

RESEARCH PAPER

Changes in growth and cell wall extensibility of maize silks following pollination

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Abstract

In response to pollination maize silks undergo an accelerated process of senescence which involves an inhibition of elongation. To gain insight into the mechanism underlying this growth response, the relationships among silk elongation kinetics, cell wall biophysical properties, pollen tube growth, and expansin protein abundance were investigated. The inhibition of silk elongation became apparent beyond 12 h after pollination. Pollinated walls were less responsive in assays of extension induced by pollen β -expansin. Expansin protein abundance and endogenous expansin activity were not considerably reduced after pollination. Silk wall plastic compliance was significantly reduced 6 h post-pollination and beyond, suggesting that the wall undergoes structural modifications leading to its rigidification in response to pollination. The reduction in the plastic compliance occurred locally and progressively, shortly after pollen tubes traversed through a region of silk. Though numerous pollen grains germinated and initiated pollen tubes at the silk tip, the density of pollen tubes gradually declined along the length of the silk and only 1–2 reached the ovary even 24 h after pollination. These results support the notion that pollination-induced cell wall rigidification plays multiple roles in maize reproduction, including inhibition of silk growth and prevention of polyspermy.

Key words: Cell wall, expansin, maize, pollen tubes, pollination, polyspermy, silk elongation, wall rigidification.

Introduction

Silks are the pollen receptive organs of maize (*Zea mays* L.) female florets, the individual flowers of the female inflorescence or ear. Numerous hairs or receptive trichomes are distributed along the length of a silk and function as the major surfaces for pollen capture and germination (Miller, 1919; Kiesselbach, 1949; Heslop-Harrison *et al.*, 1984). After germination, pollen tubes enter the trichome and subsequently reach the transmitting tracts within the main axis of the silk. The transmitting tract comprises a specialized tissue that facilitates pollen tube growth towards the ovary and spans the entire length of the silk to terminate in the upper ovary wall (Miller, 1919; Kiesselbach, 1949; Kroh *et al.*, 1979; Heslop-Harrison *et al.*, 1984, 1985). After further growth within the ovary, usually a single pollen tube grows through the micropyle into the embryo sac to participate in double fertilization (Miller, 1919; Heslop-

Harrison *et al.*, 1985; Marton *et al.*, 2005). Several mechanisms, including competition to reach transmitting tracts (Heslop-Harrison *et al.*, 1985) and the local guidance of a pollen tube through the micropyle (Marton *et al.*, 2005; Lausser *et al.*, 2010), are involved in the control of the number of pollen tubes reaching the embryo sac thereby preventing polyspermy. In flowering plants polyspermy can result in an aberrant ratio of maternal to paternal genomes in the endosperm, a condition often lethal to the endosperm (Kermicle, 1971; Lin, 1984; Haig and Westoby, 1991).

During early development silks remain enclosed by several layers of husks and, after a period of elongation within these protective layers, silks emerge beyond them, an event referred to as silking. After silking, unpollinated silks continue to elongate for several days through cell elongation (Kiesselbach, 1949; Heslop-Harrison *et al.*, 1984;

Westgate and Boyer, 1985; Fuad-Hassan *et al.*, 2008). However, the rate of elongation progressively decreases after silking (Schoper and Martin, 1989; Bassetti and Westgate, 1993a, b; Anderson *et al.*, 2004; Fuad-Hassan *et al.*, 2008), and indeed the elongation of exposed silk regions is arrested (Fuad-Hassan *et al.*, 2008). Silks naturally begin to senesce ~8–10 days after emergence beyond the husks (Bassetti and Westgate, 1993a).

Pollination inhibits silk elongation. Though this is not a novel observation from a qualitative perspective, there is very little published literature on the phenomenon. Carcova *et al.* (2003) reported a decrease in silk elongation after pollination. However, to date the mechanism(s) underlying the pollination-induced inhibition of silk elongation has not been reported. Enlargement of plant cells is a complex process in which several aspects of the plant cell wall are of key importance (Cosgrove, 2005). One aspect is the abundance and activity of cell wall-loosening agents. A second aspect is the compositional and structural features of the cell wall itself, allowing it to respond to wall-loosening activity by cell wall extension. These two aspects, in turn, are affected by cell wall pH, synthesis of wall polymers, and their assembly and cross-linking *in muro*.

