Theor Appl Genet (1985) 71:263–267

Covalently bound wall proteins of pollen grains and pollen tubes grown in vitro and in styles after self- and cross-pollination in *Lilium longiflorum*

Li Yi-qin and T. H. Tsao

Department of Biology, Peking University, Beijing, China

Received January 15, 1985; Accepted January 22, 1985
Communicated by H. F. Linskens

Summary. A method was worked out using trifluoromethanesulfonic acid (TFMS) as a reagent to split the covalently bound proteins, which are NaCl insoluble, from pollen tube walls of *Lilium longiflorum*, leaving the peptide bonds essentially intact. After electrophoretic separation, comparisons were made among these proteins from pollen grains and pollen tubes grown in vitro and in styles after self- and cross-pollination. It was found that a) the patterns of covalently bound wall proteins were different between tubes grown in vitro and in vivo; b) fewer bands were found in covalently bound wall proteins than that in noncovalently bound proteins; c) the bands remained almost the same no matter whether the tubes had been cross pollinated or self pollinated, indicating that while the noncovalently bound proteins were involved in incompatibility as shown in the previous paper, the covalently bound proteins may only serve as a structural component, having little to do with incompatibility.

Key words: Covalently bound wall proteins – Pollen – Pollen tubes – Tube growth – Incompatibility – Lily – *Lilium longiforum*

Introduction

There are two kinds of wall-bound proteins (Li et al. 1983; Li and Linskens 1983a) namely, noncovalently bound (about 22–26%) and covalently bound. While the former is soluble in NaCl solution, the latter is NaCl insoluble. Clear differences have been observed between the noncovalently bound proteins of pollen tubes grown in vitro and in vivo. The patterns of the proteins after self- and cross-pollination were found to be affected by both incompatibility and by the substrate on which the pollen germinated and where the tubes grew-either in a stigmatic and stylar exudate or in an artificial medium.

A study of tightly bound wall proteins, which are assumed to be covalently bound to the polysaccharides of the walls, is confronted with great difficulties. So far no successful procedure has been reported for releasing, intact, the proteins from the cell walls. Proteins have been detached from the cell wall network by enzymes, and by drastic chemical means, e.g., alkalis, hydrazine (Lamport 1969, Lamport and Miller 1971, Heath and Northcote 1971, Bailey and Kauss 1974; Voigt 1984). Mort and Lamport (1977), however, employed the method with which glycoproteins were exposed to anhydrous hydrogen fluoride, a reagent which splits all the linkages of neutral and acidic sugars while leaving the peptide bonds intact. A similar procedure but with an anhydrous hydrogen fluoride-pyridine mixture instead of anhydrous hydrogen fluoride has been developed (Glassman and Todd 1978; Sairam and Schiller 1979; Coudron et al. 1980). None of these methods was satisfactory in our investigation of covalently bound pollen tube wall proteins. Recently, Edge and his coworkers (1981) introduced the use of TFMS for the deglycosylation of fetuin and several other glycoproteins. It was found that under suitable conditions the peptide backbone is left intact. The reagent is safer than anhydrous hydrogen fluoride and does not require special equipment.

We adopted the TFMS method for the present study of covalently bound wall proteins of lily pollen and pollen tubes. The patterns of the proteins were studied by electrophoresis and comparisons were made between in vitro and in vivo growing, self- and cross-polinated pollen tubes.

Material and methods

Plant material

Plants of *Lilium longiflorum*, cvs. 'Arai No. 5' (self-incompatible) and 'Mount Everest' (pseudo-self-incompatible) were

grown at 15–17 °C in the greenhouse with artificial light (photoperiod of 16 h). Flower buds of the two cultivars were detached from the plants one day before anthesis, placed in jars of water at room temperature, and the anthers were removed from those flowers to be cross-pollinated. Fresh pollen was collected on the day of anthesis, either directly pollinated onto corresponding stigmas or dried above P_2O_5 and stored for in vitro germination.

Incubation of pollen tubes in vitro and in vivo

Pollinations were grouped into 'Arai 5'×'Arai 5' (A×A), 'Arai 5'×'Mount Everest' (A×M), 'Mount Everest'×'Arai 5' (M×A), and 'Mount Everest'×'Mount Everest' (M×M). Twenty-four hours after anthesis, the flowers were pollinated with fresh pollen and the pistils were incubated at 25 °C for 72 h. Pollen tubes were collected from the pistils, immediately plunged into liquid nitrogen, and then stored at −80 °C (for details, see Li and Linskens 1983 a).

