The Plant Cell, Vol. 1, 501–510, May 1989 © 1989 American Society of Plant Physiologists

Inhibition of in Vitro Pollen Tube Growth by Isolated S-Glycoproteins of *Nicotiana alata*

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Pollen from three S-genotypes of *Nicotiana alata* was grown in vitro in the presence of S-glycoproteins isolated from styles of the same three genotypes. Pollen germination was not affected by the presence of the S-glycoproteins, but pollen tube growth of all genotypes was inhibited. S_2 pollen was preferentially inhibited by the S_2-glycoprotein and S_3 pollen by the S_3-glycoprotein. The S_6-glycoprotein preferentially inhibited growth of both S_2 and S_6 pollen over S_3 pollen. Heat treatment dramatically increased the inhibitory activity of the S-glycoproteins as inhibitors both of pollen germination and tube growth; after heat treatment, S-allele specificity of pollen tube inhibition was not detected.

INTRODUCTION

The gametophytic self-incompatibility system of *Nicotiana alata* and the isolation of the style glycoproteins which are the likely products of the *S*-gene have been described in two preceding papers (Anderson et al., 1989; Jahnen et al., 1989). If the *S*-allele-associated glycoproteins are indeed *S*-gene products, and if they act directly in vivo on incompatible pollen to inhibit tube growth, it may be possible to demonstrate the inhibitory effect of isolated style glycoproteins on pollen tube growth in vitro. Past attempts (e.g., Harris, Weinhandl, and Clarke, 1989 and references therein) to demonstrate specific inhibition have been hampered by the difficulties of designing suitable in vitro assays, as well as the problems of isolating the *S*-glycoproteins in an active form and in sufficient quantities for testing.

We report here the results of experiments on in vitro growth of several genotypes of *N. alata* pollen in an improved incubation medium and in the presence of *S*-allele-associated glycoproteins isolated by cation exchange chromatography (Jahnen et al., 1989).

RESULTS

Pollen Tube Growth in Vivo

In a compatible pollination, for example $S_2S_2 \times S_3S_3$, pollen germinated and pollen tubes grew through the styles such that many reached the ovary within 72 hr. The styles were 50 mm to 60 mm long, giving an average growth rate of 0.7 mm hr^{-1} for this period. In incompatible pollinations, such as $S_3S_3 \times S_3S_3$, pollen germinated but the tubes had lower growth rates within the style, typically 0.1 mm hr^{-1} for the same period.

Conditions for in Vitro Culture of Pollen

The growth rate of tubes from pollen of genotypes S_2S_2, S_3S_3, and S_6S_6 was greater in the polyethylene glycol (PEG) 4000 medium (Medium A) which contained 2% sucrose, than in the medium described by Harris, Weinhandl, and Clarke (1989), which is based on 10% sucrose with no addition of PEG (Figure 1). The rate of pollen tube growth in the PEG 4000 medium averaged 0.08 mm hr^{-1} over the first 12 hr of incubation; that is, about the same rate as incompatible tubes grown in vivo, but about one-tenth that of compatible tubes in vivo. The germination rate of pollen of all *S*-genotypes was greater than 80% in both media.

Adjusting the osmolarity of the 2% sucrose medium (Medium F) to that of the PEG 4000 medium with either PEG 400 (Medium B) or by increasing the concentration of sucrose (Medium C) did not mimic the effect of PEG 4000, in that the length of pollen tubes after 12-hr incubation was less than that in the PEG 4000 medium (Figure 2). The same was true of adjusting the viscosity of the 2% sucrose medium (Medium F) to that of the PEG 4000 medium with either PEG 20,000 (Medium D) or gum arabic (Medium E); that is, the pollen tubes were shorter than they were in the PEG 4000 medium (Figure 2). Pollen tubes grew longer in 2% sucrose (Medium F) than they did in 9% sucrose (Medium C) (means for the three pollen genotypes were 285 μm cf. 232 μm in 2% and 9% sucrose, respectively, $P<0.001$). PEG of molecular weights 400, 4000, and 20,000 (15% weight/volume) enhanced growth, with PEG 4000 being the most effective. (Means for the

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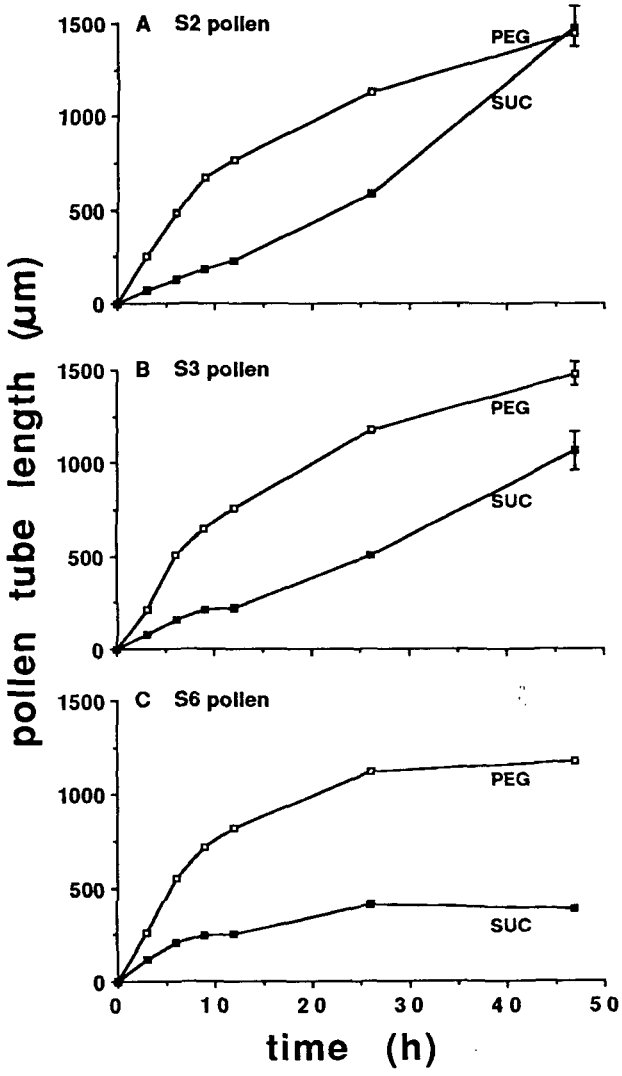

Figure 1. Growth Curves of Pollen Tubes.

