzuki (1981) showed that the major monosaccharide components of the intine of *Camellia* were Ara, Gal, Glc, and uronic acid. Our results from polysaccharide linkage analysis

extend this, showing that walls of *N. alata* pollen grains contain predominantly arabinan, xyloglucan, and cellulose; the presence of Rha residues is consistent with the presence of acidic pectins. The intine of *N. alata* grains is thus similar to typical primary walls of dicotyledonous plant cells (Bacic et al., 1988).

After a 3-h lag, which corresponds to the time taken for a hydrated pollen grain to germinate and develop its full length of cytoplasm, growing *N. alata* pollen tubes deposit carbohydrate at a linear rate. Linkage analysis showed that only three components were accumulated, namely callose (81 mol% of the neutral polysaccharide), cellulose (10%), and arabinan (9%). The carbohydrate composition of walls of *N. alata* pollen tubes grown for 6 h in the complex culture medium used in this study resembled that of tubes grown for 24 h in a suboptimal medium (Rae et al., 1985), probably because the tubes had grown to about the same length in both cases and residual polysaccharides from the pollen grain contributed similarly to both analyses. In most cells, extension growth under turgor pressure occurs over large areas of the cell surface by modulating the interaction of xyloglucan with cellulose fibrils in an elastic matrix of pectin (Carpita and Gibeaut, 1993). Pollen tubes, on the other hand, extend by tip growth (Steer and Steer, 1989), but polysaccharides from the growing tip contribute only a small amount to bulk wall analyses. The callosic layer is a secondary wall, laid down behind the tip in a region where extension growth ceases, and the composition of pollen-tube walls thus gives little indication as to the mechanism of tip extension.

The rate of glucan deposition, 18 μg Glc h^{-1} in tubes from 1 mg of pollen, was equal to approximately 0.45 ng Glc h^{-1} for individual pollen tubes. In comparison, the maximal rate of cellulose deposition in 30-mm-long cotton fibers at 28 d postanthesis was measured as 170×10^{-10} g d^{-1} mm^{-1} fiber length (Meinert and Delmer, 1977), which equals 21 ng Glc h^{-1} for individual fibers. However, pollen tubes synthesize callose only over a portion of their overall length; the length of tube that contains cytoplasm is approximately 180 μm, and the length of the region behind the growing tip where callose is deposited in the wall and as callosic plugs is probably not much longer than this. The maximal rate of cellulose deposition in individual cotton fibers can be calculated as 0.15 ng Glc h^{-1} over a length of 180 μm, and pollen tubes thus deposit callose at about three times this rate per unit length of cell.

Cellular Levels of UDP-Glc

UDP-Glc was the predominant nucleotide sugar in pollen tubes, as has been described in other plant cells (Wagner and Backer, 1992, and refs. therein), and it was present in considerably larger amounts in pollen tubes than in suspension-cultured cells. Extraction in BFA (Olempska-Beer and Bautz Freese, 1984) was fast and simple and did not involve freeze-clamping or mechanical breakage of the cells, neutralization of the extract, or extraction in boiling ethanol as required for other methods (Carpita and Delmer, 1981; ap Rees et al., 1984; Meyer and Wagner, 1985b). The anion-exchange HPLC profile of metabolites extracted from pollen tubes was simpler than that of metabolites from suspension-cultured cells, probably because pollen tubes contain low levels of phenolics. Phenolics can be removed from extracts with phenyl-Sepharose (Meyer and Wagner, 1985b), but we did not find this necessary for either cell type because the PropacPA-1 column gave sufficient chromatographic resolution for direct quantitation of UDP-Glc. Addition of KF to inhibit phosphodiesterases (Fry and Northcote, 1983) was essential for good recoveries of UDP-Glc from suspension-cultured cells, but the level of these enzymes in pollen tubes was not sufficient to affect the recovery of extracted phosphorylated metabolites.