Expansins are one of the best characterized cell wall-loosening agents and are implicated in diverse plant developmental processes including growth, fruit ripening, abscission, and pollen tube penetration into silk tissue (Cosgrove *et al.*, 1997; Rose *et al.*, 1997; Valdivia *et al.*, 2009). An extensive body of experimental evidence supports the notion that expansins are involved in plant cell enlargement and hence growth stimulation. First, expansins have the ability to mediate acid-induced extension of isolated cell walls (McQueen-Mason *et al.*, 1992; Li *et al.*, 2003), a process which in general correlates with plant growth rate (Cosgrove, 1989). Secondly, addition of expansins to live cells stimulates their enlargement (Fleming *et al.*, 1997, 1999; Link and Cosgrove, 1998; Cosgrove *et al.*, 2002). Further evidence is provided by studies on native gene expression patterns and transgenic manipulation of expansin expression (Cosgrove, 2005, and references therein). Consequently down-regulation of the synthesis or activity of expansins may lead to silk growth inhibition.

Evidence for an association among expansin, pollen tube growth, and silk elongation was first reported by Valdivia *et al.* (2006, 2007, 2009) who studied a maize line having a Mutator (*Mu*) transposon insertion in the pollen β -expansin *ZmEXPB1*. The protein encoded by *ZmEXPB1* (Zea m1) has been shown to possess wall extension activity characteristic of expansins (Cosgrove *et al.*, 1997; Li *et al.*, 2003). Valdivia *et al.* (2007, 2009) reported a significant decrease in the *in vivo* growth rate of the pollen tubes carrying the *expb1::mu* allele compared with the wild type. However, the *in vitro* growth of the pollen tubes was not affected by the mutation. Zea m1 is therefore proposed to have an *in vivo* wall-loosening function which facilitates pollen tube penetration into and growth through the silks. Moreover, in the mutant line, silks continued to elongate for longer periods after pollination, and silk senescence was delayed (Valdivia *et al.*, 2006).

Rigidification or reduction in extensibility of the cell wall has been shown to be associated with plant growth cessation in many but not all instances. According to Kutschera (1996), the cessation of cell elongation in rye coleoptiles involves a loss of cell wall plastic extensibility. The growth inhibition of the subapical regions of maize root under water stress accompanies a decrease in cell wall extensibility (Fan *et al.*, 2006). Cosgrove and Li (1993) and Wu *et al.* (1996) also provided evidence that reduced rates of plant growth involve decreased cell wall susceptibility to expansin-induced extension.

In order to elucidate the mechanism involved in the pollination-induced inhibition of silk elongation, the effect of pollination on silk elongation kinetics, expansins, and cell wall biophysical characteristics were investigated. The results demonstrate that the down-regulation of silk growth is associated with a significant reduction in cell wall extensibility which results from local signals due to the presence and/or movement of pollen tubes. Moreover, evidence in support of the notion that silk cell wall rigidification serves a role in preventing polyspermy in maize is presented.

Materials and methods

Plant material and growth conditions

All experiments were conducted using Pioneer hybrid 34M94. Field experiments were conducted at the Pennsylvania State University Agricultural Experimental Station at Rock Springs, Pennsylvania, USA during the summer. For greenhouse experiments, plants were grown under standard conditions (27–29 °C daytime temperature, supplementary lighting from 1000 W metal and sodium lamps, 12 h photoperiod) during the summer.

Controlled pollinations

In both field and greenhouse experiments, ears were covered with glassine bags prior to silk emergence. The ears thereafter were monitored daily and the date of silk emergence recorded. Two days after silk emergence the silks and the surrounding husks were cut 3 cm back from the tip of the longest husk. On the third day after silk emergence two types of controlled pollinations were performed: (i) full-ear pollinations in field-grown plants where all silks on an ear were pollinated using freshly collected pollen; and (ii) half-ear pollinations in greenhouse plants where approximately half the silks on an ear were pollinated; the rest of the silks were left as controls. In the case of (i), a separate group of plants/ears (with silks trimmed as described) in which no silks were pollinated were left as controls. After pollination ears were again covered with glassine bags to prevent further pollination. When photography was conducted, further pollination was prevented by bagging the tassels.

