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Role of a callose synthase zymogen in regulating wall deposition in pollen tubes of *Nicotiana alata* Link et Otto

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Abstract. The callose synthase (CalS) activity of membrane preparations from cultured *Nicotiana alata* Link & Otto pollen tubes is increased several-fold by treatment with trypsin in the presence of digitonin, possibly due to activation of an inactive (zymogen) form of the enzyme. Active and inactive forms of CalS are also present in stylar-grown tubes. Callose deposition was first detected immediately after germination of pollen grains in liquid medium, at the rim of the germination aperture. During tube growth the 3-linked glucan backbone of callose was deposited at an increasing rate, reaching a maximum of 65 mg h^{-1} in tubes grown from 1 g pollen. Callose synthase activity was first detected immediately after germination, and then also increased substantially during tube growth. Trypsin caused activation of CalS throughout a 30-h time course of tube growth, but the degree of activation was higher for younger pollen tubes. Over a 10-fold range of callose deposition rates, the assayed CalS activity was sufficient to account for the rate of callose deposition without trypsin activation, implying that the form of CalS active in isolated membranes is responsible for callose deposition in intact pollen tubes. Sucrose-density-gradient centrifugation separated a lighter, intracellular membrane fraction containing only inactive CalS from a heavier, plasma-membrane fraction containing both active and inactive CalS, with younger pollen tubes containing relatively more of the inactive intracellular enzyme. The increasing rate of callose deposition during pollen-tube growth may thus be caused by the transport of inactive forms of CalS from intracellular membranes to the plasma membrane, followed by the regulated activation of these inactive forms in this final location.

Key words: Callose – Callose synthase – Cell wall – *Nicotiana alata* – Pollen tube – Zymogen

Introduction

The properties of individual cell wall polysaccharides depend on the precise chemical structures determined during their synthesis (Delmer and Stone 1988; Gibeaut and Carpita 1994). However, the mechanisms that control the timing, location and extent of wall polysaccharide deposition during growth and development are not well known. In tip-growing cells such as pollen tubes, new wall synthesis takes place at a distinct cellular location, which allows relatively simple measurement of wall changes occurring during growth.

Callose, a 1,3-β-D-glucan with some 1,6-linked branches, is deposited as the major component in the wall of growing pollen tubes, but is confined to the inner wall layer behind the tube tip, and to transverse walls named plugs (Rae et al. 1985; Meikle et al. 1991; Schlüpmann et al. 1994; Geitmann et al. 1995; Ferguson et al. 1998). The control of callose deposition may be related to the mechanisms controlling tip-growth of pollen tubes and their polar cytoplasmic organisation (Heslop-Harrison 1987; Steer and Steer 1989; Cresti and Tiezzi 1990; Taylor and Hepler 1997). Pollen tubes of *Nicotiana* contain an active callose synthase (CalS; uridine-diphosphate glucose: 1,3-β-D-glucan 3-β-D-glucosyl transferase, EC 2.4.1.34) enzyme, that is distinguished from the wound-activated CalS of somatic tissues in being Ca^{2+}-independent (Schlüpmann et al. 1993). In addition, pollen-tube CalS is activated by trypsin in the presence of digitonin, and by some detergents such as Chaps (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate) (Li et al. 1997).

We have previously postulated that pollen tubes contain a large population of inactive (zymogen) CalS molecules (Schlüpmann et al. 1993, 1994), with the

Abbreviations: BSA = bovine serum albumin; CalS = callose synthase; Chaps = 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate; HE = Hepes-EDTA buffer; PBS = phosphate-buffered saline; UDP-Glc = UDP-glucose

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increase in CalS activity after treatment with trypsin or Chaps being due to activation of this inactive form of CalS. This hypothesis is supported by data obtained with the active-site-directed reagent UDP-pyridoxal (Li et al. 1997). The chitin synthases of yeast provide a precedent for activation of polysaccharide synthase zymogens by partial proteolysis (Sburlati and Cabib 1986; Choi et al. 1994), and the plasma-membrane H^+-ATPase of higher plants can also be activated in the same way (Palmgren et al. 1990a).

Here we investigate changes in CalS activity and rates of callose deposition during a 30-h time course of pollen-tube growth, and provide further evidence for the existence of a separate, intracellular CalS zymogen. The activation of this zymogen form after its transfer to the plasma membrane may control both the rate of callose deposition and its spatial location within the plasma membrane.

Materials and methods

Plant materials. Plants of *Nicotiana alata* Link & Otto (self-incompatibility genotypes S_2S_2, S_2S_3 and S_6S_6) were grown in a greenhouse as previously described (Schlüpmann et al. 1993). Pollen was collected and either stored under liquid nitrogen or used immediately. The growth medium and culture conditions were as described by Li et al. (1997).

Chemicals. Digitonin was from Serva (Heidelberg, Germany). 1,3-β-D-Glucan-specific monoclonal antibody was from Biosupplies (Parkville, Vic., Australia). Affinity-purified goat anti-mouse IgG conjugated to 15-nm colloidal gold was from Electron Microscopy Services (Washington, USA). Pectinase was from Calbiochem (La Jolla, Calif., USA). Uridine 5′-diphospho-[U-^{14}C]glucose (11 GBq/mmol, 302 mCi/mmol) was from Amersham (Buckinghamshire, UK). Cellulase, sheep anti-mouse IgG antiserum conjugated to Cy3, and all other reagents were from Sigma (St. Louis, Mo., USA), and were analytical grade (AR) except where otherwise specified.

Preparation of membranes. Membranes were prepared from cultured pollen tubes as described by Li et al. (1997). Membranes of stigmas, styles and stylar-grown tubes were obtained from S_6S_6 styles pollinated with compatible S_2S_3 pollen. Pollinated styles, including stigmas, were homogenised in a pestle and mortar in 50 mM Hepes-NaOH (pH 7.8), 10 mM EDTA (HE buffer) containing 5% sucrose, then sonicated and membranes prepared as for cultured pollen tubes. Membranes were similarly prepared from unpollinated S_2S_2 styles and stigmas collected from non-emasculated flowers. Fractionation of membranes by sucrose density gradient centrifugation, and identification of membrane fractions according to their equilibrium density and the presence of marker enzymes (cytochrome-*c* reductase for endoplasmic reticulum, Triton-dependent IDPase for Golgi, and cytochrome-*c* oxidase for mitochondria), was performed according to Turner et al. (1998).

Protein determination. Protein was determined by a Coomassie blue dye-binding assay (Bio-Rad, Hercules, Calif., USA). Bovine serum albumin was used as a standard.