The pollen grains were germinated at 25 °C for 20 h in a medium composed of 0.29 mM calcium nitrate, 0.16 mM boric acid and 0.99 mM potassium nitrate at pH 5 (Dickinson 1968) in a mass culture vessel (Schrauwen and Linskens 1967). The concentration of pollen in the medium was 4 gl^{-1}. After incubation the culture was filtered through a sieve (pore width 0.6 mm) to remove ungerminated pollen grains and the germinated pollen was resuspended in cold 0.05 M phosphate buffer for the preparation of cell walls.

Preparation of cell walls

Pollen tubes, germinated pollen and pollen grains were ruptured, centrifuged and the walls were washed as described earlier (Li et al. 1983; Li and Linskens 1983 a). The pellet thus obtained was suspended in 1 M NaCl and stirred at 0 °C for 2 h. The extraction procedure was repeated once more. After centrifugation, the pellet was washed with distilled water five times at 0 °C and lyophilized.

Extraction of covalently bound proteins

The lyophilized samples were suspended in a mixture of anisole and TFMS (purchased from Merck-Schuchardt) (v/v = ½) pre-cooled in an ice-water bath. Nitrogen was bubbled through the suspension to replace oxygen and the reaction tube was sealed with a teflon lined screw cap. With continuously magnetic stirring the samples were digested at 0 °C or room temperature for varying lengths of time. The proteins were freed of reagents and low molecular weight sugars by the following procedure: the reaction mixture was diluted with two volumes of diethyl ether pre-cooled to −40 °C. An equal volume of ice-cold 50% (v/v) aqueous pyridine was added to the clear solution, the mixture was shaken thoroughly and then allowed to stay at 0 °C for 4 h. The ether phase was discarded. After repeating the ether extraction of the aqueous phase, this portion was dialyzed against 2 mM pyridine acetate buffer pH 5.5 at 0°–4 °C for two days (Edge et al. 1981).

Electrophoresis

Sodium dodecyl sulfate (SDS) gel electrophoresis and Coomassie blue and silver staining of the proteins have been described elsewhere (Li and Linksens 1983 a).

Results

Covalently bound wall proteins were extracted under various conditions with TFMS, and the proteins were separated by SDS gel electrophoresis. When the reaction was carried out at 0 °C, no substantial difference in the pattern of proteins was observed (Fig. 1) between the samples digested for 1 h and those for 3 h although when the digestion time was longer, darker protein bands were revealed and, at the same time, some low molecular weight bands appeared. Then the reaction time was prolonged to 24 h, the protein bands in the high molecular weight region either disappeared or lightened; Conversely, low molecular weight bands darkened or appeared. If the temperature was raised to about 20 °C (room temperature) only a few very light bands in the low molecular weight region were observed when the reaction time was shortened to only 1 h. It is obvious that prolonged reaction time and high temperature not only cleaved the linkage between the