Pollen was cultured in either PEG 4000 medium (open symbols) or 10% sucrose medium containing antibiotics (closed symbols). Each point represents the mean length of 100 pollen tubes; bars on the points show the mean ± SE where this was large enough to be depicted.

three genotypes were 344 μm, 717 μm, and 594 μm in PEG 400, 4000, and 20,000, respectively; the least significant difference of the means at $P<0.001$ was 37 μm.)

Pollen tubes growing in the PEG 4000 medium differed morphologically from those grown in medium containing sucrose as the main component. Tubes growing in the PEG 4000 medium were less twisted, fewer had swollen tips, and less callose was deposited along the length of the pollen tube as judged by the intensity of staining (Figure 3). The generative cell in tubes cultured in the PEG 4000 medium divided normally within 12 hr (Figure 3).

Pollen tube growth in the PEG 4000 medium increased with increasing concentrations of pollen up to 500 pollen grains/well (10^4 ml^{-1}) for the S_2 and S_3 pollen and up to 1000 grains/well for S_6 pollen (Figure 4).

Effect of Isolated *S*-Glycoproteins and Other Glycoproteins on in Vitro Pollen Tube Growth

Pollen tube growth was routinely assayed under conditions that produced the most rapid pollen tube growth; that is, PEG 4000 medium with a pollen concentration of about 1000 grains/well (20,000/ml). Pollen germination was high (>80%) in all treatments.

In the PEG 4000 medium and in the same medium with added BSA or ovalbumin, the distributions of pollen tube lengths were statistically normal (Figures 5A to 5C). In the presence of bromelain, the tube lengths were scattered more widely (e.g., Figure 5D). The distributions of lengths in the presence of the *S*-glycoproteins were normal in this experiment (e.g., Figure 5E); however, in some experiments, skewed or more scattered distributions were obtained.

The presence of BSA or ovalbumin (1 mg ml^{-1}) in the culture medium produced differences in the average pollen tube length for S_2 pollen of less than 5% (Figures 5A to 5C) with no statistically significant differences in the variability of growth as judged by the standard deviations of the means. A similar result was obtained with S_3 and S_6

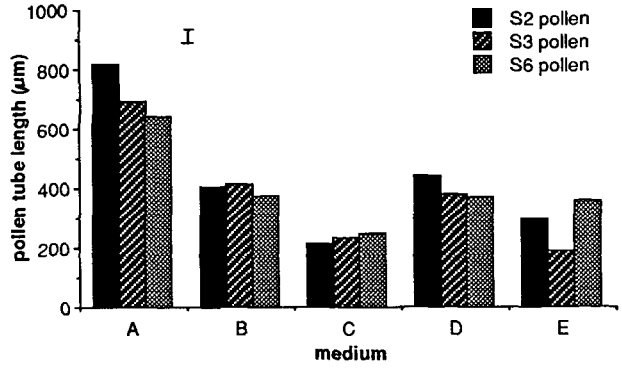

Figure 2. Pollen Tube Length after 12-hr Incubation in Media of Osmolarity (B,C) or Viscosity (D,E) Equivalent to That of the PEG 4000 Medium (A).

For (B), the osmolarity was adjusted with PEG 400 and for (C) with sucrose. For (D) the viscosity was adjusted with PEG 20,000 and for (E) with gum arabic. Values are the means of two replicates each of 50 pollen tubes. The bar indicates the least significant difference between values ($P<0.05$) based on analysis of variance.

