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Wall biosynthesis and wall polysaccharide composition in pollen tubes of *Malus domestica* growing at low rate after monensin treatment

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SUMMARY

Tube growth rate, wall biosynthesis and wall polysaccharide composition were studied in pollen of *Malus domestica* Borkh. germinating in the presence of monensin, a monovalent ionophore which is known to cause swelling of Golgi apparatus cisternae in plant and animal cells. 125 nM monensin decreased tube growth rate, inhibiting tube elongation by 50% within 2 h germination. Wall biosynthesis was reduced in the treated tubes: label incorporation decreased to 61% (acid-soluble fraction) and 54% (alkali-soluble fraction) with respect to controls. Protein content of the wall ionic fraction was also decreased in the presence of the ionophore. Moreover, the sugar composition of acid or alkali-soluble wall fractions was shown to be partially affected by monensin; this could result from a decrease in number and reactivity of the large vesicles derived from dictyosomes, which was previously observed in treated apple pollen tubes.

Key-words: Malus domestica, sodium ionophore, tip growth, tube wall polysaccharides.

INTRODUCTION

Pollen-tube growth, resulting from a complex network of various mechanisms, is characterized by high environmental sensitivity and flexibility. The use of inhibitors is therefore interesting, and the ionophores seem to be the most suitable: in fact, polar movements of ions and electrical currents play important roles both in early polarization of the grain and during tube elongation (Sievers & Schnepf 1981; Weisenseel & Jaffe 1976; Weisenseel *et al.* 1975). It is known that apical growth of pollen tubes is associated with the presence of large numbers of vesicles in the tip: the role of vesicles as the source of wall polysaccharides is well understood (Heslop-Harrison 1987). However, observations on liliaceous and other pollens seem to indicate that some materials to be transferred into the growing tube are not vesiculate, and not all arise from dictyosomes (Heslop-Harrison 1987). The use of a ionophore (monensin) able to interfere with Golgi apparatus and intracellular transport of secretory vesicles (Tartakoff 1983a,b) therefore, may be useful to improve our understanding of the actual role of dictyosome activity in the mechanism of pollen tube growth.

The carboxylic ionophore monensin forms lipid-soluble complexes that transport ions across biological membranes, with a tenfold affinity preference for Na^+ over K^+; morphological and physiological consequences, e.g. swelling of the Golgi cisternae and perturbation of vesicular traffic, have been described in animal as well as plant cells (Heupke & Robinson 1985; Melroy & Jones 1986; see Tartakoff 1983a for review). The swelling response of Golgi apparatus cisternae to monensin is even used as an assay because it is reduced by cell injury (Mollenhauer *et al.* 1992). Growth effects of monensin were studied in *Candida albicans* (Pancaldi *et al.* 1985), *Pellia epiphylla* (Morré *et al.* 1986) and *Zea mays* (Sticher & Jones 1988).

After studying dose-response and dictyosome alteration in monensin-treated apple pollen (Speranza & Calzoni 1992; Ciampolini *et al.* 1993), the present paper was aimed to investigate the actual relevance of the Golgi activity to normal tube elongation in apple. Therefore, data on impact of monensin on either tube wall polysaccharide synthesis and composition or rate of tube growth, are presented.

MATERIAL AND METHODS

Plant material

Pollen was collected from *Malus domesticas* Borkh. cv. 'Starkrimson' grown in experimental plots near Ravenna, Italy. Handling and storage were as described by Calzoni *et al.* (1979).

In vitro *pollen germination*

Pollen was rehydrated at 30°C and 100% relative humidity for 30 min. Germination was performed in glass Petri dishes by suspending the pollen in medium containing 0·2 M sucrose, 3·2 mM boric acid and 1·3 mM calcium nitrate at pH 6·6, obtained with McIlvaine citrate-phosphate buffer 100-fold diluted. Apart from controls, 125 nM monensin taken from a stock solution in ethanol (0·1 μg ml^{-1}) was added to the basal medium; ethanol concentration was 0·086% in both treated pollen and controls. The cultures were incubated at 30°C in the dark. Tube length was measured on at least 80 randomly chosen pollen tubes, at 15-min intervals up to 120 min. A *t*-statistic was used to compare means and standard deviations.