Standard assay of CalS. Incorporation of radioactivity from UDP-[U-^{14}C]glucose into product insoluble in 66% (v/v) ethanol was used to assay CalS activity. Assays contained 50 μg ml^{-1} membrane protein (10 μg in 200 μl assay volume), 1 mM UDP-glucose (UDP-Glc) and 0.02% digitonin as well as other standard components (Li et al. 1997).

Activation of CalS. Trypsin activation of CalS was carried out by preincubating membrane fractions with trypsin (usually 1:1 trypsin:membrane protein by weight) in the presence of 0.1% (w/v) digitonin for 30 min on ice, then assaying as above. The inclusion of digitonin ensured that no issues of membrane impermeability confounded the analysis (Wu and Wasserman 1993; Li et al. 1997). Activation of CalS by Chaps was determined by including 0.15–0.25% (w/v) Chaps in the assay (Li et al. 1997).

Immunofluorescence microscopy. Pollen grains were hydrated and tubes cultured in 0.25 ml medium in wells of 24-well tissue culture plates (Greiner 'Labortechnik') for 1–4 h, then transferred to Eppendorf tubes. Grains and tubes were washed twice by centrifugation and resuspension in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), then digested in cellulase (10 U in 100 μl) plus pectinase (10 U in 100 μl) for 10–20 min. Grains and tubes were then rinsed twice in PBS, then incubated in anti-(1,3)-β-D-glucan-specific antibody (1:100 dilution) at room temperature for 2 h, then washed in PBS and incubated with anti-mouse IgG antiserum conjugated to Cy3 (1:20 dilution). Controls used PBS instead of primary antibody.

Immunogold electron microscopy. Pollen grains were hydrated and cultured as for light microscopy, then fixed, embedded, sectioned and labelled with anti-(1,3)-β-D-glucan-specific antibody followed by affinity-purified goat anti-mouse IgG conjugated to 15-nm colloidal gold, according to Ferguson et al. (1998).

Preparation and analysis of pollen-tube walls. Pollen-tube walls were prepared according to Schlüpmann et al. (1994). Neutral carbohydrate determinations were performed according to Dubois et al. (1956) with glucose as a standard, then adjusted to account for the proportion of uronic acids determined by linkage analysis, and total carbohydrate expressed as mg g^{-1} pollen. Linkage analysis of wall polysaccharides was performed by methylation using the NaOH method (Ciucanu and Kerek 1984; Schlüpmann et al. 1993) with hydrolysis in 2.5 M trifluoroacetic acid for 8 h at 100 °C, and permethylated alditol acetates were separated, identified, and quantified by gas chromatography-mass spectrometry as described by Lau and Bacic (1993). Carboxyl reduction before linkage analysis was performed according to Sims and Bacic (1995), except that smaller amounts of pollen-tube walls (50–200 μg) were used in the analysis. Glycosyl residue types were allocated to different polymers according to the logic of Shea et al. (1989), then the amount of individual polymers was deduced from the weight of total wall carbohydrate plus the linkage composition. Subtraction of the contribution of previously synthesised polysaccharides was performed according to Schlüpmann et al. (1994).

Results

Activation of CalS in membranes from pollinated styles. The CalS enzyme of *N. alata* pollen tubes grown in liquid culture is distinguished from the wound-activated CalS of other cell types by its activity in the absence of Ca^{2+}, by its activation by trypsin in the presence of digitonin and by its activation by Chaps. Membranes were prepared from *N. alata* styles pollinated with compatible pollen to investigate whether the CalS of stylar-grown pollen tubes displayed similar kinetics. The membranes from pollinated styles contained a CalS active in EDTA, and the level of activity (10.4 nmol min^{-1} from 50 styles pollinated with an estimated 5 mg compatible pollen) was similar to that present in membranes from tubes cultured from a similar mass of pollen (9.8 nmol min^{-1}; Table 1). A low level of CalS activity in unpollinated styles may have resulted from a

Table 1. Callose synthase activity of pollen tubes grown in styles and in culture[a]

	Activity (nmol min^{-1})		
	No activators	+Trypsin	+Chaps
Unpollinated styles (50)	2.3	3.2	3.4
Pollinated styles (50)[b]	10.4	24.2	26.6
Activity due to stylar-grown pollen tubes[c]	8.1	21.0	23.2
Activation (fold)		2.6	2.8
Pollen (5 mg) in culture[d]	9.8	29.2	31.6
Activation (fold)		3.0	3.2

[a]Data from one of three similar experiments
[b]Styles (50) were pollinated with approximately 5 mg compatible pollen and harvested after 16 h
[c]Activity in pollinated styles minus activity in unpollinated styles
[d]Pollen (5 mg) was germinated in liquid culture medium and grown for 16 h

small amount of self-pollination and subsequent slow growth of incompatible pollen tubes (Lush and Clarke 1997). The CalS of pollinated styles was activated 2.6 to 2.8-fold by trypsin or Chaps, similar to the activation from membranes of cultured pollen tubes (Table 1). Three separate experiments gave similar results. The pollen-tube CalS present in pollinated styles therefore displays kinetics similar to the enzyme isolated from cultured pollen tubes, and consistent with the presence of active and inactive forms of CalS in both cases.

Activity of CalS during growth of pollen tubes in culture. No CalS activity (< 10 nmol min^{-1} g^{-1} pollen, assayed at 1 mM UDP-Glc) was detected in membranes prepared from pollen grains hydrated in HE buffer containing 5% sucrose and extracted immediately, or from pollen grains hydrated in culture medium for 1 h (Fig. 1 inset). Activity was first detected 2 h after hydration (Fig. 1), a time at which most grains had just germinated as defined by production of a tube at least as long as half the grain diameter (Fig. 2A,B). The CalS activity then increased substantially during pollen-tube growth, reaching after 20 h a level of 4610 nmol min^{-1} in tubes grown from 1 g pollen (Fig. 1).