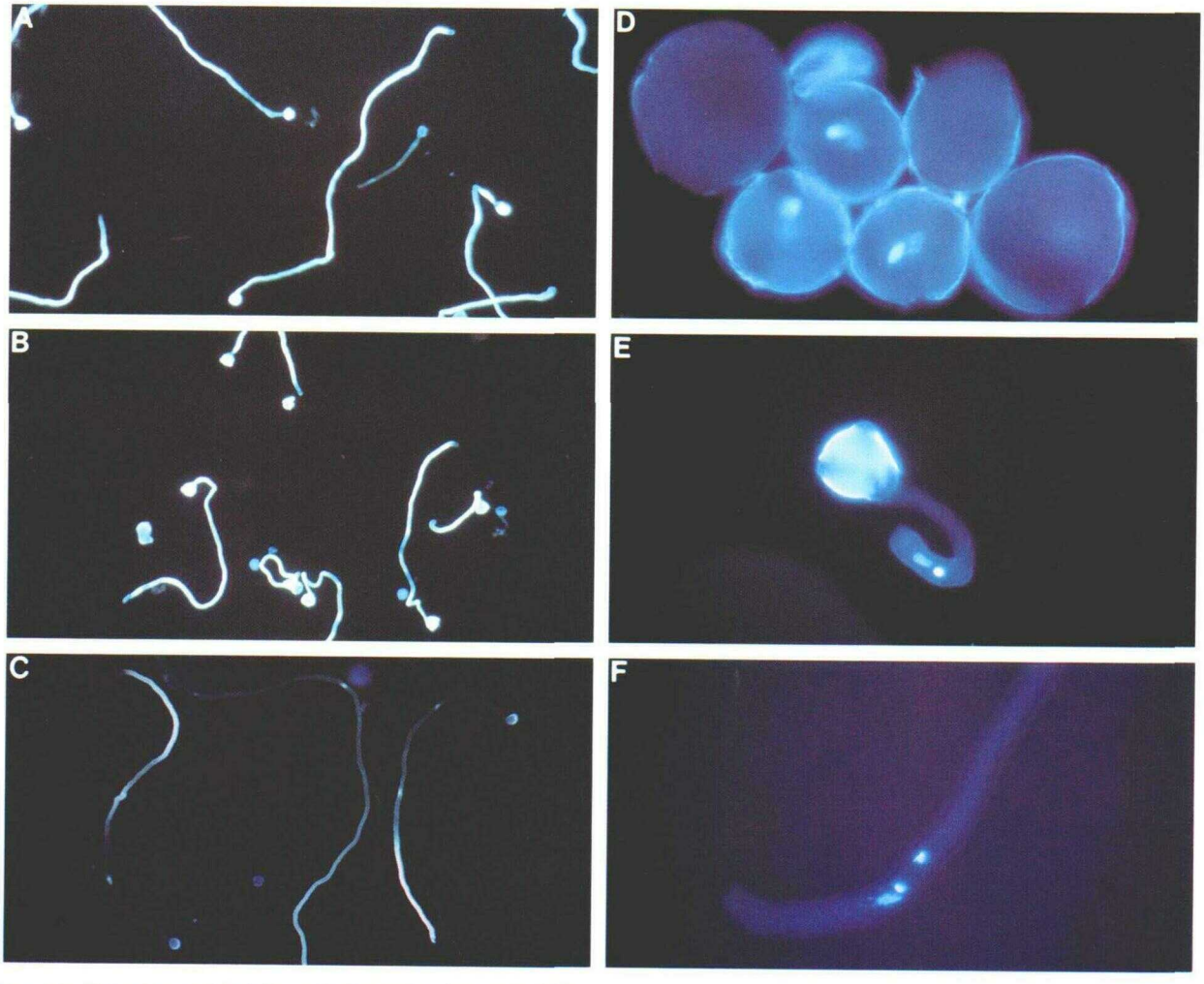

Figure 3. Histochemical Staining of Pollen Tubes Grown in Vitro.

(A) Pollen was grown for 12 hr in medium containing macronutrients, boron, and MES buffer with the addition of 2% sucrose.
(B) Pollen was grown for 12 hr in medium containing macronutrients, boron, and MES buffer with the addition of 9% sucrose.
(C) Pollen was grown for 12 hr in medium containing macronutrients, boron, and MES buffer with the addition of 15% PEG 4000.
(D) Pollen was grown for 0.5 hr in medium containing macronutrients, boron, and MES buffer with the addition of 15% PEG 4000.
(E) Pollen was grown for 6 hr in medium containing macronutrients, boron, and MES buffer with the addition of 15% PEG 4000.
(F) Pollen was grown for 12 hr in medium containing macronutrients, boron, and MES buffer with the addition of 15% PEG 4000.
(A) to **(C)** The pollen was stained with aniline blue specific for callose.
(D) to **(F)** The pollen was stained with the DNA-specific fluorochrome, DAPI.

pollen (data not shown). The effect of bromelain on the average pollen tube length was of the same order, but there was a striking increase in the variability of pollen tube growth for all three genotypes. This increase in variability was evident in the distribution of pollen tube lengths about the mean (e.g., Figure 5D), but because the distributions were not statistically normal, the standard deviations of the means were not appropriate measures of variability. Instead, the effect of bromelain on the variability of growth was assessed in terms of the distribution of lengths in the quartile ranges 0 to 500 μm, 500 to 1000 μm, 1000 to 1500 μm, and 1500 to 2000 μm. Ninety-eight percent of the measurements of S_2 pollen tube lengths lay between 500 μm and 1500 μm when pollen was grown in the PEG 4000 medium, whereas only 70% of the lengths lay within that range when S_2 pollen was incubated in the presence of bromelain (Figures 5A and 5D).

In this experiment (Figure 5), the mean length of S_2 pollen tubes grown in the presence of isolated S_2-glycoprotein was reduced by more than 50% (Figure 5E), with

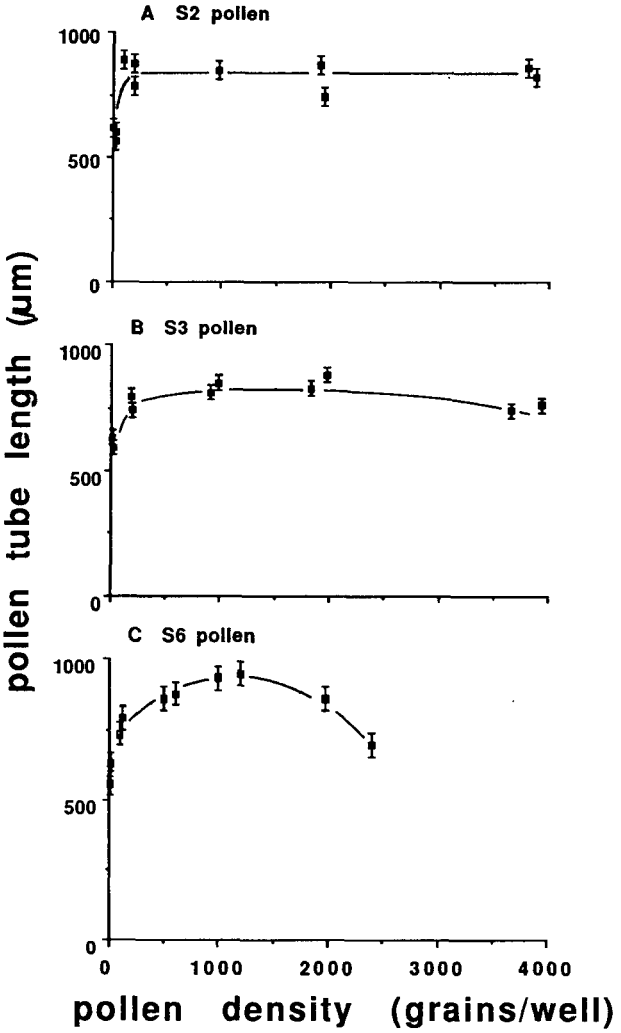

Figure 4. Effect of Pollen Concentration in the Growth Medium on Pollen Tube Growth after 12-hr Incubation.