Cellular levels of UDP sugars expressed relative to fresh weight varied between different cell types and in cells of different physiological states: for example, suspension-cultured cells of *Nicotiana tabacum* contained more UDP-Glc at the beginning of their proliferative phase, and generally contained more UDP-Glc (20–137 nmol g^{-1} fresh weight) than did cells from leaf (23 nmol g^{-1} fresh weight) or root (9 nmol g^{-1} fresh weight; Meyer and Wagner, 1985a, 1985b). We measured similar levels of UDP-Glc (30 nmol UDP-Glc g^{-1} fresh weight) in exponentially growing suspension-cultured cells of *N. alata*. This large range of values expressed relative to fresh weight could be due to changes in the relative cytoplasmic content of the cells and their degree of vacuolation, as well as to changes in metabolic activity. Since most of the nucleotide sugars are found in the cytoplasm (Dancer et al., 1990), expression of results in terms of cytoplasmic concentrations allows a more relevant comparison, especially for pollen tubes, which maintain a constant volume of cytoplasm during active growth and for which fresh weight measurements are not readily made. Using an estimated value for the cytoplasmic volume of suspension-cultured cells as 10% of the packed cell volume (Carpita and Delmer, 1981), we calculated the cytoplasmic concentration of UDP-Glc in suspension-cultured *N. alata* cells to be approximately 0.3 mM.

Actively growing pollen tubes from 1 mg of *N. alata* pollen contained a constant 1.6 nmol of UDP-Glc from 4 h after hydration. The approximately cylindrical shape of pollen-tube cytoplasm allowed accurate determination of its volume: each pollen tube contained 11.5 pL of cytoplasm, and the UDP-Glc concentration was thus calculated as 3.5 mM. This value is much higher than that measured for suspension-cultured cells, but is similar to the 5 mM UDP-Glc reported in the cytoplasm of rapidly growing cotton-fiber cells 24 d postanthesis (Carpita and Delmer, 1981). The content of UDP-Glc in cultured pollen tubes of *Camellia japonica*, determined enzymically (Nakamura et al., 1980), followed kinetics similar to those of the UDP-Glc content of *N. alata* pollen tubes, increasing during the first 2 to 3 h after hydration and then remaining constant; in *C. japonica*, the maximal amount of UDP-Glc attained was 3 to 4 nmol UDP-Glc mg^{-1} pollen. The lower amounts of UDP-Glc observed during the first few hours of pollen-tube growth in both *N. alata* and *C. japonica* may reflect the smaller cytoplasmic volume of younger pollen tubes rather than a lower UDP-Glc concentration, and may not limit the rate of wall deposition during this period.

The high cytoplasmic UDP-Glc concentration in actively growing pollen tubes correlates with the low affinity for UDP-Glc of the Ca^{2+}-independent callose synthase in *N. alata* pollen-tube membranes. This enzyme has a K_m for UDP-

Glc of 1.8 to 2.5 mM, and is not saturated by UDP-Glc concentrations as high as 16 mM (Schlüpmann et al., 1993). In contrast, the low cytoplasmic UDP-Glc concentration in suspension-cultured cells of *N. alata* is consistent with the much lower K_m (0.25–0.6 mM) of the wound-induced, Ca^{2+}-dependent callose synthase of suspension-cultured cells and other tissues (Hayashi et al., 1987; Meikle et al., 1991; Schlüpmann et al., 1993).

Carbohydrate Metabolism in Growing Pollen Tubes

Comparison of the measured amount of UDP-Glc (1.6 nmol in tubes from 1 mg of pollen) and the rate of deposition of the callose backbone (1.4–1.7 nmol 3-linked and 3,6-linked Glc min^{-1} in tubes from 1 mg of pollen) gives a calculated minimum turnover time for the UDP-Glc pool of approximately 1 min. UDP-Glc is also a precursor of cellulose, of UDP-Ara for arabinan synthesis, and of a range of other glucosylated products (Helsper, 1979; Wagner and Backer, 1992), and thus the actual turnover time would be even shorter. Metabolic labeling with external [^{14}C]Suc required a relatively high pollen concentration and lowering of the Suc concentration to 0.5%, and under these conditions UDP-Glc was labeled to constant specific radioactivity in less than 1 min; the turnover of the UDP-Glc pool was too rapid to determine its rate of labeling more accurately. These measurements are thus consistent with UDP-Glc being the precursor for callose deposition in actively growing pollen tubes.