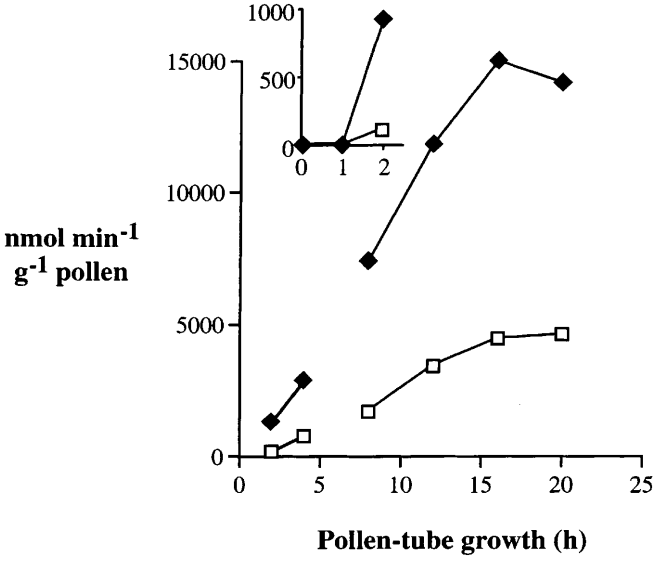


Fig. 1. Callose synthase activity during pollen-tube growth. The CalS activity of isolated membranes was assayed without (–□–) or with (–◆–) trypsin treatment, and is expressed relative to the initial mass of pollen grains as nmol min^{-1} g^{-1} pollen. Main graph (2–20 h) and inset (0–2 h) are from three separate experiments. Several other experiments gave similar results

Callose synthase in membranes from pollen tubes of all culture times could be activated by trypsin (Fig. 1), and the enzyme was similarly activated by Chaps (data not shown). The degree of activation was lower in membrane preparations from older pollen tubes, with trypsin activation (assayed at 1 mM UDP-Glc) decreasing from 9.4-fold in 2-h tubes, to 4.5-fold in 8-h tubes, and 3.3-fold in 16-h tubes (Fig. 1). This suggests that younger pollen tubes contain relatively larger proportions of the form of CalS that can be activated with trypsin or Chaps.

Immuno-localisation of callose. Sites of callose deposition in cultured grains and tubes were located by both light and electron microscopy using a (1,3)-β-D-glucan-specific monoclonal antibody. Treatment with cellulase and pectinase, which presumably makes the inner callosic layer of the tube wall more accessible to antibody, was required for strong and even staining of intact tubes under light microscopy. Observations of populations of germinating grains showed that callose appeared initially around the germinal aperture between 1 and 2 h after hydration, and at the base of the emerging pollen tube (Fig. 2A). In the absence of primary antibody, only autofluorescence of the pollen grain was observed (Fig. 2B). Subsequently, labelling of callose was observed along the tube but not at the growing tip (Fig. 2C), with control incubations again only showing non-specific autofluorescence (Fig. 2D).

Post-embedding immuno-labelling of thin sections showed labelling to be specific to the inner wall layer of the pollen tubes. No callose was present at initial emergence of a germinal bud, which can occur as early as 10 min after hydration, but callose first appeared immediately after germination when a growing tube tip was produced (Fig. 3A). Callose deposition became more dense during the process of tube wall formation, being distributed evenly through a thick inner wall layer (Fig. 3B). Both microscopy methods therefore imply that callose deposition is initiated immediately after germination of individual pollen grains.

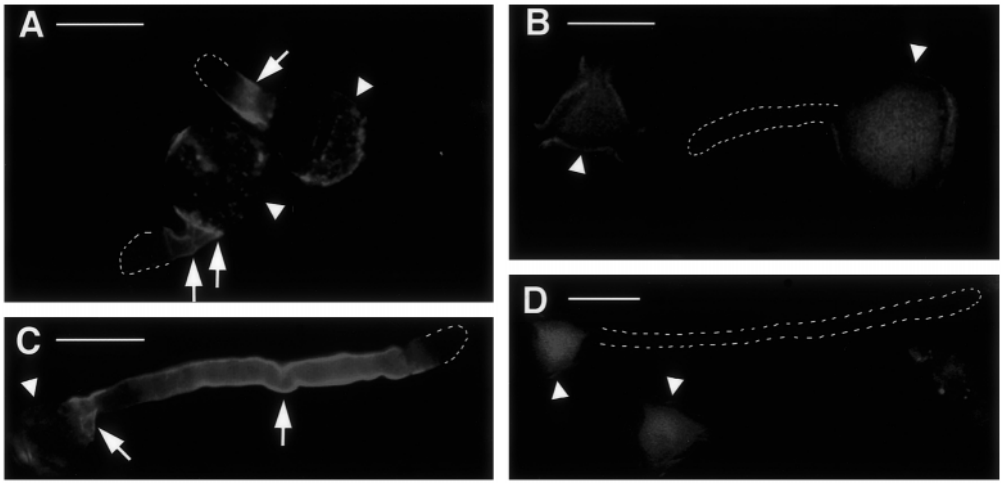


Fig. 2A–D. Immunofluorescence microscopy of germinating pollen grains. Non-specific autofluorescence of grain (*arrowheads*), specific fluorescence due to callose (*arrows*). Positions of pollen tubes are outlined. **A** 2 h after hydration, incubated in anti-(1,3)-β-D-glucan monoclonal antibody, followed by secondary antibody conjugated to Cy3. Callose deposition at the site of germination and base of emerging tube. Two grains are at different focal planes. ×1150; bar = 10 μm. **B** 2 h after hydration, control incubated only in secondary antibody. ×1150; bar = 10 μm. **C** 4 h after hydration, incubated in anti-(1,3)-β-D-glucan monoclonal antibody, followed by secondary antibody conjugated to Cy3. Callose deposition along the tube wall. ×1150; bar = 10 μm. **D** 4 h after hydration, control incubated only in secondary antibody. ×950; bar = 10 μm

Chemical analysis of pollen-tube wall polysaccharides. The timing of initial callose deposition was also determined chemically. Wall carbohydrate was present at 17 mg g^{-1} pollen both in dry pollen grains and in grains hydrated for 1 h, but began to increase at 2 h, concomitant with germination and tube growth. Rapid carbohydrate deposition occurred after germination, with total carbohydrate levels reaching 2010 mg g^{-1} pollen in 32-h pollen tubes (Fig. 4A). Linkage analyses on isolated walls were performed after reduction of uronic acids to include these residue types in the compositional profile (Table 2), and amounts of individual polysaccharides were then calculated from the linkage compo-

Fig. 3A,B. Immuno-gold electron microscopy of germinating pollen grains. All sections incubated in anti-(1,3)-β-D-glucan monoclonal antibody, followed by secondary antibody conjugated to colloidal gold. **A** Emerging pollen tube, 1.5 h after hydration. The pollen-tube tip is immediately to the right of the field of view. Callose deposition (*arrowheads*) initiated. ×20,000, bar = 0.5 μm. **B** Wall layers behind the pollen-tube tip, 4 h after hydration. The thick inner wall contains callose (*arrowheads*). ×45,000; bar = 0.25 μm