Silk elongation measurements

In full-ear pollination experiments, silks were destructively sampled at floret position 24 from the base of the ear. The length of 2–10 silks from each of five control ears and five fully pollinated ears was measured to determine the average length of pollinated and control silks. In half-ear pollination experiments, each ear was photographed at 15–30 min intervals and the lengths above the husks of the three longest control and pollinated silks were separately determined at intervals after pollination. Spot-advanced software (Diagnostic Instruments, Inc., Sterling Heights, MI,

USA) was used for length determination in digital images. Length data from five half-ear pollinated plants were used to calculate the average length of pollinated and control silks.

Silk sampling for cell wall biophysical assays and protein extraction

Silk samples for wall biophysical assays (stress/strain analysis, wall susceptibility to expansin, acid-induced extension, and wall stress relaxation) were collected from full-ear pollination experiments conducted under field conditions. Mid and basal silk segments were sampled from floret position 24 (counting from the base of the ear) at different times after pollination. At equivalent times control samples were also collected in parallel. For protein extraction, silks were sampled from floret positions 15–24 (counting from the base of the ear) at 24 h post-pollination. In this case, the entire length of silks except the segments protruding beyond the husks was collected. The removal of the exposed regions of silks was a precaution to prevent contamination with pollen. Upon harvest, silks were quick-frozen in liquid nitrogen and stored at -80°C until used.

Stress/strain analysis

Stress/strain analyses were conducted as described by Yuan *et al.* (2001). Frozen, thawed silk samples were abraded using a carborandum slurry and pressed between two glass slides under weight to express cell sap. Subsequently they were kept on ice bathed in 50 mM HEPES buffer (pH 6.8) until the analysis was complete. Silk segments were clamped (5 mm between clamps) in a tensile tester and extended in two cycles at 3 mm min^{-1} up to a load limit of 4.0 g (for mid silk samples) or 5.0 g (for basal silk samples) and immediately returned to their original length and extended a second time. In the case of each extension, a second-degree polynomial was fitted to the stress/strain data to calculate the slope (i.e. compliance) at the end of the cycle. Plastic and elastic compliances are expressed as percentage extension per 100 g force.

Wall susceptibility to expansin (wall reconstitution)

Methodology as described by Cosgrove *et al.* (1997) was followed. Briefly, silk wall samples were prepared as described for stress/strain analysis and microwaved in 100 ml of distilled water for 90 s to inactivate endogenous expansins. Heat-treated samples were then clamped (a 5 mm silk segment between clamps) on a custom-made extensometer under a constant tension of 7.5 g force. Initially a sample was bathed in 50 mM sodium acetate pH 4.5 buffer and wall extension measured by a position transducer attached to the lower clamp of the extensometer. Once the rates of extension were stabilized, the bathing buffer was replaced by a protein extract (total protein concentration: 0.34 mg ml^{-1}) prepared from maize pollen (see below for details on protein extraction from pollen). Wall extension was monitored for a total of 150 min.

Acid-induced extension (native wall creep)

Experiments were conducted according to Cosgrove (1989). Silk wall samples were prepared as described above (without heat inactivation) and $\sim 5\text{ mm}$ silk segments were then clamped in a custom-made extensometer under a constant tension of 7.5 g force. Initially the samples were bathed in 50 mM HEPES, pH 6.8 and, once the rates of extension were stabilized, the buffer was changed to 50 mM sodium acetate, pH 4.5. Wall extension was monitored using a position transducer attached to the lower clamp of the extensometer.

Wall stress relaxation analysis

Methodology was based on Cosgrove *et al.* (1997). In brief, silk samples were processed as described above (without heat inactivation) and treated with 50 mM sodium acetate, pH 4.5 for

20 min at room temperature and then kept on ice until the analysis was complete. A silk sample (5 mm) was clamped in a tensile tester and rapidly extended to reach a force of 5 g. The wall sample was then held at constant length and the decay of force on the sample monitored for the next 5 min. The change in force (dF) against the change in log time (dlogt) was plotted to generate stress relaxation spectra.