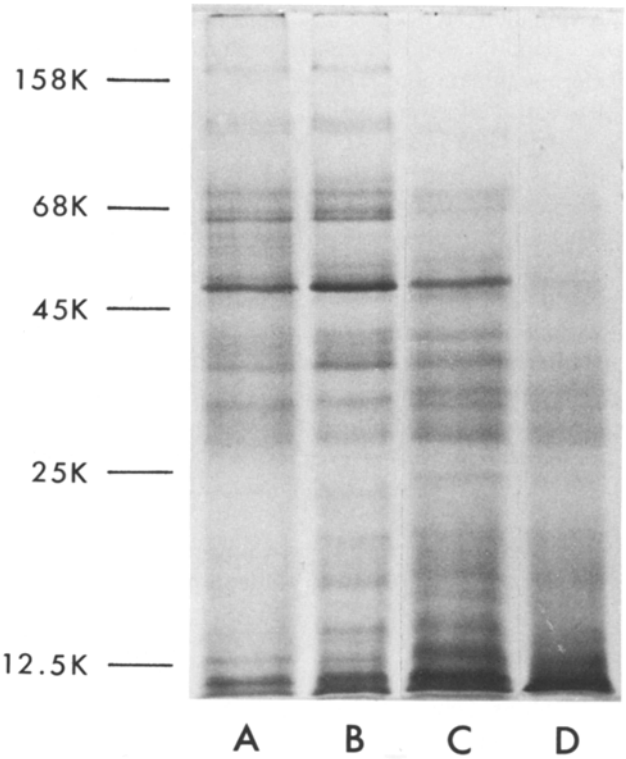

Fig. 1. SDS gel electrophoresis of covalently bound wall proteins of lily pollen tubes grown in vitro. The proteins were extracted under various conditions sith TFMS: *A* at 0 °C, 1 h; *B* at 0 °C, 3 h; *C* at 0 °C, 24 h; *D* at 20 °C, 1 h. All proteins were electrophoresed on the same 11% polyacrylamide gel and visualized by Coomassie blue R250. Cytochrome C, chymotrypsinogen A, hen egg albumin, bovine serum albumin and aldolase (Combithek calibration proteins, Boehringer Mannheim GmbH; Biochemica) were used as molecular weight markers

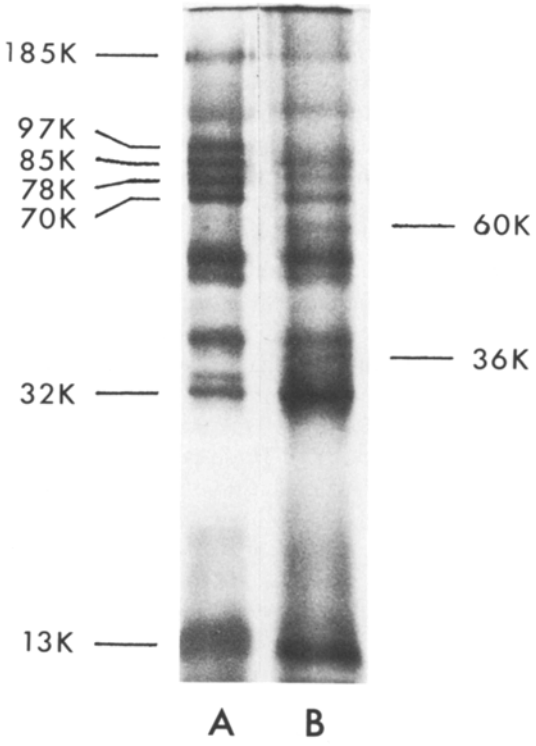

Fig. 2. SDS gel electrophoresis of covalently bound wall proteins extracted by TFMS at 0 °C for 2 h. *A* pollen grains; *B* germinated pollen. All proteins were electrophoresed on the same 11% polyacrylamide gel and visualized by silver staining

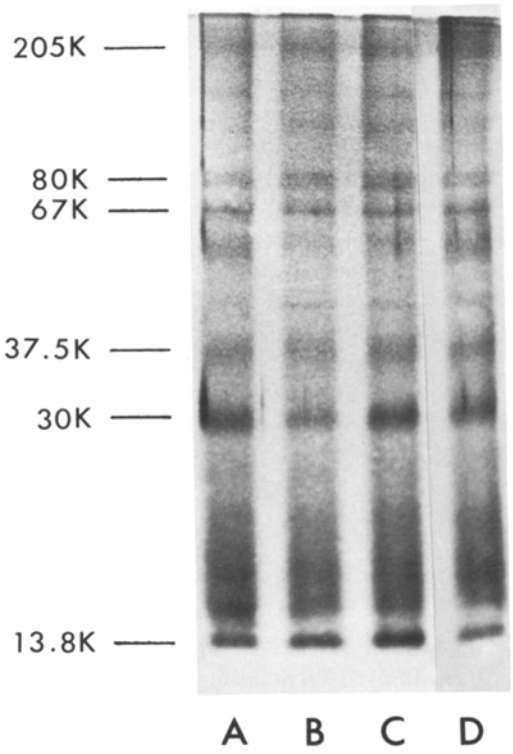

Fig. 3. SDS gel electrophoresis of covalently bound wall proteins of lily pollen tubes after self- and cross-pollination. All proteins extracted by TFMS at 0 °C for 2 h were electrophoresed on the same 11% polyacrylamide gel and visualized by silver staining. *A* M×A; *B* A×A; *C* A×M; *D* M×M

protein and polysaccharide but also broke down peptide bonds. Nevertheless, appropriate conditions could be chosen, i.e. at 0 °C between 1–3 h, to produce satisfactory results for the SDS-PAGE studies of pollen tube wall proteins. Therefore, in the present investigation, the wall samples were all treated at 0 °C for 2 h. The proteins obtained in this way, determined by the Lowry method after dialysis, accounts for 70% of the NaCl insoluble proteins.