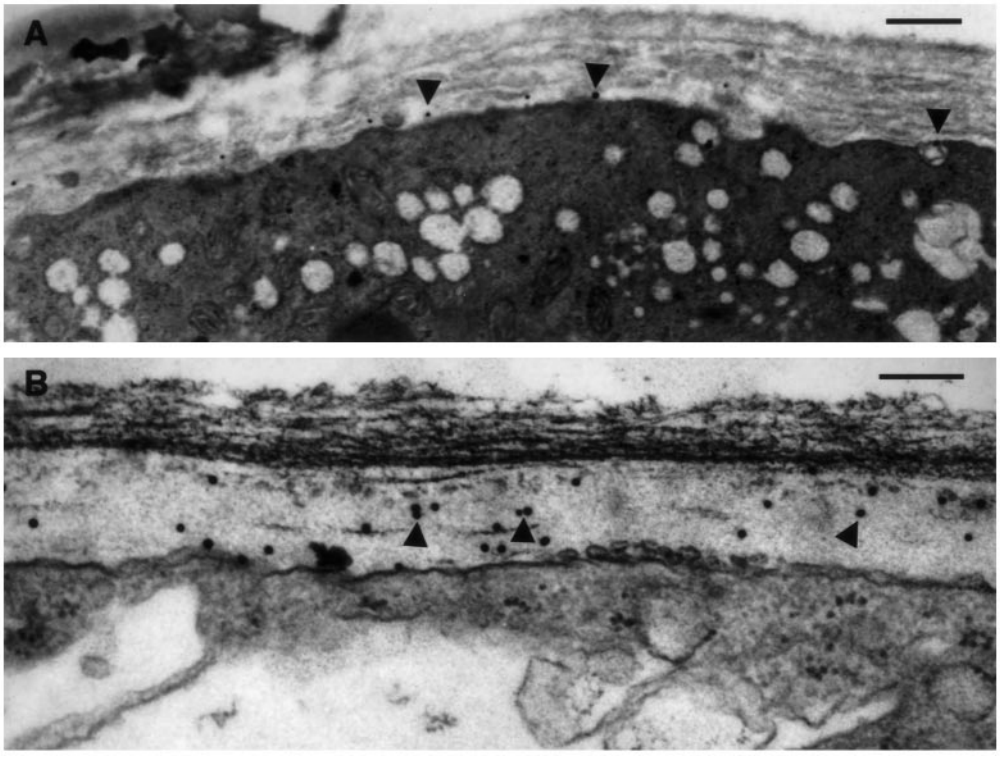


Table 2. Glycosyl-linkage composition (mol%) of pollen-tube walls[a]

Glycosyl residue	Deduced glycosyl linkage	Pollen-tube growth (h)							Deduced polymer
		0	1	2	4	8	16	32	
Rha*p*	2,4-	2	1	1	–	–	–	–	RG
Fuc*p*	terminal	1	2	–	–	–	–	–	XG
Ara*f*	terminal	1	4	6	5	3	2	1	arabinan
	2-	1	–	–	–	–	–	–	arabinan
	3-	1	1	–	–	–	–	–	arabinan
	5-	19	18	23	20	12	5	5	arabinan
	2,5-	1	2	3	3	2	–	–	arabinan
Ara*p*	terminal	2	2	1	–	–	–	–	other[b]
Xyl*p*	terminal	4	2	2	–	–	–	–	XG
	2-	2	2	–	–	–	–	–	XG
	4-	2	1	1	–	–	–	–	other[b]
Gal*p*	terminal	4	4	2	–	–	–	–	XG
	2-	2	1	1	–	–	–	–	XG
	4-	3	2	2	2	1	–	–	other[b]
	6-	3	3	3	2	1	–	–	other[b]
Glc*p*	terminal	1	1	3	7	10	10	11	callose
	3-	–	3	10	30	47	60	62	callose
	4-	24	22	22	13	7	5	5	XG, cellulose
	3,6-	–	–	1	5	7	9	9	callose
	4,6-	11	9	6	3	1	–	–	XG
	2,3-	–	–	–	1	2	3	2	callose
GalA*p*[c]	4-	13	11	10	6	6	6	5	galacturonan/RG
GalA*p* Me-ester[c]	4-	1	2	2	2	ND	ND	ND	galacturonan/RG
GlcA*p*[c]	4-	2	2	1	1	1	–	–	other[b]
Neutral carbohydrate (mg g^{-1} pollen)		14	14	23	45	182	1040	1900	
Total wall carbohydrate (mg g^{-1} pollen)		17	17	27	50	197	1110	2010	

[a]Data are the mean of three experiments
[b]Other could include arabinogalactan-protein, Type I galactan and xylan
[c]GalA*p*, GalA*p* Me-ester and GlcA*p* linkages are deduced after reduction of carboxyl groups
Ara*f* = arabinofuranose; Ara*p* = arabinopyranose; Fuc*p* = fucopyranose; Gal*p* = galactopyranose; GalA*p* = galacturonopyranose; Glc*p* = glucopyranose; GlcA*p* = glucuronopyranose; ND, not determined; Rha*p* = rhamnopyranose; RG, rhamnogalacturonan; XG, xyloglucan; Xyl*p* = xylopyranose

sitions plus total carbohydrate levels. These analyses thus gave quantitative information on the rate of deposition of the individual wall polysaccharides detected, namely callose, cellulose, arabinan, galacturonan and xyloglucan (Fig. 4).

No callose was detected in pollen grains (< 0.2 mg g^{-1} pollen), but the linkage type indicative of the callose backbone, 3-linked glucopyranose (Glc*p*), was first detected at low levels at 1 h, with 3-linked Glc*p* and the residue types derived from callose branches, 3,6-linked Glc*p* and terminal Glc*p*, increasing thereafter (Table 2, Fig. 4A). After 4 h of growth, callose already constituted 40% (w/w) of the total wall carbohydrate, at 22.4 mg g^{-1} pollen, and then increased over 70-fold to reach 1700 mg g^{-1} pollen by 32 h (Fig. 4A). Cellulose, characterised by 4-linked Glc*p*, was one of the major components in pollen grains (3.8 mg g^{-1} pollen, 22% of grain wall carbohydrate) and over 32 h of tube growth increased steadily to 100 mg g^{-1} pollen (Fig. 4B). Arabinan, characterised by 5-linked and other arabinofuranose (Ara*f*) residues, was another major grain component (3.4 mg g^{-1} pollen, 20% of grain wall carbohydrate), and also increased over 32 h of tube growth to 100 mg g^{-1} pollen (Fig. 4B). Galacturonan, mostly unesterified and characterised by 4-linked galacturonopyranose (GalA*p*), was present at 2.7 mg g^{-1} pollen (16% of grain wall carbohydrate) but increased with tube growth to reach 108 mg g^{-1} pollen after 32 h (Fig. 4B). Xyloglucan, with a 4-linked and 4,6-linked Glc*p* backbone plus branches including xylose residues, was present at 4.5 mg g^{-1} pollen (27% of grain wall carbohydrate), but the level of xyloglucan did not change or decreased slightly during tube growth, and was not detectable after 8 h growth against the increasing background of other polysaccharides (Fig. 4B, inset).