The rate of incorporation of radioactivity from external [^{14}C]Suc into the walls of pollen tubes grown for 6 h was equivalent to only 52% of the measured rate of total glucan deposition. Possible experimental reasons for this discrepancy include a lower rate of wall synthesis at the reduced labeling temperature of 20°C, a reduced growth rate caused by handling and the lower concentration of Suc, and dilution of the added [^{14}C]Suc by nonradioactive Suc not fully washed from the tubes during transfer. However, the decrease in growth rate of *N. tabacum* pollen tubes between 24.5°C and 20.1°C was only 12% (Read et al., 1993a), and under the conditions of labeling used here the deposition of wall carbohydrate continued linearly for over 30 min and the size of the UDP-Glc pool was unaltered, indicating that growth was not impaired. In addition, Deshusses et al. (1981) showed that the K_m for Suc uptake by *Lilium* pollen tubes was 1.8 mM, well below the 14.6 mM Suc in our low-Suc medium. Finally, the specific radioactivity of [^{14}C]Suc extracted from the tubes was equal to that supplied exogenously, even after labeling times as short as 1 min, which implies that no dilution of the added [^{14}C]Suc occurred.

Therefore, we conclude that there is a real difference between the rate of glucan synthesis and the rate of incorporation of radioactivity from exogenous [^{14}C]Suc in pollen tubes grown for 6 h. This difference is probably due to the use for wall synthesis of reserves stored in the pollen grain, which can include Suc, starch, phytic acid, and lipids in pollen of different species (Baker and Baker, 1979; Nakamura et al., 1980; Jackson et al., 1982; Knox, 1984); the changing rate of incorporation of radioactivity from [^{14}C]Suc into the tube wall over the first 8 h after pollen germination implies that the contribution of these internal reserves to wall synthesis decreases during tube growth. This conclusion was substantiated by determination of the specific radioactivity of UDP-Glc extracted from pollen tubes that had been grown for 6 h and then incubated with [^{14}C]Suc, which was 55% of the specific radioactivity of the hexoses in the [^{14}C]Suc supplied; the relative contributions of nonradioactive internal reserves and exogenous [^{14}C]Suc to wall synthesis could thus be measured directly from the specific radioactivity of UDP-Glc. Both the rate of saturation of the UDP-Glc pool and the final specific radioactivity attained were therefore consistent with UDP-Glc being the precursor for wall glucan synthesis in vivo.

Metabolic labeling studies with excised cotton fibers incubated in medium containing [^{14}C]Glc established that the rate of UDP-Glc turnover was also consistent with a role for UDP-Glc as the precursor for cellulose synthesis in these cells (Carpita and Delmer, 1981). In cotton fibers, however, the turnover time of the UDP-Glc pool was longer than in pollen tubes and can be calculated as 9.5 min (from a pool size of 7.1 nmol UDP-Glc and a transfer coefficient of 45 nmol h^{-1} in the fibers from one ovule). In addition, the pools of Glc-P and UDP-Glc in cotton fibers did not saturate with radioactivity until 2 h of incubation in [^{14}C]Glc, probably due to the rapid exchange of cytoplasmic [^{14}C]Glc with a large vacuolar Glc pool. Exchange with large internal stores of Suc, and slow utilization of exogenous Suc for synthesis of wall polysaccharide, was also found in *Ricinus* cotyledons (Komor, 1977). This contrasts with the rapid utilization of Suc for cell-wall synthesis in pollen tubes of *N. alata*, where the incorporation of exogenous [^{14}C]Suc into Glc-P, UDP-Glc, and glucan occurred without saturation with radioactivity of the large and presumably vacuolar Glc and Fru pools. Similar rapid utilization of external Suc for wall synthesis was observed in pollen tubes of *Lilium* (Deshusses et al., 1981), but might not be expected in pollen that stores large quantities of Suc, such as pollen from *Camellia* (Nakamura et al., 1980). Suc is present in large amounts in the stylar extracellular fluid in members of the Solanaceae (Konar and Linskens, 1966; H. Schlüpmann, unpublished data), and our results are consistent with metabolic adaptation of *Nicotiana* pollen tubes to rapid heterotrophic growth through the style.