Protein extraction from silk cell walls

Cell wall protein extraction using 1 M NaCl was done as described by McQueen-Mason *et al.* (1992) and Wu *et al.* (1996) with slight modifications. Silks were homogenized in buffer containing 25 mM HEPES, 3 mM sodium metabisulphite, and 2 mM EDTA (pH 7.0) at 4°C . Cell wall material was recovered by filtration through nylon mesh and washed twice in the same buffer. Wall proteins from the recovered cell wall material were extracted overnight at 4°C using the same buffer containing 1 M NaCl (pH 7.0). After concentration and buffer exchange with 10 mM sodium acetate with 10 mM dithiothreitol (DTT; pH 4.5) using Amicon Ultra centrifugal devices (Millipore, Billerica, MA, USA), proteins were quantified using the Coomassie plus protein assay reagent (Thermo Scientific, Rockford, IL, USA). Cell wall material first extracted with 1 M NaCl was washed well with deionized water to remove salt and homogenized in 1% (w/v) SDS using a pestle and mortar. The homogenized suspension was then heated at 100°C for 5 min and filtered using nylon mesh (Lee and Choi, 2005). Total protein in the filtrate was quantified using the BCA protein assay kit (Thermo Scientific) following the manufacturer's instructions. Similar amounts of total protein per unit dry silk mass were extractable from control and pollinated samples using salt or SDS.

Electrophoresis and immunoblot analysis

Protein samples were separated by SDS-PAGE on a 13% (w/v) polyacrylamide gel using a minigel apparatus (Bio-Rad Laboratories, Hercules, CA, USA) following methods described by Laemmli (1970). After SDS-PAGE, proteins were transferred on to a Protran BA nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories). Protein transfer was performed using a solution of 192 mM glycine, 25 mM TRIS, and 20% (v/v) methanol at 25 V cm^{-2} for 100 min. Membranes containing the transferred proteins were then washed in phosphate-buffered saline (PBS) and blocked with 10% horse serum in PBS containing 0.05% (v/v) Tween-20 and 5 mM sodium azide for 1 h. Afterwards, the membranes were incubated with rabbit primary antibody [1:1000 (v/v) dilution for anti-CsEXPA1 and 1:5000 (v/v) dilution for anti-ZmEXPB8 and anti-OsEXPB3] for 1 h in the same solution (PBS with Tween, horse serum, and sodium azide) and washed twice (5 min each) with PBS containing 0.05% (v/v) Tween-20 and 5 mM sodium azide, and twice (5 min each) with TRIS-buffered saline (TBS) containing 0.05% (v/v) Tween-20 and 5 mM sodium azide. Subsequently the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG [1:8000 (v/v)] in TBS with 0.05% (v/v) Tween-20 and 5 mM sodium azide, washed four times with TBS containing 0.05% (v/v) Tween-20 and 5 mM sodium azide, and developed with 0.1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate and 0.2 mg ml^{-1} nitroblue tetrazolium (Sigma-Aldrich, Inc., St. Louis, MO, USA) in buffer (pH 9.5) containing 100 mM TRIS-HCl, 100 mM NaCl, and 5 mM MgCl₂.

Purification of Zea m1 from maize pollen

Protein extraction from maize pollen was done as essentially described by Li *et al.* (2003). Maize pollen was extracted with 50 mM sodium acetate pH 4.5 for 1 h at 4°C and the suspension centrifuged at $15\,000\text{ g}$ at 4°C . The resultant supernatant was loaded onto a CM-Sepharose Fast Flow column (GE Healthcare)

equilibrated in 20 mM sodium acetate pH 4.5 and the bound proteins subsequently released using a linear gradient of NaCl (0–500 mM in 5 h). All Zea m1 isoforms (β -expansin) (see Li *et al.*, 2003) were collected and buffer exchanged with 50 mM sodium acetate pH 4.5. This protein preparation was used in the wall susceptibility analyses described above.