Each point represents the mean length of 50 pollen tubes derived from a single well; bars on the points show the mean ± SE where this was large enough to be depicted.

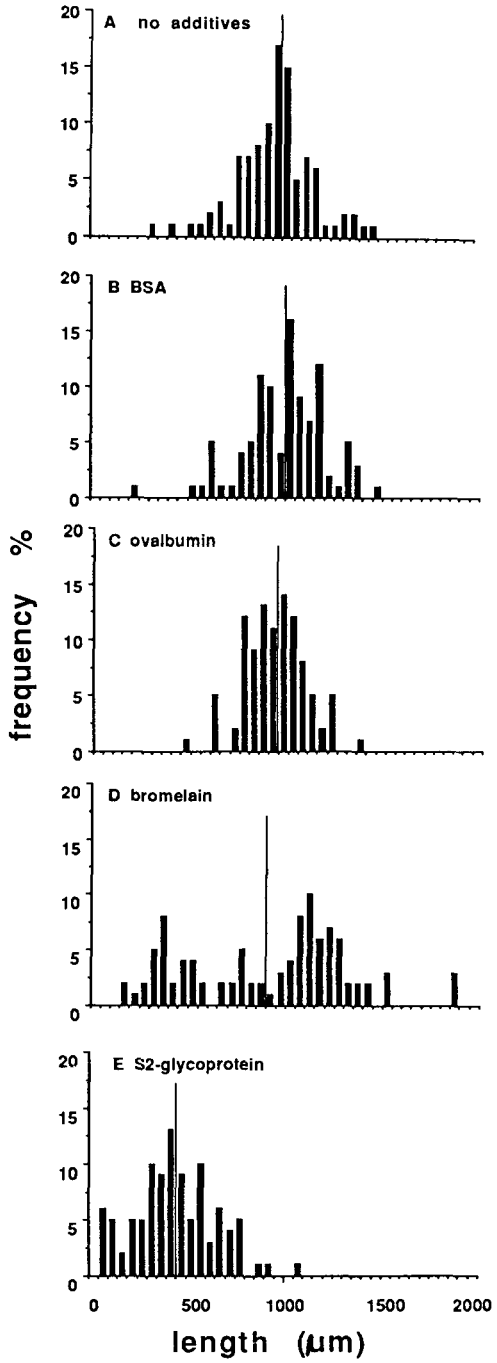

Figure 5. Frequency Distributions of S_2 Pollen Tube Lengths after Incubation for 12 hr in PEG 4000 Medium.

(A) The medium contained no additives.
(B) The medium contained BSA (1 mg ml^{-1}).
(C) The medium contained ovalbumin (1 mg ml^{-1}).
(D) The medium contained bromelain (1 mg ml^{-1}).
(E) The medium contained S_2-glycoprotein (0.6 mg ml^{-1}).
The data represent the combined measurements of two replicates each of 50 pollen tubes. Means are shown by fine lines.

no change in the variability of growth. In further experiments, the concentration dependence of the inhibition of pollen tube growth was examined. The extent of inhibition increased with increasing concentration of *S*-glycoprotein over the range 0 mg ml^{-1} to 0.2 mg ml^{-1}, but the point of apparent saturation of inhibition varied from 0.2 mg ml^{-1} to >0.4 mg ml^{-1} in different experiments.

Specificity of the Effects of the *S*-Glycoproteins

In experiments in which pollen of the S_2-, S_3-, or S_6-genotypes was grown in the presence of the isolated S_2-,

S_3-, or S_6-glycoproteins, growth was inhibited in most pollen/glycoprotein combinations. In addition to the general inhibition of pollen tube growth caused by all S-glycoproteins, there was a specific inhibition in the homologous pairs. The specificity of inhibition was most apparent after 12-hr incubation (Figure 6B). At this time, for example, S_2, S_3, and S_6 pollen tubes grown in the PEG 4000 medium grew 860 μm to 1020 μm long. Addition of S_2-glycoprotein (0.6 mg ml^{-1}) to the incubation medium resulted in reduced growth of the tubes of all three genotypes (mean lengths ranged from 390 μm to 600 μm; probability of differences between these lengths and those of controls occurring by chance <0.001), but the growth of the S_2 pollen tubes was inhibited most. Viewed another way, growth of S_3 and S_6 pollen was reduced in the presence of isolated S_2-glycoprotein to 68% of growth in controls, whereas growth of S_2 pollen was reduced to 38% of that in controls (Figure 6B). Addition of the S_3-glycoprotein (0.3 mg ml^{-1}) diminished the growth of all pollen genotypes to between 610 μm and 940 μm (probability of the differences between these means and those of controls occurring by chance <0.05); in this case the greatest inhibition was toward S_3 pollen, the growth of which was reduced to 67% of that in controls, while growth of S_2 and S_6 pollen was reduced to 91% and 83%, respectively, of that in controls. The S_6-glycoprotein reduced the growth of S_6 pollen to 77% of that in controls (from 860 μm to 660 μm, probability of the difference occurring by chance <0.001); it also inhibited S_2 pollen (P<0.05) but did not inhibit S_3 pollen.