Radioactivity incorporation

Either control or 125 nM monensin-treated pollen were fed with 37·0 kBq mg^{-1} pollen of D-[6-^{3}H] galactose (Amersham, UK, specific activity 944 GBq $mmol^{-1}$) at the concentration of 39 nM, and with unlabelled galactose up to 39 μM. The ethanol from the labelled galactose solution was previously removed by gentle nitrogen streaming. Ethanol concentration was adjusted to 0·086% (v/v) in the medium of both treated pollen and controls. After 120-min incubation the pollen tubes were separated from the medium by Millipore filtration (5 μm in pore diameter size) and washed with fresh medium containing 390 μM unlabelled galactose. Radioactivity incorporation was measured by a Beckman LS 1800 liquid scintillation counter provided with automatic quench correction.

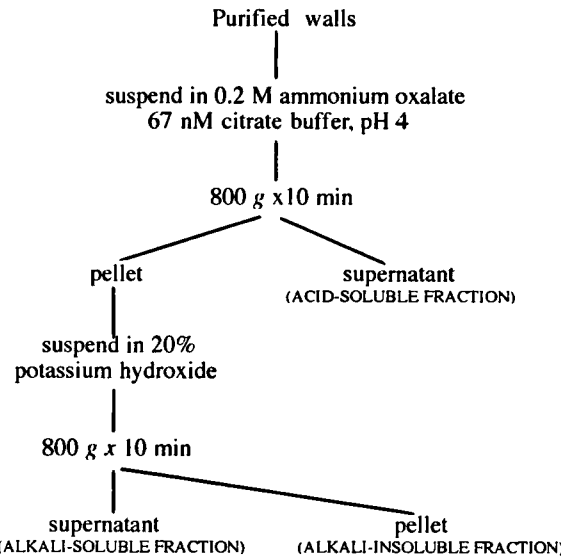

Fig. 1. Fractionation protocol of purified walls of pollen tubes of *Malus domestica* after 120 min incubation in the presence or absence of 125 nM monensin.

Pollen homogenization and wall protein extraction

Either control or 125 nM monensin-treated pollen tubes grown for 120 min were separately suspended in cold 50 mM phosphate buffer, pH 6·0, and ruptured by passing through a French Press. The homogenate was centrifuged at 1000 *g* and 0°C for 15 min to separate cell wall from the cytoplasm. The pelleted cell walls were prepared according to Li *et al.* (1983), by exhaustive washings with phosphate buffer, water, 1% Triton QS 100, and water in sequence.

The wall ionically bound proteins were then extracted twice in 1 M sodium chloride for 3 h at 4°C under magnetic stirring; the salt extracts were separated by centrifugation, pooled and ultrafiltered with an Amycon system using YM 100 membranes, cut-off 10 000.

Protein concentration was determined according to Lowry *et al.* (1951).

Cell wall preparation and fractionation

In order to eliminate tightly bound proteins, the walls were suspended in protease solution (from papaya, Sigma: 5 mg ml^{-1} in 50 mM phosphate buffer, pH 7·0) in the presence of 0·2% (w/v) sodium azide, and incubated at 30°C for 24 h. In order to eliminate starch, the wall material was treated with α-amylase according to Nevins *et al.* (1977). The pelleted walls were then washed with 90% ethanol, acetone and petroleum spirit, respectively (Carrington & Firn 1985).