The patterns of the covalently bound wall proteins of pollen grains and pollen tubes germinated in vitro are shown in Fig. 2. The molecular weights of the proteins ranged from 185 k–13 k. In comparing the patterns of these proteins with those of the noncovalently bound proteins reported in the previous paper (Li and Linskens 1983 a), clear differences could be seen either in number or in distribution of protein bands. Although the covalently bound wall proteins of the pollen grains and pollen tubes appear to ressemble each other, some differences of the proteins could still be detected between grains and tubes. Firstly, the staining density of the protein bands shows that the proteins which are abundantly present in grain, wall and tube wall are not the same, e.g. proteins 85 k, 78 k and 70 k are found less frequently in the tube wall. Secondly, protein 97 k found in the grain wall is absent in the tube wall. Thirdly, proteins 60 k and 36 k are newly incorporated into tube wall during pollen germination and tube growth.

Figure 3 shows the patterns of covalently bound wall proteins of pollen tubes grown in vivo. The molecular weights of the predominant proteins which are densely stained by the silver staining method are 205 k, 80 k, 67 k, 37.5 k, 30 k and 13.8 k. The composition of these covalently bound proteins is much simpler than that of the noncovalently bound wall proteins of the same samples (Li and Linskens 1983 a) since fewer protein bands were found in the former case. Moreover, it can also be seen that covalently bound wall proteins of pollen tubes grown in vivo differ from those of pollen tubes grown in vitro. A similar difference was also reported for noncovalently bound proteins (Li and Linskens 1983 a).

No distinction was found in protein patterns among the samples M×A, A×A, A×M and M×M. This means that covalently bound wall proteins of pollen tubes remain the same, no matter whether self-pollination or cross-pollination took place.

Discussion

The insolubility of cell wall proteins is a major obstacle in their study. As for pollen tube wall proteins, although some preliminary data about noncovalently bound proteins extractable by NaCl are at hand, the covalently bound proteins which constitute about 70% of the total wall proteins remain an unknown quality. It is almost impossible to obtain electrophoretic patterns of these proteins after hydrolysis with harsh alkalis and acids. This implies that no intact peptides have been obtained. In the present study, TFMS, a reagent for deglycosylation, was used successfully in isolating covalently bound wall proteins. As there are many possible cell wall linkages, e.g. polysaccharide to protein links, protein to protein links and so on, (Lamport 1970; Lamport and Epstein 1983), changes in the length of time and temperature of the hydrolysis by TFMS could cleave the linkages to different degrees. That was why various peptides could be observed in Fig. 1. If the optimal condition can be worked out, TFMS can be employed in isolating covalently bound wall proteins.

The pollen grain wall is formed in the anther during the development of the pollen. The wall is composed of exine and intine and originates from the diploid tapetum and haploid male gametophyte (Linskens 1964, 1969), but the pollen tube, growing at the tip, is formed in a medium after pollen germination. The covalently bound wall proteins of the pollen grain are not the same as those of the pollen tube. This implies that the protein-polysaccharide structure of the cell wall is dependent upon the pathways and sites of wall synthesis, and on the roles of the wall as well. The differences between covalently bound wall proteins of pollen grains and pollen tubes grown in vitro are not so pronounced as those of noncovalently bound proteins. This is likely related to their respective functions. However, there is something in common with both types of proteins: some proteins are broken down and some new ones are incoporated into the tube walls during pollen germination and tube growth.

For in vitro and in vivo growing pollen tubes, differences were observed in their covalently bound wall proteins as well as in their noncovalently bound wall proteins. In addition, it was reported in our previous study that neutral sugar compositions of pollen tube walls grown in vitro and in vivo were different. The in vitro growing pollen tube walls have a slightly lower content of arabinose, galactose and glucose, and a higher content of xylose than in vivo growing pollen tubes (Li and Linskens 1983 b).

Considering all these aspects one may draw the conclusion that walls of pollen tubes grown in vitro and in vivo are different in structure (Kroh 1967). This means that the substrates, including carbohydrates from the artificial medium and the secretion products of stigma and style, contribute to the growth of pollen tubes in a different manner.

It is remarkable that there are striking resemblances between covalently bound wall proteins of pollen tubes after self- and cross-pollination. On the contrary, noncovalently bound wall proteins of pollen tubes after self- and cross-pollination differ from each other and are related to the incompatibility reaction. From these facts it might be that covalently bound wall proteins of pollen tubes serve the wall in a structural function in order to form a coherent network (King 1966; Lamport 1965; Lamprt 1980; Lamport and Catt 1981; Cooper and Varner 1983; Fry 1982). These latter proteins are primarily concerned with pollen tube growth and have little to do with the incompatibility reaction.