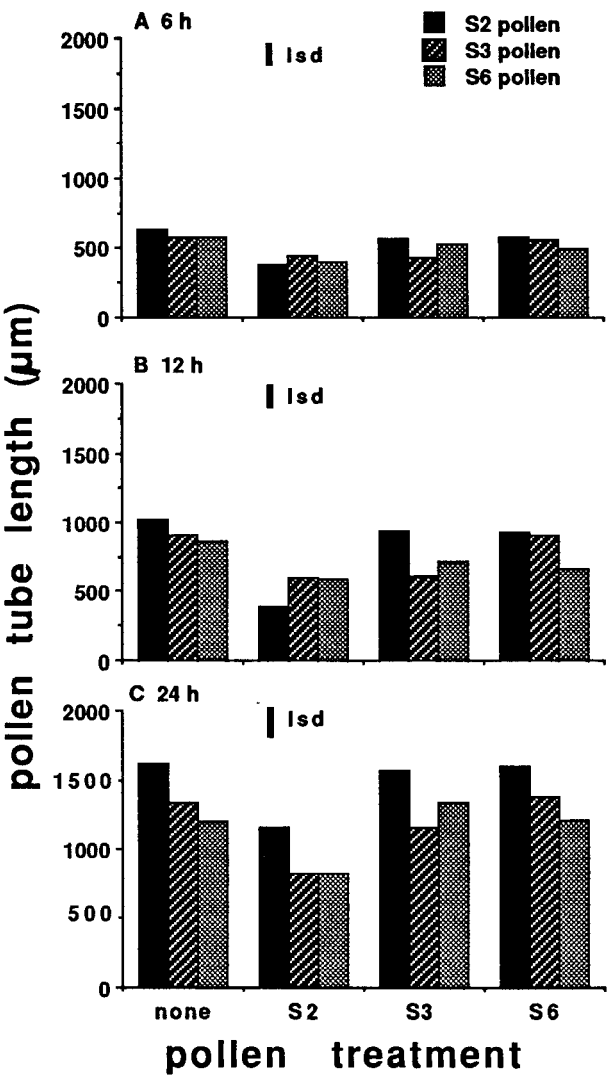

Figure 6. Pollen Tube Lengths after Incubation for 6 hr, 12 hr, or 24 hr in the PEG 4000 Medium and in the Presence of S_2- (0.6 mg ml^{-1}), S_3- (0.3 mg ml^{-1}), and S_6- (0.4 mg ml^{-1}) Glycoproteins.

Data represent the mean of two replicates each of 50 pollen tubes; bars show the least significant difference (P<0.05) based on analysis of variance.

The variability of pollen tube lengths was increased in all pollen/S-glycoprotein combinations. In the presence of the S_2-glycoprotein, the greatest increase in the standard deviation of lengths occurred in S_2 pollen (SD = 366 μm cf. 263 μm in controls, P<0.005 assuming the populations being compared are normally distributed). In the presence of the S_3-glycoprotein, the growth of S_3 pollen was most variable (SD = 308 μm cf. 175 μm in controls, P<0.001). In the medium containing the S_6-glycoprotein, the growth of S_2 pollen was most variable (SD = 388 μm cf. 263 μm in controls, P<0.001).

Thus, after 12-hr incubation, there were three effects of adding isolated style S-glycoproteins to the medium for in vitro pollen tube growth. First, there was a general inhibition of tube growth for pollen of heterologous S-genotypes; second, there was an increase in the variability of pollen tube length; and third, there was a predominant inhibition of the tube growth of homologous S-genotypes.

Pollen tube lengths after 6-hr incubation showed similar but less pronounced patterns of general inhibition as well as specific inhibition in homologous combinations of S-glycoprotein and pollen (Figure 6A). In this experiment the general inhibition by the S_2-glycoprotein, but not by the S_3- and S_6-glycoproteins, was maintained for 24 hr; in contrast, the specific inhibition of the S_3-glycoprotein but not of the S_2- or S_6-glycoproteins was maintained (Figure 6C). Pollen tube growth in the presence of S-glycoproteins remained more variable than in controls, but the differences were less marked, mainly because pollen tube lengths in the controls themselves had become considerably more variable during this extended period of incubation.

The effects of isolated S-glycoproteins were studied on three occasions using freshly prepared S-glycoproteins and pollen, and on a further three occasions using frozen or azide-stored S-glycoproteins with fresh or frozen pollen (see Methods). The general inhibitory effects of the S-glycoproteins on pollen tube growth and the associated

increase in the variability of growth were observed in each experiment. If specific inhibition is defined as an effect in which the percent inhibition relative to controls of a particular *S*-glycoprotein is greatest toward the homologous pollen genotype, then, taken as a whole, our results indicate specificity of action by the S_2- and S_3-glycoproteins (Table 1). The result was less clear for isolated S_6-glycoprotein; of a total of six experiments, three showed greatest inhibition of S_6 pollen and three showed greatest inhibition of S_2 pollen. Thus, there appeared to be a preferential inhibition by S_6-glycoprotein of S_2 and S_6 pollen over S_3 pollen.

Biological Activity of Heat-Treated Glycoproteins

Pollen germination in the presence of heat-treated *S*-glycoproteins was always reduced, sometimes to as little as 1% of total pollen numbers. The growth of the pollen tubes of those grains that did germinate was greatly inhibited, particularly in the presence of heat-treated S_2- and S_3-glycoproteins (Figure 7). The allele-specificity of inhibition was lost following heat treatment (Figure 7). The variability of growth, as measured by the standard deviation, decreased (data not shown).

The effect of BSA or ovalbumin on pollen tube growth was not altered by pretreating these proteins at 100°C (data not shown). Heat treatment of bromelain resulted in an increase in mean pollen tube length after 12 hr (from an average for the three pollen genotypes of 854 μm in the presence of bromelain that was not heat treated to 1016 μm, $P<0.001$). The extended distribution of pollen tube lengths found in the presence of bromelain (Figure 5D) did not occur in the presence of heat-treated bromelain. Using the quartile ranges defined above for bromelain, 95% of the measurements of pollen tube grown in the presence of heat-treated bromelain occurred between 500 μm and 1500 μm cf. 70% in the presence of bromelain that was not heat-treated and 98% in controls.