Between 8 and 32 h, callose was deposited into pollen-tube walls at a net rate of 65 mg h^{-1} g^{-1}, accompanied by cellulose at 3.6 mg h^{-1} g^{-1}, arabinan at 3.0 mg h^{-1} g^{-1} and galacturonan at 4.0 mg h^{-1} g^{-1}. Similar rates, and no detectable deposition of xyloglucan, were also observed in pollen tubes of *N. tabacum* (data not shown). The wall deposited by growing *N. alata* pollen tubes therefore contains 86% callose, 5% cellulose, 4% arabinan and 5% galacturonan.

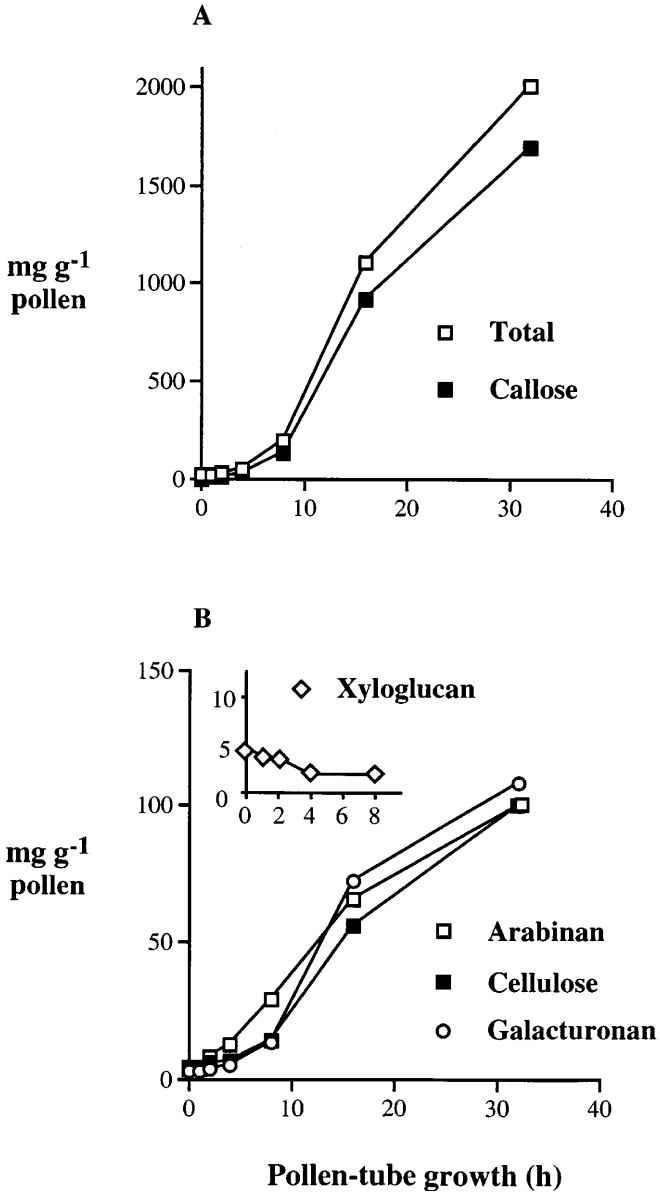


Fig. 4A,B. Deposition of polysaccharides into pollen-tube walls during growth. Neutral carbohydrate was determined according to Dubois et al (1956), then adjusted to account for the proportion of uronic acids determined by linkage analysis, and total wall carbohydrate then expressed as mg g^{-1} pollen. Amounts of individual polysaccharides were calculated from linkage analysis plus total carbohydrate levels (see Table 2), and expressed as mg g^{-1} pollen. Note different scales on vertical axes

This rate of callose deposition calculated from the linkage analysis of walls from cultured tubes was compared to the CalS activity assayed in isolated membranes from the same batch of pollen (Table 3). Callose synthase was assayed at 1 mM UDP-Glc with and without treatment with trypsin, V_{max} values were then calculated using the K_m values measured for untreated (2.5 mM) and trypsin-treated (1.3 mM) CalS (Schlüpmann et al. 1993; Li et al. 1997), and the activity as nmol Glc residues min^{-1} mg^{-1} protein was recalculated as mg callose h^{-1} g^{-1} pollen to allow comparison with polysaccharide deposition rates. Thus, the 20-h CalS activity of 4610 nmol min^{-1} g^{-1} pollen at 1 mM UDP-Glc was equivalent to a V_{max} of 174 mg h^{-1} g^{-1} pollen. Over a time-course from 2 to 30 h, a period when the rate of callose deposition increased over 10-fold then reduced slightly, the calculated CalS V_{max} as assayed without treatment with trypsin was 1.1 to 3.8-fold greater than that required to account for the observed rate of callose deposition (Table 3). The CalS activity in vivo may be a little lower than these calculated V_{max} values because intracellular UDP-Glc concentrations in the growing region are only 3.5 mM and may not always saturate the enzyme (Schlüpmann et al. 1994). Indeed, the larger discrepancy at longer culture times suggests that more of the CalS enzyme is not saturated with substrate UDP-Glc in older portions of the tube. The calculated V_{max} as assayed after treatment with trypsin was 4.1 to 7.8-fold greater than required to account for the observed rate of callose deposition (Table 3). The CalS assayable in vitro without trypsin treatment is thus always sufficient to account for the rate of callose deposition in vivo.

Separation of active and inactive CalS. Membranes isolated from pollen tubes cultured for different times were separated by centrifugation on linear 30–50% (w/w) sucrose gradients. In all cases, maximal CalS activity was present in heavier membranes (fractions 8–11, density approximately 1.18 g ml^{-1}; Fig. 5), representing the plasma membrane (Turner et al. 1998). Treatment of these fractions with trypsin increased their CalS activity 2 to 4-fold, with higher levels of activation in membranes from younger tubes.

However, treatment with trypsin also revealed an appreciable amount of CalS activity in lighter membranes (typically fractions 3–7, with densities from 1.13 g ml^{-1} to 1.17 g ml^{-1}), representing intracellular membranes (Fig. 5). Pollen-tube membranes containing an endoplasmic-reticulum marker equilibrate in fractions 2–4 on these gradients, at approximately 1.11 g ml^{-1}, whereas membranes containing a Golgi marker equilibrate in fractions 4–6, at approximately 1.15 g ml^{-1} (Turner et al. 1998). These lighter membranes were therefore probably Golgi-derived, and their low level of CalS assayed in the absence of trypsin probably represents contamination by plasma membrane. These intracellular membranes thus effectively contain only zymogen forms of CalS.