Fractionation protocol is summarized in Fig. 1. The purified walls were suspended in 0·2 M ammonium oxalate, 67 mM citrate buffer, pH 4, into screw-topped glass test tubes (Carrington & Firn 1985; Mankarios *et al.* 1980). The stoppered tubes were placed in a boiling water bath on a magnetic stirrer/hot plate, with 30-min continuous stirring. After centrifuging at 800 *g* for 10 min at 4°C, the supernatant was removed and the pellet rinsed with the same buffer. The combined supernatants represented the acid-soluble extract.

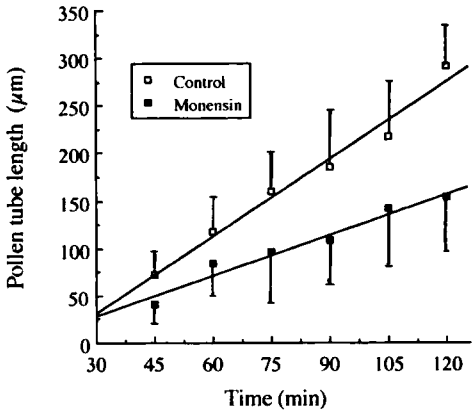

Fig. 2. Mean length of pollen tubes of *Malus domestica* during 120 min incubation in the presence or absence of 125 nM monensin. Bars represent standard deviations. Correlation coefficient was 0·99 (control) and 0·98 (monensin-treated).

The wall residues were suspended in 20% (w/v) potassium hydroxide and stirred overnight on a magnetic stirrer in a nitrogen-filled dessicator (Carrington & Firn 1985). After centrifuging at 800 *g* for 10 min at 4°C, the supernatant was removed and the pellet rinsed with the same solution. The combined supernatants represented the alkali-soluble extract. The residual pellet represented the alkali-insoluble extract.

Wall polysaccharide composition

Aliquots of the acid or alkali-soluble fractions were ethanol-precipitated and the pellets hydrolized by 1·5 N methanolic HCl for 4 h at 100°C. After removing the HCl and drying in nitrogen streaming, silane-derivatives of monosaccharides were prepared by reacting for 1 h at 60°C with a mixture composed of pyridine, trimethylchlorosilane, bis-trimethylsililacetamide, and trichlorofluorethane. The silane-derivatives were analysed by a Varian 3600 gas chromatography on a 0·53 mm inner diameter-30 m long SPB-1 capillar column coated with methylsilicone, using a flame ionizing detector with an automatic electronic integrator (Varian Star). The initial column temperature was 120°C with a ΔT of 4°C min^{-1} reaching a final temperature of 200°C (injector temperature: 240°C; detector temperature: 300°C). Helium was the carrier gas at the flow rate of 2·2 ml min^{-1} and splittless mode was chosen. The attenuation was 16, range 11. Sorbitol purchased from Merck was used as internal standard.

RESULTS

3.1. *Inhibition of pollen tube growth*

The time-course of pollen-tube growth in the presence of 125 nM monensin and in controls is presented in Fig. 2. Although in both cases the tubes lengthened linearly with time, the regression coefficient of the curve for the treated pollen dropped from 2·7 (controls) to 1·4. Tube elongation was significantly decreased ($P<0·01$) starting just after 45 min incubation.