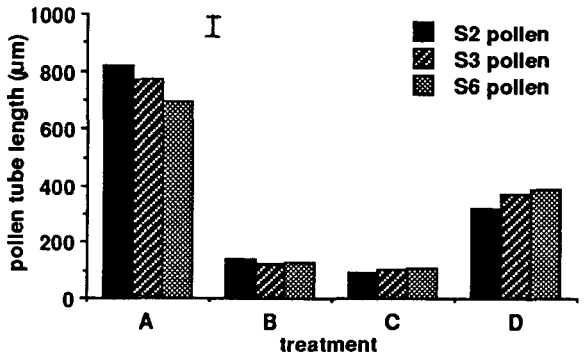

Figure 7. Pollen Tube Lengths after Incubation 12 hr in PEG 4000 Medium and in the Presence of Boiled S_2- (0.4 mg ml^{-1}), S_3- (0.5 mg ml^{-1}), and S_6- (0.5 mg ml^{-1}).

Data represent the mean of two replicates each of 50 pollen tubes; the bar shows the least significant difference ($P<0.05$) based on analysis of variance.

Table 1. Specificity of Action of the *S*-Glycoproteins toward Homologous and Heterologous Pollen

Pollen Genotype	Glycoprotein					
	S_2		S_3		S_6	
	Fresh	Stored	Fresh	Stored	Fresh	Stored
S_2	2	3	0	0	1	2
S_3	1	0	3	2	0	0
S_6	0	0	0	1	2	1

The table shows the number of experiments in which a particular combination of *S*-glycoprotein and pollen produced the greatest inhibition of pollen growth relative to controls. Two sets of experiments, each set made up of three experiments, were performed. In the first set, *S*-glycoproteins were used immediately after purification, and in the second, the *S*-glycoproteins were stored before use.

DISCUSSION

Attempts to reproduce the differential growth of compatible and incompatible pollen tubes that characterizes gametophytic incompatibility have resulted in both stimulation (Sharma and Shivanna, 1986; Harris, Weinhandl, and Clarke, 1989) and inhibition (Straub, 1947; Shivanna, Heslop-Harrison, and Heslop-Harrison, 1981; Williams et al., 1982) of growth. The growth of pollen tubes in vitro is usually considerably slower than that in vivo, and reflects in part the non-ideal nature of the incubation media. It is therefore not surprising that crude extracts stimulate pollen growth when added to some incubation media; nor is it surprising that crude extracts inhibit growth in certain media, as pollen tube growth is extremely sensitive to low concentrations of many compounds (Pfahler, 1981).

A critical test of the biological role of *S*-glycoproteins is that they should inhibit pollen growth with *S*-genotype specificity. Specific inhibition has been demonstrated using crude pistil extracts in some genotypes of *Petunia*, *Primula*, *Nicotiana*, and *Papaver* (Brewbaker and Majumder, 1961; Shivanna, Heslop-Harrison, and Heslop-Harrison, 1981; Sharma and Shivanna, 1986; Franklin-Tong, Franklin, and Franklin, 1988). Advances on work with crude extracts were the demonstrations that a purified *S*-glycoprotein preparation from *Brassica oleracea* specifically inhibited in vivo growth of pollen from the same genotype (Ferrari, Bruns, and Wallace, 1981), and that the purified S_2-glycoprotein from *N. alata* specifically inhibited in vitro growth of S_2 pollen (Harris, Weinhandl, and Clarke, 1989). Even

where apparent specific inhibition has been recorded, it is possible that it in fact reflects genetic variability of growth in different media. For example, in the present study, removal of PEG 4000 from the growth medium produced an effect that could be interpreted as "specific inhibition" of S_2 pollen tube growth, in that growth of S_2 pollen was inhibited more than the growth of S_3 or S_6 pollen (Figure 2).

In establishing in vitro bioassays, it is thus important to use purified preparations of style components and to reduce the chances of obtaining false-positive results by testing more than one allelic product. Purified preparations of the S_2- and S_3-glycoproteins specifically inhibited the growth of homologous pollen (Table 1). The isolated S_6-glycoprotein preferentially inhibited S_2 pollen on some occasions and S_6 pollen on others; it never preferentially inhibited S_3 pollen (Table 1). This inhibition by the S_6-glycoprotein may reflect homology in the mode of action of the S_2- and S_6-glycoproteins; although the S_2- and S_6-glycoproteins are not more closely related in amino acid sequence than S_2- and S_3- or S_3- and S_6-glycoproteins (Anderson et al., 1989), the possibility of homology in functional regions is not precluded.

Several explanations can be advanced for the less marked specificity of inhibition of pollen tubes in vitro by comparison with in vivo observations. First, there is the problem of preserving biological activity throughout purification and subsequent storage. The biological activity of our preparations was maintained during storage at −70°C and at 4°C in the presence of azide, but the less pure preparations of Shivanna, Heslop-Harrison, and Heslop-Harrison (1981) lost activity when stored. Second, the concentration of isolated S-glycoproteins is lower in the in vitro tests than occurs in vivo; in the work reported here, the concentration of the S-glycoproteins was in the order of 2% to 10% of in vivo concentrations, due to the difficulty of isolating sufficient material to use at higher concentrations. In other bioassays, the concentrations of S-glycoproteins were even lower (e.g., Harris, Weinhandl, and Clarke, 1989). Third, there are the growth characteristics of the pollen itself. Inhibition in vivo is not obvious until the pollen has reached a critical growth stage related to either pollen tube length (Straub, 1946; Herrero and Dickinson, 1981) or time since pollination (Brewbaker and Majumder, 1961). The timing of inhibition can be allowed for in bioassays, but it is difficult to reproduce in vivo pollen tube lengths. In *N. alata*, specific inhibition became apparent 4 hr to 6 hr after the start of growth in vivo or in vitro (Figure 6), but at this time in vitro pollen tubes were only about one-quarter of the length of in vivo pollen tubes.