Observation of pollen tubes within silks and ovaries

A modified staining protocol based on Martin (1959) was followed. Silks and ovaries were destructively sampled at different times after pollination. Silks were divided into serial segments along their entire length. Samples were fixed in FAA (50% ethanol, 10% formaldehyde, 5% acetic acid) overnight at 4 °C and then serially rinsed with 100, 75, 50, 30, and 0% ethanol. Samples were left in each solution for ~15 min. Afterwards they were cleared with 8 M NaOH for 40–45 min and washed three times with distilled water. After storing overnight in distilled water at 4 °C, samples were stained with 0.05% (w/v) aniline blue in 0.033 M K_3PO_4 in the dark for ~20 min. Ovaries were bisected prior to staining. Ovary and silk samples were observed by UV-fluorescence microscopy.

Results

Silk elongation is considerably reduced beyond 12 h after pollination

Silk elongation kinetics were characterized using two experimental strategies. In one approach using greenhouse-grown plants, approximately half the silks on an ear were pollinated and the rest left as controls (half-ear pollinations). Unpollinated silks continued to elongate during the entire period of measurement (56 h), with a pronounced increase in length during the night and early morning (Fig. 1, left y-axis). Compared with controls, pollinated silks were considerably shorter beyond 12 h after pollination. Results also indicated a trend of decreasing silk elongation even before 12 h post-pollination. Statistically significant decreases in length were noted at 24 h after pollination ($P=0.02$) and beyond.

Since silks are partly covered by husks, the photographic method used in the above experiments does not allow the determination of entire lengths of silks; that is, from the ovary–silk junction to the tip of the silk. Moreover, it was noted that the silks originating at the top of the ear are shorter than those originating at the bottom (data not shown). In the photography method it is not possible to ascertain the origin (i.e. floret position) of a silk being measured. Therefore, silk elongation was measured by destructive sampling in field experiments in which all silks were collected at floret position 24 counting from the bottom of the ear. During the course of 42 h, pollinated silks elongated only 1.3 cm compared with 3 cm in controls (Fig. 1, right y-axis). Significant decreases in silk length were observed at 18 h ($P=0.02$), 24 h ($P=0.03$), and 42 h ($P=0.02$) post-pollination. Though the variability in the data did not allow a precise time-course, a general tendency of decreasing silk elongation which becomes more obvious 12 h after pollination was noted. The trend of decreasing silk elongation prior to 12 h noted in the photography

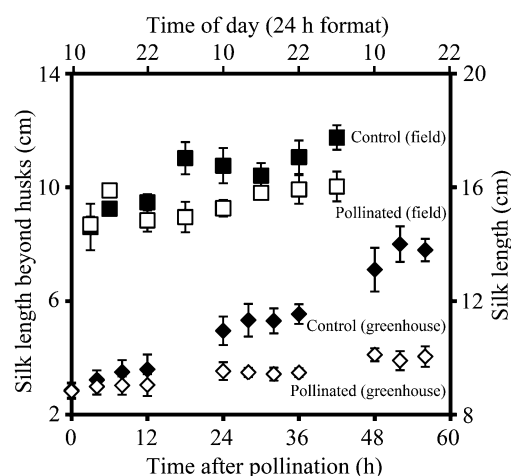


Fig. 1. Effect of pollination on silk elongation. The left y-axis denotes the silk length beyond the husks determined in half-ear pollination experiments in the greenhouse. In each ear, approximately half of the silks were pollinated (open diamonds) and the others left unpollinated (controls, filled diamonds). At the times shown, the length beyond the husks of the three longest control silks and pollinated silks in a given ear was separately determined by digital photography. Data shown are means $\pm$ SE ($n=5$). The right y-axis shows the entire silk length measured in full-ear pollination experiments in the field. All silks on an ear were either pollinated (open squares) or left as controls (filled squares). At each time point, silks were destructively sampled from floret position 24 (counting from the base of the ear) and the length measured. Means $\pm$ SE ($n=5$) are shown.

method was not evident in the destructive sampling experiment. Apart from that difference, both experimental methods demonstrated essentially the same silk elongation pattern. Taken together, the results of both greenhouse and field experiments support the general conclusion that a major reduction in silk elongation occurs 12 h after pollination.