The relative proportions of the various forms of CalS were calculated from the V_{max} activity values of pooled fractions (Fig. 6). While the total CalS activity increased during pollen-tube growth (Fig. 1), the proportion of CalS present as an inactive intracellular zymogen decreased from 46% to 20%, and the proportion present as zymogen in the plasma membrane also decreased from 28% to 19%. At the same time, the proportion present as active plasma-membrane enzyme increased from 20% to 49% (Fig. 6). Recently germinated tubes thus contained relatively more of the inactive form of

Table 3. Rate of callose deposition during pollen-tube growth

Pollen-tube growth (h)	Callose deposition (mg h^{-1} g^{-1} pollen)		
	From carbohydrate analysis[a]	From CalS assay[b]	
		No trypsin	+Trypsin
grain	–	<0.3	<0.3
1	1.83	<0.3	<0.3
2	4.24	4.66	23.9
4	11.9	26.1	69.0
8	44.5	62.5	183
16	63.5	133	294
20	45.2	174	351
30	39.8	123	242

[a]Calculated from the slope of a plot of callose deposition against time (Fig. 4A)
[b]CalS V_{max} values recalculated in mg $h^{-1}g^{-1}$ pollen are the means of three separate experiments

CalS, both in intracellular membranes and the plasma membrane, which explains the higher degree of CalS activation by trypsin in unfractionated membranes from younger tubes (Fig. 1).

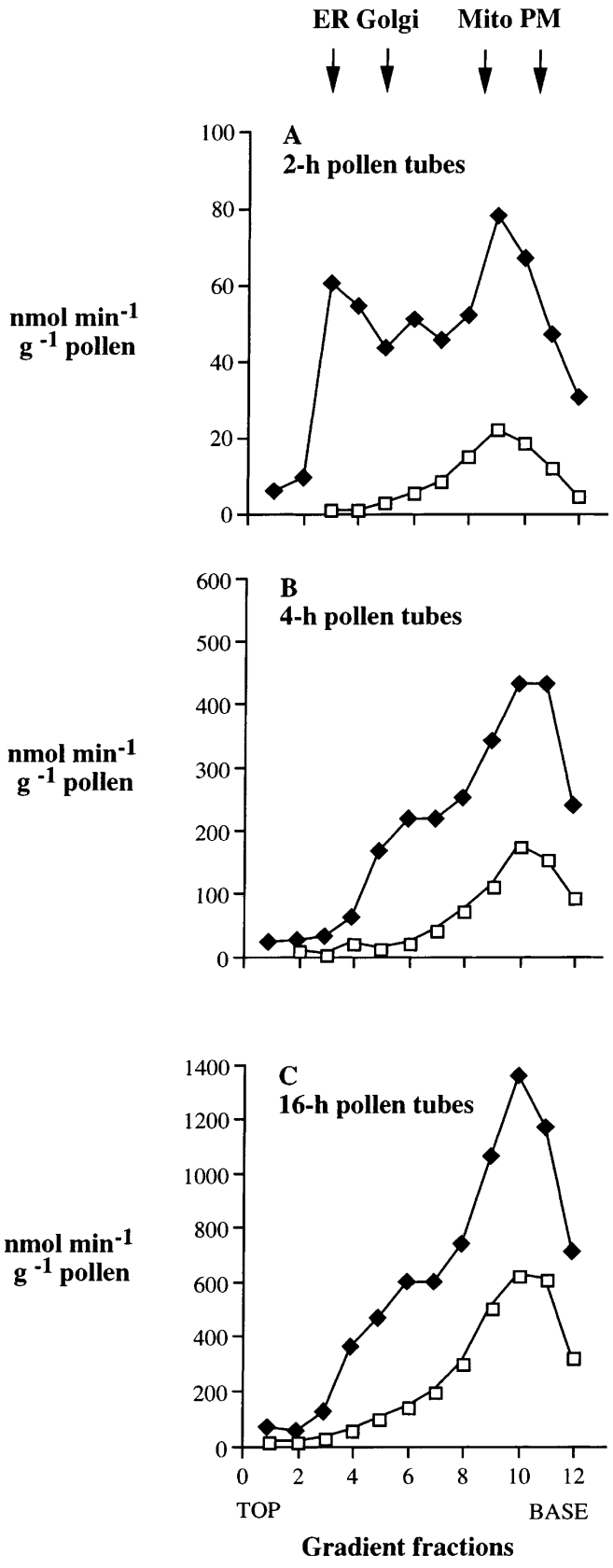
Discussion

Most pollen grains of *N. alata* germinate between 1 and 2.5 h after hydration (Read et al. 1993). There is no CalS activity in membranes isolated from ungerminated grains, while membranes isolated from growing pollen tubes exhibit a high activity. This CalS activity can be quantitatively related to the rate of callose deposition in intact tubes over the time-course of tube growth. Here we present evidence to support our model that changes in CalS activity over time are linked to transport and activation of inactive (zymogen) forms of the enzyme.

The initiation of callose deposition during germination and tube growth was investigated by both immunocytochemistry and chemical analysis. Staining intact pollen tubes with a (1,3)-β-D-glucan-specific monoclonal antibody required that tubes be first treated with wall-degrading enzymes, consistent with the localisation of callose to the inner wall layer (Meikle et al. 1991; Ferguson et al. 1998). Callose first appeared at the plasma membrane around a pollen grain aperture immediately after emergence of the tube, which can vary from 1–2 h after hydration in different grains, and was subsequently deposited behind the tip in increasing amounts to create an inner wall layer. The 3-linked glucose residue characteristic of the callose backbone was also first detected at 1 h after hydration. These data imply that an active CalS enzyme is first present in the plasma membrane at germination. In agreement with this, CalS activity was detected in isolated membrane preparations only 2 h after hydration.

Previous measurements have shown that the neutral components of the *N. alata* tube wall deposited between 4 and 8 h after germination comprised mostly callose, with some cellulose and arabinan (Schlüpmann et al. 1994). Here we obtained an accurate measure of the rate of callose deposition over a 32-h period, using carboxyl-reduction linkage analysis to ensure that uronic acid residues were included in the compositional profile. Callose was not detectable in pollen grains, but constituted over 80% (w/w) of total wall carbohydrate after 16 h of pollen-tube growth. Subtraction of polysaccharides present in the grain allowed calculation of the true composition of the wall deposited during pollen-tube growth as 86% (w/w) callose, 5% cellulose, 4% arabinan and 5% polygalacturonan. No xyloglucan was deposited in this wall, or in the wall of *N. tabacum* pollen tubes (data not shown), which is in agreement with the hypothesis that tube growth is controlled by de-esterification of methyl-esterified polygalacturonan in the tube tip (Derksen et al. 1995), rather than by regulation of cellulose-xyloglucan interactions as is the case in other cells. The rate of callose deposition was calculated from the linkage analysis data plus total wall carbohydrate levels, and reached 65 mg h^{-1} g^{-1} pollen. These calculations are based on the assumption that there is no substantial turnover, metabolism or loss of polysaccharides from the pollen-tube wall, and thus this value represents a minimum rate of synthesis.