We experimented with the growth medium used in earlier work (Harris, Weinhandl, and Clarke, 1989) to try to overcome any limitations to the sensitivity of the bioassay inherent in the relatively slow growth of pollen in vitro. By reducing the concentration of sucrose from 10% to 2% and adding 15% PEG 4000, we succeeded in obtaining a threefold increase in initial growth rates (Figure 1). This enhanced growth is consistent with reports that PEG is superior to sucrose as the major component of pollen incubation media (Hong-Qi and Croes, 1982; Subbaiah, 1983).

It is not clear how PEG interacts with pollen to produce more rapid tube growth. For pollen tube growth to occur, the growth medium should be nutritionally adequate and should also enable the pollen tube to maintain turgor at a level sufficient for cell wall extension but insufficient for rupture of the wall. PEG was included in the growth medium on the assumption that it would function as an osmoticum and hence regulate pollen tube turgor (Hong-Qi and Croes, 1982). However, results with media of differing osmotic potentials indicate that pollen tube turgor is not limiting growth over the range used (Figure 2). PEG also increased the viscosity of the growth medium; many style secretions are viscous, due at least in part to their content of arabinogalactan proteins (Gleeson and Clarke, 1979). Viscosity, however, did not appear to be an important factor in pollen tube growth, as growth was depressed in media with the same viscosity as the PEG 4000 medium, but in which PEG 4000 was replaced by PEG 20,000 or gum arabic (Figure 2). PEG 4000 stimulated pollen tube growth more than the higher and lower molecular weight PEGs used. It is possible that PEG 4000 affects growth by influencing the extensibility of the cell wall at the growing tip of the pollen tube, since the incidence of tip swelling and deposition of callose (Figure 3) were both reduced in the presence of PEG 4000. Experiments with *Gladiolus* pollen tubes showed that the porosity of the cell walls was approximately equal to the diameters of molecules of PEG with molecular weights between 1000 and 4000 (Hoggart and Clarke, 1982). If the same is true for *N. alata* pollen tubes, then PEG 4000 may enter the cell walls, whereas PEG 20,000 is probably excluded and PEG 400 probably passes through to the cell membrane.

To maximize pollen tube growth, we also optimized pollen concentration. The concentration required to achieve the highest growth rates of *N. alata* pollen was 10,000 to 20,000 grains ml^{-1}, similar to the 20,000 grains ml^{-1} needed for maximal growth of *Petunia* pollen (Brewbaker and Majumder, 1961). By analogy with *Petunia*, the stimulation of growth may be caused by a water-soluble compound(s) present in the pollen which supplement growth in nutritionally deficient media (Brewbaker and Majumder, 1961).

The mechanism of the inhibitory action of the S-glycoproteins is not known, but it is not a consequence of changes in the osmotic potential or viscosity of the growth medium. The increase in the variability of growth in the presence of the S-glycoproteins suggests that some pollen escapes inhibition. Escape is unlikely to be caused by uneven access to the pollen tubes, as the incubation times were long and the pollen was thoroughly dispersed in the medium and distributed in a single layer in wells of the culture plates, which were shaken periodically. Escape

does occur in vivo, in that a few tubes in incompatible pollinations grow considerably longer than the majority. The weakening of the *S*-glycoprotein inhibition during prolonged growth in vitro suggests either that the *S*-glycoproteins are degraded or that pollen tubes can eventually overcome the inhibition.

We were surprised to find that the *S*-glycoproteins became more potent, although non-specific, inhibitors of pollen tube growth after heat treatment. The action of the heat-treated *S*-glycoproteins was to inhibit dramatically both germination and pollen tube growth; in this respect, their behavior was like that of *S*-glycoproteins in sporophytic incompatibility systems. Heat treatment enhanced inhibition on all occasions, regardless of whether the *S*-glycoproteins were heat treated in water, MES buffer, or the PEG 4000 medium. We had anticipated that there would be a loss of function as is found for biologically active proteins in general, and for style extracts in particular (Brewbaker and Majumder, 1961; Franklin-Tong, Lawrence, and Franklin, 1988; Harris, Weinhandl, and Clarke, 1989). Neither ovalbumin nor BSA, either before or after heat treatment, affected pollen tube growth, and heat treatment of bromelain resulted in the expected loss of biological activity. At present, we have no explanation of the effect of heat treatment on the biological activity of the *S*-glycoproteins other than to suggest that it may induce conformational change and that it might be an important clue to the nature of the incompatibility reaction.

METHODS

Plant Material

Pollen and styles were obtained from plants (*Nicotiana alata* Link et Otto) of the self-incompatible genotypes S_2S_2, S_3S_3, and S_6S_6 from sources described in Anderson et al. (1989).

Isolation of Stylar *S*-Glycoproteins

S-Glycoproteins were isolated by cation exchange chromatography (Jahnen et al., 1989) from styles of the genotypes S_2S_2 (100 styles; yield 1 mg), S_3S_3 (200 styles; yield 0.5 mg), and S_6S_6 (100 styles; yield 1 mg). The *S*-glycoproteins had <5% contaminating material. This was assessed by rechromatography using FPLC and SDS-PAGE and establishing the ratio of the major peak to the total of the minor peaks. The *S*-glycoproteins were dialyzed overnight into 20 mM MES buffer, pH 6.0, and either used immediately or after storage at −70°C or storage at 4°C in the presence of 0.02% NaN_3 and subsequent dialysis into water before use.

In Vivo Pollen Tube Growth

S_2S_2, S_3S_3, and S_6S_6 styles were cross- and self-pollinated with pollen from the same genotypes. Flowers to be used as female parents were collected soon after opening. The pedicels were recut under distilled water and held in distilled water thereafter. The flowers were emasculated by removing the upper half of the corolla, to which the stamens were attached. Anthers from the genotypes to be used as pollen parents were placed in Eppendorf tubes and vortexed to remove the pollen. Stigmas were pollinated by dipping them into mounds of pollen on glass slides. The pollinated flowers were placed in the dark in a box lined with wet paper at 25°C. The flower pedicels were recut under water every 24 hr.