Wall structural changes due to pollination: silk cell walls become less extensible after pollination

In order to examine whether pollination caused structural modifications in silk cell walls, wall extensibility was measured by stress/strain analysis. In this method, both elastic (reversible Δ length/ Δ force) and plastic (irreversible Δ length/ Δ force) compliances of a wall sample are determined (Cosgrove, 1993; Yuan *et al.*, 2001). In one set of experiments, mid silk samples collected from floret position 24 were used. Compared with controls, the plastic compliance of pollinated silks was significantly reduced 6 h after pollination ($P=0.004$) and beyond (Fig. 2A). The reduction ranged from 33% to 41% at different time points. There was a small (~5%) but statistically significant reduction in the elastic compliance 12 h after pollination ($P=0.002$) and beyond (Fig. 2B).

Taking another approach, the effect of pollination on the extension response of heat-inactivated silk walls to maize pollen extract containing β -expansin protein was tested. As shown in Fig. 3, compared with controls, the extension response of pollinated walls was significantly reduced 16 h post-pollination ($P=0.02$) and beyond. The results of stress/strain analysis and susceptibility to pollen β -expansin collectively show that pollination causes a decrease in silk cell wall extensibility (i.e. the walls become more rigidified).

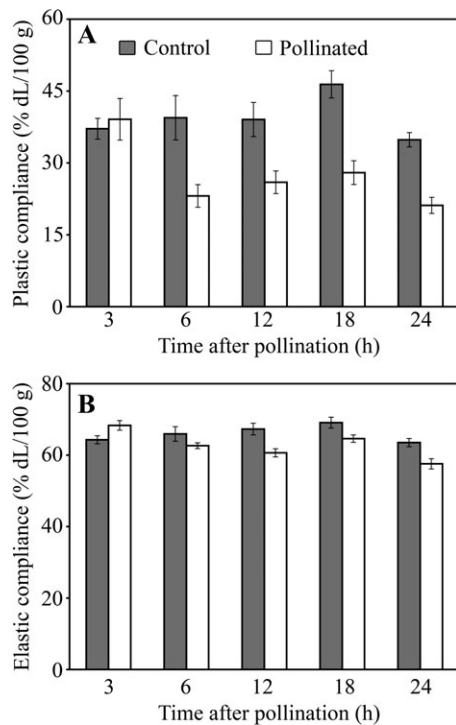


Fig. 2. Stress/strain analysis of control and pollinated wall samples collected from the mid silk region: (A) plastic compliance, (B) elastic compliance. Data are means $\pm$ SE ($n=9-21$).

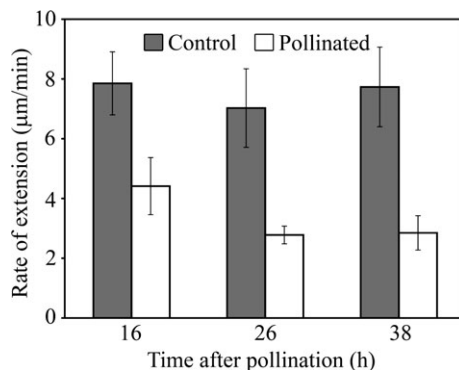


Fig. 3. Extension response of heat-inactivated silk walls to maize pollen extract containing β -expansin protein. For each sample the average rate of extension during the time period 40–60 min after the addition of the pollen extract was calculated. These averages were used to calculate means for control and pollinated samples at different times after pollination. Means $\pm$ SE ($n=7-8$) are shown.

The reduction in wall extensibility occurs only after pollen tubes have traversed a given region of silk

Results of stress/strain analyses raised the question of whether the reduction in wall compliances measured here is related to pollen tube movement along the silk. To address this issue the presence of pollen tubes along the length of silks and within ovaries was mapped. Silks and ovaries were sampled from floret position 24. Though numerous pollen tubes were present at the tip of a silk, in most instances only one reached the ovary even as late as 24 h after pollination (Fig. 4). A plot of the number of pollen tubes along the length of silk indicated two major regions where the number of pollen tubes decreased sharply. The first location was the tip—most 0.5 cm of the silk and the second the basal—most 2 cm proximal to the silk–ovary junction. In between these two regions there was a more gradual decrease in the number of pollen tubes.