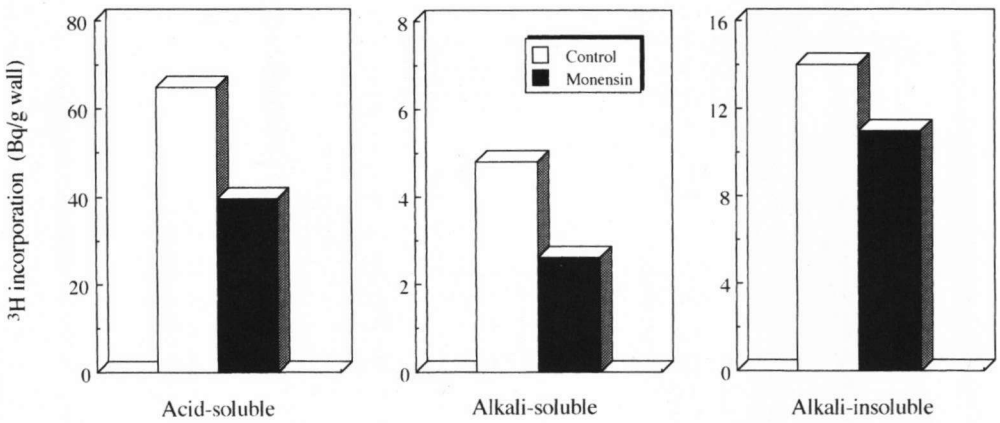

Fig. 3. [6-^{3}H]-galactose incorporation into different fractions of pollen tube walls of *Malus domestica*, after 120 min incubation in the presence or absence of 125 nM monensin.

It may be noted that satisfactory tube growth rate can be achieved by our system *in vitro* (see control of Fig. 2), i.e. about 70% on average of the rate measured *in vivo* into apple style (Heslop-Harrison 1987).

3.2. *^{3}H-gal incorporation*

Pollen grown either in the presence or absence of 125 nM monensin was fed with ^{3}H-galactose. In order to follow the fate of the label in wall polysaccharides *de novo* synthesized, different fractions of walls were separated after 120-min germination. The protocol followed to fractionate the cell walls (Fig. 1) according to Carrington & Firn's method (1985), was substantially similar to that of Nakamura & Suzuki (1981) for pollen of five species. The fractions described of apple pollen wall might be identified as mainly pectic (acid-soluble), or mainly hemicellulosic (alkali-soluble), the residual (alkali-insoluble) containing cellulose.

The values for radioactivity incorporated into the wall fractions were much lower in the presence of monensin compared with the control, especially for the acid- and alkali-soluble fractions (Fig. 3).

Moreover, since galactose units are exclusively added in the Golgi apparatus during oligosaccharide processing to the complex form, ^{3}H-gal incorporation was also studied in weakly bound glycoproteins of the tube wall. The radioactivity increased, whereas the whole protein titre was markedly lower in the treated pollen than in the controls (Table 1).

3.3. *Sugar composition of wall polysaccharides*

The sugar composition of hydrolysed wall polysaccharides of acid- or alkali-soluble fractions of treated apple pollen tubes and controls is shown in Tables 2 and 3. Within each sample, sugar amounts are expressed as relative percentage of the whole sugar content. In both fractions, either in the treated pollen and controls, the main wall sugar was glucose. Arabinose, galacturonic acid and rhamnose decreased in the acid-soluble fraction of tubes grown in the presence of monensin. Rhamnose decreased also in the

Table 1. Titre and radioactivity incorporation of ionically bound proteins of *Malus domestica* pollen tube wall, after 120-min growth in the presence or absence of 125 nM monensin

	Protein ($\mu g\ g^{-1}$ wall)	^3H-incorporation ($Bq\ g^{-1}$ wall)
Control	508 ± 68	204 ± 22
Monensin	370 ± 44	275 ± 8

Table 2. Percentage sugar composition of the acid-soluble fraction of the *Malus domestica* pollen tube wall after 120-min growth in the presence or absence of 125 nM monensin

	Amount (% of total sugars)	
	Control	Monensin
Glucose	60·20	73·45
Arabinose	17·60	11·00
Galacturonic acid	11·10	7·26
Galactose	4·47	3·03
Rhamnose	2·05	1·24
Xylose	0·96	0·65
Others	3·22	2·97

Table 3. Percentage sugar composition of the alkali-soluble fraction of the *Malus domestica* pollen tube wall after 120-min growth in the presence or absence of 125 nM monensin

	Amount (% of total sugars)	
	Control	Monensin
Glucose	70·30	65·30
Arabinose	9·39	8·21
Galactose	5·39	5·01
Xylose	4·52	5·40
Fucose	1·71	3·07
Rhamnose	1·17	0·86
Galacturonic acid	1·02	4·00
Others	6·50	8·15

alkali-soluble fraction of the same treated tubes; by contrast, mainly fucose and galacturonic acid increased in percentage. No other significant differences appeared with respect to controls.