Acknowledgements. The plant material was produced by the Botanical Garden, University of Nijmegen; we thank Mr. A. H. Glaap for caring for the plants.

References

Bailey RW, Kauss H (1974) Extraction of hydroxyproline-containing proteins and pectic substances from cell walls of growing and non-growing mung bean hypocotyl segments. Planta 119:233–245

Cooper JB, Varner JE (1983) Insolubilization of hydroxyproline rich cell wall glycoprotein in aerated carrot root slices. Biochem Biophys Res Commun 112:161–167

Coudron C, Ellis K, Philipson L, Schwartz N (1980) Preliminary characterization of a xylose acceptor prepared by hydrogen fluoride treatment of proteoglycan core protein. Biochem Biophys Res Commun 92:618–623

Dickinson DB (1968) Rapid starch synthesis with increased respiration in germinating lily pollen. Plant Physiol 43:1–8

Edge ASB, Faltynek CR, Hof L, Reichret LE, Weber P (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal Biochem 118:131–137

Fry SC (1982) Isodityrosine, a new cross-linking amino acid from cell wall glycoprotein. Biochem J 204:449–455

Glassman JHS, Todd CN, Shiveley JE (1978) Chemical deglycosylation of carcinoembryonic antigen for amino acid sequence studies. Biochem Biophys Res Commun 85:209–216

Heath MF, Northcote DH (1971) Glycoprotein of the wall of sycamore tissue-culture cells. Biochem J 125:953–961

King NJ, Bayley ST (1966) A preliminary analysis of the proteins of the primary walls of some plant cells. J Exp Bot 16:294–303

Kroh M (1967) Fine structure of *Petunia* pollen germinated in vivo. Rev Palaeobotan Palynol 3:197–203

Lamport DTA (1965) The protein of primary cell walls. In: Preston RD (ed) Adv bot res vol 2. Academic Press, London New York, pp 151–218

Lamport DTA (1969) The isolation and partial characterization of hydroxyproline rich glycoproteins obtained by enzymic degradation of primary cell walls. Biochemistry 8:1155–1163

Lamport DTA (1980) Structure and function of plant glycoproteins. In: Preiss J (ed) Biochemistry of plants, vol 3. Academic Press, New York, pp 501–541

Lamport DTA, Catt JW (1981) Glycoproteins and enzymes of the cell wall. Encyl Plant Physiol 13 B: 130–141

Lamport DTA, Miller DH (1971) Hydroxyproline arabinosides in the plant kingdom. Plant Physiol 48:454–456

Lamport DTA, Epstein L (1983) A new model for the primary cell wall: a concatenated extension-cellulose network. In: Randall DD (eds) Curr top plant biochem physiol symp, vol 2. University of Missouri, Columbia, pp 73–83

Li Yi-qin, Linskens HF (1983a) Wall-bound proteins of pollen tubes after self- and cross-pollination in *Lillium longiflorum.* Theor Appl Genet 67:11–16

Li Yi-qin, Linskens HF (1983b) Neutral sugar composition of pollen tube walls of *Lilium longiflorum.* Acta Bot Neerl 32:437–445

Li Yi-qin, Croes AF, Linskens HF (1983) Cell wall proteins in pollen and roots of *Lilium logiflorum:* extraction and partial characterization. Planta 158:422–427

Linskens HF (1964) Pollen physiology. Annu Rev Plant Physiol 15:255–270

Linskens HF (1969) Fertilization mechanism in higher plants. In: Metz C, Monroy A (eds) Fertilization, vol 2. Academic Press, New York, pp 189–254

Mort AJ, Lamport DTA (1977) Anhydrous hydrogen fluoride deglycosylates glycoproteins. Anal Biochem 82:289–309

Sairam MR, Schiller PW (1979) Receptor binding, biological, and immunological properties of chemically deglycosylated pituitary eutropin. Arch Biochem Biophys 197:294–301

Schrauwen JAM, Linskens HF (1967) Mass culture of pollen tubes. Acta Bot Neerl 16:177–179

Voigt VJ (1984) A study of cross-links between the glycoprotein subunits within the insoluble inner cell wall layer of *Chlamydomonas reinhardii.* Mitt Inst Allg Bot, Hamburg 19:83–98