The CalS activity of isolated membranes also increased substantially during tube growth. The increasing activity during the early stages of tube growth is probably related to an increasing area of active plasma membrane, followed by the onset of callose plug deposition 6–8 h after germination (Read et al. 1993). The assayable activity of CalS, measured without trypsin treatment, was sufficient to account for the measured rate of callose deposition into pollen-tube walls at all stages from 2 to 30 h, even though the deposition rate increases over 10-fold during this period. Schlüpmann et al. (1994) reached the same conclusion but used data derived from only the 4–8 h time period. These calculations assume that CalS is saturated with UDP-Glc in vivo, and would not be invalidated if there were an efficient flow of glucose units directly to CalS from sucrose synthase. The level of CalS activity in membranes from older pollen tubes is noticeably greater than that required to account for the rate of callose deposition at those time points, even without trypsin treatment of the membranes. This excess activity may



represent CalS molecules in regions of the plasma membrane behind the callose plug closest to the tip, and not saturated with substrate UDP-Glc in vivo: these

Fig. 5A–C. Fractionation of pollen-tube membranes by sucrose density gradient centrifugation. *Arrows* show fractions containing peak marker-enzyme activity in a representative gradient (*ER*, cytochrome-*c* reductase for endoplasmic reticulum; *Golgi*, Triton-dependent IDPase for Golgi; *Mito*, cytochrome-*c* oxidase for mitochondria) as well as (*PM*) the deduced location of plasma membrane (Turner et al. 1998). Callose synthase activity was assayed without (–□–) or with (–◆–) trypsin treatment, and is expressed as nmol min^{-1} g^{-1} pollen. **A** 2-h pollen tubes; **B** 4-h pollen tubes; **C** 16-h pollen tubes. Note different scales on vertical axes

regions often retain an apparently intact plasma membrane but little cytoplasm (Ferguson et al. 1998, 1999), and therefore presumably have low concentrations of UDP-Glc, resulting in no new deposition of callose in vivo (Fig. 7) but high CalS activity in vitro. These older regions are not required for continued growth at the tube tip (Jauh and Lord 1995).

In general, however, callose deposition is correlated with the assayable CalS activity of isolated membranes throughout grain germination and tube growth, providing quantitative evidence for developmental regulation of pollen-tube CalS. The form of the CalS enzyme that is active in isolated membranes thus represents the population of CalS molecules that are active in intact pollen tubes. This validates our standard assay conditions, but leads to the question of the origin and function of the extra activity revealed after treatment of isolated membranes with trypsin or Chaps.

There are several pieces of evidence that suggest that the extra CalS activity of isolated membranes assayed

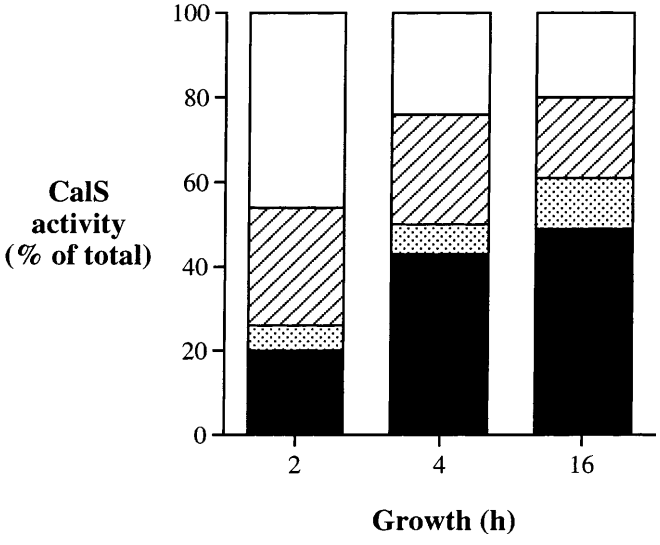


Fig. 6. Changes in proportions of CalS activity in various fractions during pollen-tube growth. Active enzyme in plasma-membrane fractions (*black column areas*), active enzyme in intracellular membrane fractions (*dotted*), zymogen in plasma-membrane (*diagonal lines*), zymogen in intracellular membranes (*clear*). Active enzyme is the CalS activity assayable without trypsin treatment, total activity is the activity assayable after trypsin treatment, and the amount of zymogen is calculated as the difference between these values. Activities are V_{max} values calculated as for Table 3. Plasma membrane was collected as fractions 8–11, and intracellular membranes as fractions 3–7, from a sucrose density gradient (see Fig. 5)

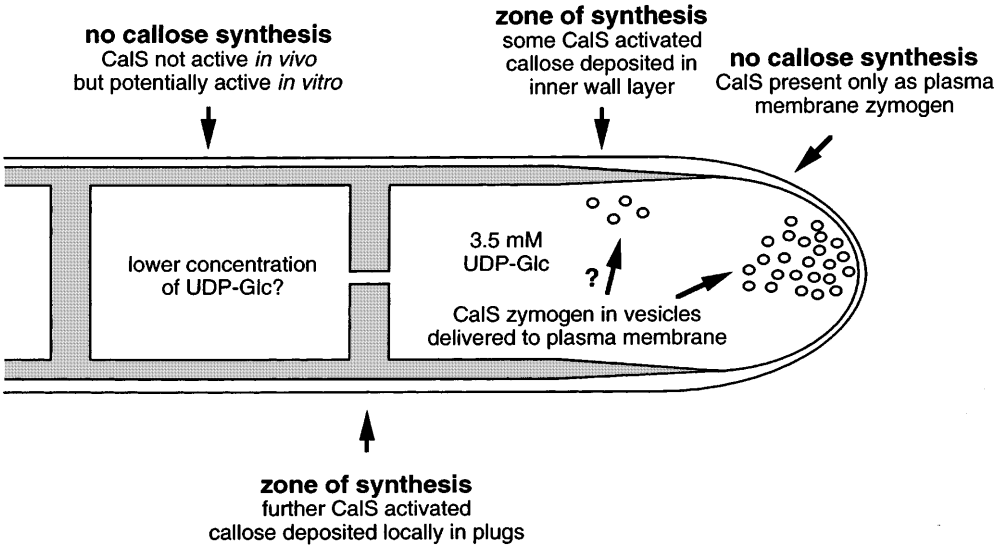


Fig. 7. Diagrammatic representation of possible mechanisms of CalS activation in growing pollen tubes. We postulate that the CalS zymogen is present in membrane vesicles that are delivered to the plasma membrane at the growing tip. Some of the zymogen molecules are then activated behind the growing tip and initiate callose deposition. Other CalS molecules remain as zymogen in the plasma membrane, and a further proportion is then activated locally when callose plugs are formed. Low concentrations of UDP-Glc in older regions of the tube could restrict callose deposition there. The population of vesicles containing CalS zymogen may not be the same as those delivering pectins to the growing tip, and it is not clear (?) whether membrane vesicles containing CalS are also delivered directly to the flanks of the tube. *Shading* represents callose