In order to investigate the relationship between pollen tube growth and changes in silk wall extensibility, mid and basal silk segments were compared based on stress/strain measurements. Since basal regions are closer to the ovary compared with mid silk regions, pollen tubes take more time to reach basal silk samples. As shown in Fig. 5A and B, the plastic and elastic compliances of basal silk samples were significantly reduced 12 h post-pollination (plastic, $P < 0.001$; elastic, $P = 0.003$) and beyond. However, the compliances of basal samples collected 6 h after pollination did not decrease significantly (plastic, $P = 0.37$; elastic, $P = 0.13$). In contrast, at 6 h post-pollination, the plastic compliance of mid silk samples was reduced by 33% (Fig. 2A). This is an important difference which demonstrates that the decrease in plastic compliance moves downward towards the ovary through the silk. Aniline blue staining showed the presence of pollen tubes in both middle and basal silk samples 6 h post-pollination (Fig. 5C, D), illustrating that the reduction in the wall extensibility occurs only after pollen tubes have traversed a given region of silk.

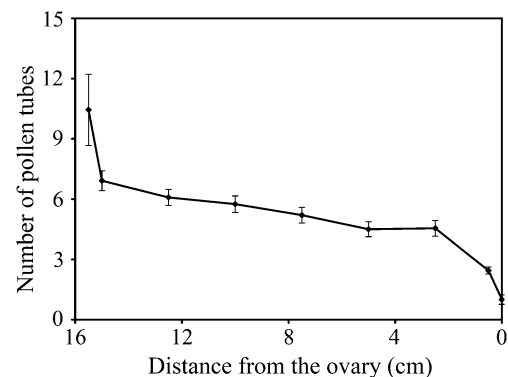


Fig. 4. Number of pollen tubes at different distances from the ovary at 24 h after pollination. Pollen tubes within silks and ovaries were observed by aniline blue staining followed by UV fluorescence microscopy. Zero distance indicates the inside of the ovary. Means $\pm$ SE ($n=5-12$) are shown.

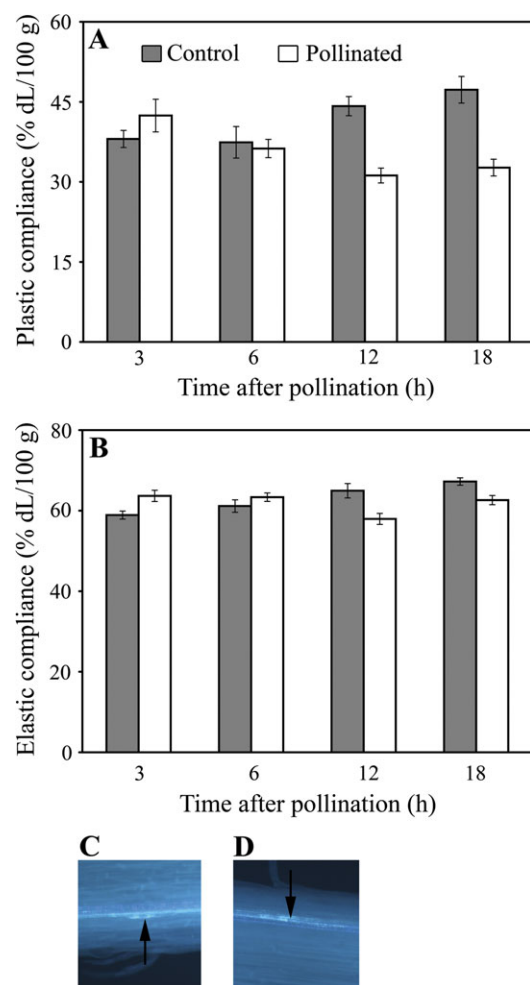


Fig. 5. Stress/strain analysis and pollen tube growth. Basal silk samples collected from floret position 24 were used in stress/strain analysis: (A) plastic compliance, (B) elastic compliance. Data are means $\pm$ SE ($n=9-15$). (C) and (D) Pollen tubes (arrows) stained with aniline blue in mid and basal silk samples collected 6 h post-pollination. (This figure is available in colour at JXB online.)