In all cases, unidentified minor peaks were found with gas chromatography.

It should be noted that sugars from the grain wall also contribute to the fractions analysed.

DISCUSSION

Early on and during the germination period, 125 nM monensin strongly inhibited apple pollen tube elongation rate; however, growth was not completely impaired, nor was the linear mode of tube lengthening affected (Fig. 2). The extent of tube growth inhibition after 120 min incubation was 50%.

Cell-wall biosynthesis was markedly affected by monensin: radioactivity incorporation into the acid- or alkali-soluble fractions failed to 60% and 54% of the control group, respectively. Moreover, a certain decrease in ^{3}H-galactose incorporation was also shown in the alkali-insoluble fraction. This might signify that normal deposition of the tube wall cellulosic layer could be, to some extent, related to the presence of pectic or hemicellulosic components; in this respect, tube wall assembling appears as a finely regulated mechanism. It should be noted that Golgi activity is also needed to send enzymatic complexes involved in cellulose biosynthesis at the plasmalemma of the growing apex.

A marked reduction in the protein content in salt-soluble fraction (Table 1), resulting from the presence of 125 nM monensin, could be related to the ionophore ability to disturb intracellular vesicle traffic (Tartakoff 1983a,b). On the contrary, neither decrease in protein titre nor alteration of electrophoretic patterns were induced in apple pollen tubes by lower monensin concentrations until 50 nM (Speranza & Calzoni 1992). Interestingly, a certain increase in radioactivity is shown in the wall glycoproteins of the 125 nM treated tubes. This seems to indicate some *qualitative* alteration of wall components, namely that a different oligosaccharide processing related to galactose (whose addition is peculiar to the Golgi) occurred in the presence of monensin.

Analysis of sugar composition of apple pollen showed that glucose and arabinose are the major neutral sugars after acid hydrolysis of the tube wall. The heterogeneous character of tube wall polysaccharide is reported by Nakamura & Suzuki (1981) and Clarke *et al.* (1985); Heslop-Harrison (1987) emphasizes the fact that, in application to pollen tubes, terms such as cellulosic, callosic, and pectic must be taken as describing broad classes of compounds of variable composition.

The alteration in the sugar composition of pollen tube walls shown by our data could be related to the behaviour of two different vesicle classes observed in apple pollen tubes after 120 min incubation in the presence of 125 nM monensin (Ciampolini *et al.* 1993). In fact, a small vesicle class (0·04–0·06 μm) remained unchanged in number and reactivity to Thiéry test; by contrast, larger vesicles (0·1–0·2 μm) decreased in number, became hardly recognizable, and did not react, or only weakly, after the polysaccharide test. Thereafter, it may be hypothesized that, in the presence of monensin, packaging and sorting of some of the wall components could have been affected; or the biosynthetic ability itself of the larger vesicles could have been altered so that different products arrived at the tip.

The present findings clearly indicate that monensin, greatly reducing apple pollen-tube growth rate, induces complex changes in tube wall synthesis and composition. Monensin inhibition seems to be not simply quantitative: a different processing of dictyosome products, such as wall glycoproteins or polysaccharides, appears to have been induced by the ionophore in apple pollen tubes. Such a monensin effect (if due to

disorganization at the dictyosome level) should then strongly testify to the relevance of Golgi apparatus activity to the intimate mechanism of apple pollen-tube growth. Anyway, a general effect of monensin on the whole tube cytoplasm (on its turgor pressure or metabolism) should not be excluded; in fact, because of its lipophilic nature, the ionophore can presumably insert into all cellular membranes (Tartakoff 1983b).

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