Measurements of pollen tube growth in vivo were based on the method of Williams and Knox (1982). Styles were fixed for 2 hr in 1:3 acetic acid:ethanol at intervals between 6 hr and 120 hr after pollination. The fixed styles were washed in distilled water and then boiled in 10% anhydrous Na_2SO_4 for 20 min to soften them. The styles were washed twice and then stained with the aniline blue fluorochrome (0.1%, British Drug House). The styles were cut in half to fit on slides and slit longitudinally, and the transmitting tissue was squashed out of the surrounding tissues with a coverslip. The length of pollen tubes was estimated using the microscope stage micrometer. Unpollinated styles were fixed as controls.

Media for in Vitro Pollen Tube Growth

All the media used were based on 20 mM MES buffer, pH 6.0, and contained the same nutrient salts (0.07% $Ca(NO_3)_2 \cdot 4H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% KNO_3, and 0.01% H_3BO_3). The standard medium also contained 2% sucrose and 15% PEG 4000 (referred to as PEG 4000 medium). The time course of pollen tube growth was investigated in the PEG 4000 medium and in a medium used by Harris, Weinhandl, and Clarke (1989) in which 10% sucrose, 0.1% casein hydrolysate (Behring Diagnostics), and the antibiotics rifampicin (5 $\mu g\ ml^{-1}$, Sigma) and nystatin (15 $\mu g\ ml^{-1}$, Sigma) were added to MES buffer and salts of the pH and concentrations shown above.

In addition, pollen growth was tested in media with the range of osmolarities and viscosities listed in Table 2. With the exception of the 9% sucrose medium, all media contained 2% sucrose. PEG of all molecular weights were from British Drug House. Gum arabic was from Sigma.

Pollen Incubation

Pollen was prepared from freshly harvested flowers as described above and used immediately or frozen in liquid nitrogen for up to 2 months before being used. Filter-sterilized (0.22 μm pore size) incubation media were pipetted (aliquots of between 100 μl and 300 μl depending upon the design of the experiment) into 1.5-ml Eppendorf tubes. Dry pollen was collected by pressing a 5-μl or 10-μl glass micropipette into a tube of pollen; the column of pollen (1 mm to 2 mm) in the bore of the pipette was blown into the incubation medium and mixed by vortexing. The mixture of pollen and medium was dispensed into 96-well culture plates (50 μl/well) after vortexing again to ensure that the pollen was suspended. There were two duplicate wells for each treatment. Where two plates were needed to accommodate an experiment, each treatment was represented once on each plate; the treatments were not randomized. The plates were incubated in the dark in a container lined with wet paper at 23°C to 25°C.

Table 2. Pollen Growth Testing Media

Medium	Constituents in Addition to Buffer, Salts, and 2% Sucrose	Osmolarity	Viscosity Relative to 2% Sucrose
		mosm kg^{-1}	
A	15% PEG 4000	327	4.7
B	7% PEG 400	320	1.3
C	9% (total) sucrose	325	1.2
D	6% PEG 20,000	110	4.6
E	9.3% gum arabic	123	4.2
F	No additives	83	1.0
G	15% PEG 400	715	1.8
H	15% PEG 20,000	212	21.0

Osmotic potential was measured with a vapor pressure osmometer (Wescor) using NaCl solutions as standards. Viscosity relative to that of the 2% sucrose medium (Medium F) was measured at 20°C with a falling head viscometer.

Media containing protein or glycoprotein were prepared by 1:1 dilutions of the protein or glycoprotein in water or 20 mM MES buffer with double-strength PEG 4000 medium or with PEG 4000 medium containing 20 mM MES but double-strength PEG, salts, and sucrose. In addition to the *S*-glycoproteins, BSA, ovalbumin (both from Sigma), and bromelain (Behring Diagnostics) were used in some experiments.

Pollen that had been stored at −70°C was used in experiments to determine the time course of pollen tube growth, the effects of pollen concentration on tube growth, and the effects of viscosity and osmotic potential of the medium on growth. Pollen stored at −70°C was also used in one experiment on growth in the presence of *S*-glycoproteins. In this experiment, the *S*-glycoprotein had also been stored at −70°C. In all other experiments, the pollen used was freshly collected.

Measurement of Tube Lengths of Pollen Grown in Vitro

Incubation of pollen was terminated by pipetting 50% glycerol in distilled water (50 μl) into each well and transferring the contents to glass slides which were then frozen (−20°C). Fifty randomly selected pollen tubes from each slide were measured using a Videoplan computerized image analysis system (Zeiss, Oberkochen, Federal Republic of Germany). Tube lengths were only measured in germinated pollen grains; germination was defined as having occurred when the length of the pollen tube was greater than or equal to the diameter of the grain.

The mean and standard deviation of each sample of 50 pollen tubes were calculated using Videoplan software; the means and standard deviations (two replicates per treatment) were then analyzed by analysis of variance (GENSTAT, Rothamsted, Great Britain). In some experiments, the data met the assumptions of analysis of variance better if values were first transformed to logarithms. These transformations, however, had little effect on the statistical significance of particular comparisons and no effect on the biological interpretation of the experiments. Consequently, we present untransformed data.

Histochemistry of Pollen

Pollen tubes were fixed in formaldehyde (2%) in phosphate buffer (0.1 M, pH 7.4) for 3 hr on ice. After rinsing with phosphate buffer (three changes, 3 min each), the pollen was stained with decolorized aniline blue fluorochrome (Stone et al., 1984) or with the DNA-specific stain, DAPI (Vergne, Delvallee, and Dumas, 1987).

ACKNOWLEDGMENTS

We thank Professor Peter Brockwell and Mr. Peter Franz for their advice on statistical analyses, Professor Bruce Knox for use of the Videoplan, and Mrs. Susan Mau for growing the plant material. This work was supported by a post-doctoral fellowship granted to W.J. by the Deutsche Forschungsgemeinschaft.

Received March 13, 1989.

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