Implications for Properties of Pollen-Tube Callose Synthase

We have shown here that actively growing *N. alata* pollen tubes accumulate 3-Glc*p* and 3,6-Glc*p* residues of the callose backbone at a rate of 1.4 to 1.7 nmol Glc min^{-1} mg^{-1} pollen. The rate of synthesis of linear 3-glucan by membrane preparations from these pollen tubes, without activation by treatment with trypsin, was 1.95 nmol Glc min^{-1} mg^{-1} pollen in the presence of 3.5 mM UDP-Glc, the cytoplasmic UDP-Glc concentration measured here, and with 20 mM cellobiose as a cofactor (Schlüpmann et al., 1993). Comparison of the in vivo and in vitro rates of a reaction is clearly difficult and necessitates many assumptions, but at the first level of analysis the activity of the nontrypsin-treated callose synthase in membrane preparations can account for total callose deposition in cultured pollen tubes provided that high levels of β-glucoside are present. The amounts and intracellular distri-

bution of β-glucosides have not been studied in pollen tubes, but in other cells it has been suggested that release of vacuolar β-glucosides is required for callose synthase activation on wounding (Ohana et al., 1992, 1993); it is possible that pollen-tube cytoplasm contains a higher level of β-glucosides than other cells.

Second, we conclude that treatment with trypsin increases the callose synthase activity of isolated pollen-tube membranes 10-fold above the level required to support callose deposition in vivo (Schlüpmann et al., 1993). This raises the question of the physiological role of proteolytic activation. One simple but attractive possibility is that intact pollen tubes contain a substantial amount of inactive callose synthase, perhaps present in an intracellular compartment and activated by either proteolysis or some other regulatory mechanism on transport to the plasma membrane; in this model, trypsin treatment of isolated membranes would reveal the activity of the zymogen form of the enzyme. The relative amounts of inactive and active enzyme could then reflect the rate of membrane turnover, which varies with pollen-tube growth rate (Steer, 1988). Pollen-tube callose synthase remains dependent on a β-glucoside activator after activation with trypsin (Schlüpmann et al., 1993), which implies that activation by β-glucosides and by proteolysis are distinct modes of regulation.

Chitin synthases from a variety of fungi also exist as inactive zymogens (Duran and Cabib, 1978; Cabib et al., 1988), and activation of chitin synthesis at growing hyphal tips may result from controlled proteolytic activation of the synthase zymogen. In the yeast *Saccharomyces*, the chitin synthases encoded by the *Ch1* and *Ch2* genes, which are responsible for localized deposition of chitin during repair processes and in the intercellular septa, respectively, occur as zymogens, whereas the chitin synthase encoded by the *Ch3* gene, which is responsible for deposition of chitin throughout the cell wall, does not (Shaw et al., 1991). Therefore, we suggest that the site of callose synthesis in actively growing pollen tubes, which is restricted to the subapical region and callose plugs, could also possibly be controlled by proteolytic activation of an inactive zymogen; low mol wt activators, such as β-glucosides, diffuse rapidly throughout the cytoplasm (Ohana et al., 1993) and cannot be responsible for regulation of the site of enzymic activity.

A possible activating system for yeast chitin synthases, consisting of a protease and a protease inhibitor, has been characterized (Ulane and Cabib, 1974). However, direct regulation by proteolytic activation of the callose synthase in intact pollen tubes remains to be proven. The interaction of pollen-tube callose synthase with such regulatory proteins, as well as with proteins mediating its interaction with both membranes and the cytoskeleton (Andrawis et al., 1993; Delmer et al., 1993), may reveal features that are specific to the control of cell-wall synthesis in tip-growing cells.

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