with trypsin or Chaps is due to activation of inactive zymogen forms of pollen-tube CalS. First, activation of the enzyme by treatment with trypsin occurs in the presence of permeabilising concentrations of digitonin (Schlüpmann et al. 1993; Li et al. 1997), indicating that trypsin activation does not involve formation of holes in the membranes (Wu and Wasserman 1993). This activation occurs in membranes prepared from pollinated styles as well as in membranes prepared from cultured pollen tubes, and the similar degree of activation of CalS by trypsin or Chaps in membranes prepared from these sources suggests that the CalS zymogen is present at similar levels in both cases. Second, treatment of membranes with the active-site-directed irreversible inhibitor UDP-pyridoxal shows that CalS activation with Chaps (Li et al. 1997) or with trypsin (data not shown) involves revealing new enzyme molecules that were completely inactive in the absence of the activating treatment. Lastly, the identification of intracellular membranes that have very little CalS activity without treatment with trypsin or Chaps, but considerable activity after such treatment, indicates that a population of inactive CalS zymogen molecules can be separated from the plasma-membrane CalS activity.

Analysis over a time-course of pollen-tube growth shows that activation of CalS involves progressive transformation of intracellular zymogen into active plasma-membrane enzyme, presumably with the plasma-membrane zymogen as an intermediate. This process would occur in addition to continued synthesis of new enzyme molecules. Solubilisation of gradient-purified plasma membranes increases CalS activity in a manner that resembles activation of CalS by trypsin or Chaps (Turner et al. 1998), consistent with zymogen activation in vivo occurring in the plasma membrane. Taken together, these data link the increasing rate of callose deposition during pollen-tube growth to the conversion of an intracellular zymogen form of CalS into an active plasma-membrane form of the enzyme (Fig. 7).

The mechanism by which the CalS zymogen is activated in vivo has not yet been clarified. Chitin, a 1,4-β-linked homopolymer of N-acetylglucosamine, is important for morphogenetic events in yeast, and deposition of chitin at specific intracellular sites and at various stages of the yeast cell cycle is regulated by the activation of numerous different chitin synthase zymogens (Bulawa 1993; Choi et al. 1994). Chitin synthase 3 is involved in formation of the ring of chitin found at the base of an emerging bud, chitin synthase 2 is responsible for construction of the chitin disc that completes the primary septum at cytokinesis, while chitin synthase 1 is proposed to have a repair function and act as a balancing factor in chitin synthesis in the wall (Cabib et al. 1989; Shaw et al. 1991; Choi et al. 1994). These chitin synthases are synthesised as inactive zymogens that can be activated by partial proteolysis, with other yeast genes presumably encoding the developmentally related proteases (Sburlati and Cabib 1986; Choi et al. 1994). The plasma-membrane H^+-ATPase of higher plants can be activated in vitro by proteolytic removal of its C-terminal autoinhibitory domain (Palmgren et al. 1990a), or by incubation with lysophospholipids (Palmgren et al. 1990b), and can be activated by treatment of intact cells with fusicoccin. Lysophospholipids and fusicoccin are suggested to act by displacing the C-terminal autoinhibitory domain that is removed in vitro by protease (Johansson et al. 1993; Rasi-Caldognov et al. 1993; Lanfermeijer and Prins 1994), with activation in vivo suggested to involve formation of a complex of 14-3-3 proteins and the phosphorylated C-

terminal region (Baunsgaard et al. 1998; Olsson et al. 1998). The activation of the CalS zymogen by Chaps and other detergents (Li et al. 1997) suggests that the in-vivo regulation of this enzyme may also involve conformational changes rather than proteolysis.

Callose synthase activity could thus be regulated at several levels: new transcription, translation of stored mRNA, or activation of inactive enzyme molecules. Growing pollen tubes are transcriptionally active (Mascarenhas 1993), and new gene expression is presumably required to account for the large increase in CalS activity during tube growth. However, pollen grains also contain stored mRNA (Frankis and Mascarenhas 1980; Stinson et al. 1987; Schrauwen et al. 1990), and the rapid initiation of protein synthesis from this stored mRNA on grain hydration (Hoekstra and Bruinsma 1979) could be responsible for the initial appearance of CalS activity and the start of callose deposition around the germinal aperture. Synthesis of CalS as an inactive zymogen, followed by its subsequent regulated activation, gives a third mechanism for increasing the rate of appearance of CalS activity in the plasma membrane, plus allowing control of the intracellular location of callose deposition within the growing tube (Fig. 7).

The intracellular membranes that contain the CalS zymogen could be equivalent to the phosphotungstic acid-stained cytoplasmic vesicles observed under the pollen-tube plasma membrane (Turner et al. 1998). The fusion of these vesicles with the plasma membrane would create the pool of plasma-membrane zymogen, which would then be activated to control callose deposition; it is, however, not clear whether all the CalS is made initially as an inactive zymogen or whether some can be synthesised directly in an active form. This activation of CalS within the plasma membrane could occur behind the growing tube tip as well as during the initiation of callose plug formation in older portions of the tube (Fig. 7). Ectopic activation of the CalS zymogen at the tube tip appears to occur in lily pollen tubes treated with a Yariv phenylglycoside reagent that prevents normal membrane flow following vesicle fusion (Roy et al. 1998), and presumably also occurs in aberrant tubes formed under certain cultural conditions or in self-incompatibility reactions (Read et al. 1993; Lush and Clarke 1997).

There is thus good evidence for the existence and controlled activation of a zymogen form of pollen-tube CalS. Regulation of the Ca^{2+}-dependent CalS responsible for cell plate formation may also involve localised activation of a zymogen (Samuels et al. 1995), and it is possible that a similar mechanism occurs during the rapid and localised activation of CalS that occurs with wounding in most cell types (Girard and MacLachlan 1987; Kauss 1996). Understanding the molecular mechanisms regulating CalS activation in the plasma membrane awaits cloning of CalS genes and identification of the relevant polypeptides.

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