Pollination and expansins: pollination does not cause a significant decrease in endogenous expansin activity or protein abundance

Control and pollinated silk walls were assayed using acid-induced extension (native wall creep) and wall stress relaxation. These two assays are considered as hallmarks of expansin activity (Li *et al.*, 2003). Figure 6 depicts the results of a time-course analysis of the acid-induced extension of isolated maize silk cell walls. At all the post-pollination time points tested, pollinated walls had lower rates of native wall creep compared with controls. Though the reduction in creep rate ranged from 13% to 27% depending on the post-pollination time point, it was not statistically significant ($P > 0.05$) at any of the individual time points. However, an analysis of variance (ANOVA) performed by pooling samples across all post-pollination times indicated a significant decrease ($P=0.02$) in the extension rates of pollinated silks. There was no significant main factor (pollination and time) interaction ($P=0.99$).

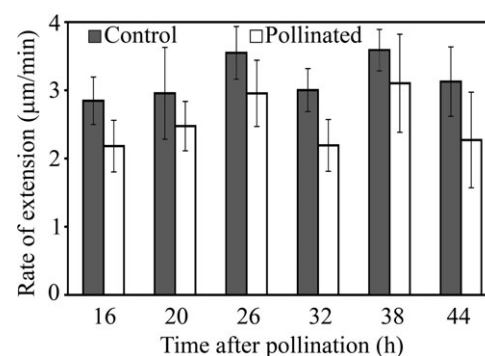


Fig. 6. Acid-induced extension (native wall creep) of control and pollinated silk cell walls (mid silk). Samples were initially bathed in neutral buffer (50 mM HEPES pH 6.8). Once the rates of extension were stabilized, low pH treatment was administered by changing the bathing buffer to 50 mM sodium acetate pH 4.5. For each sample the average rate of extension during the time period 50–70 min after low pH treatment was determined. These averages were used to calculate means for control and pollinated samples at the different post-pollination times indicated. Means $\pm$ SE ($n=5-11$) are shown.

Wall stress relaxation data collected 12, 24, and 30 h post-pollination are shown in Fig. 7. There were no significant differences ($P > 0.05$) in the rates of relaxation of control and pollinated silk walls (over the entire time scale of measurement) at any of the post-pollination times tested.

To determine whether the lack of a pollination-induced reduction in expansin activity indicated by the biophysical assays is reflected in protein abundance, both α - and β -expansin protein levels were investigated by wall extraction followed by immunoblot analyses. α -Expansins are typically extracted from plant cell walls with salt (1 M NaCl). In contrast, β -expansins require much harsher extraction methods which use chaotropic reagents such as SDS (Lee and Choi, 2005). Immunoblot analysis of NaCl-extracted silk wall proteins using anti-CsEXPA1 antibodies (Li *et al.*, 1993) detected two bands of ~ 28 kDa, indicating that at least two proteins related to α -expansins are extractable from maize silks. There was an $\sim 12\%$ increase in the intensities of the immunoblot signals of these two protein bands 24 h after pollination (Fig. 8).

Two antibodies (anti-ZmEXPB8 and anti-OsEXPB3) were used to evaluate β -expansin abundance in two different types of maize silk cell wall protein preparations: NaCl extracts and 1% (w/v) SDS extracts. Since only low levels of total soluble protein could be extracted per g of silk, walls were sequentially extracted first with 1 M NaCl and then with 1% (w/v) SDS. For a given type of extract, both antibodies showed a similar banding pattern and therefore only the results obtained with anti-ZmEXPB8 are shown (Fig. 9).

Immunoblot analysis of NaCl extracts using anti-ZmEXPB8 antibodies showed a complex banding pattern which included two bands of ~ 33 kDa (Fig. 9A, arrow), a molecular mass estimate similar to that of OsEXPB3 (Lee and Choi, 2005). Compared with controls, protein extracts from pollinated silks produced stronger immunoblot signals

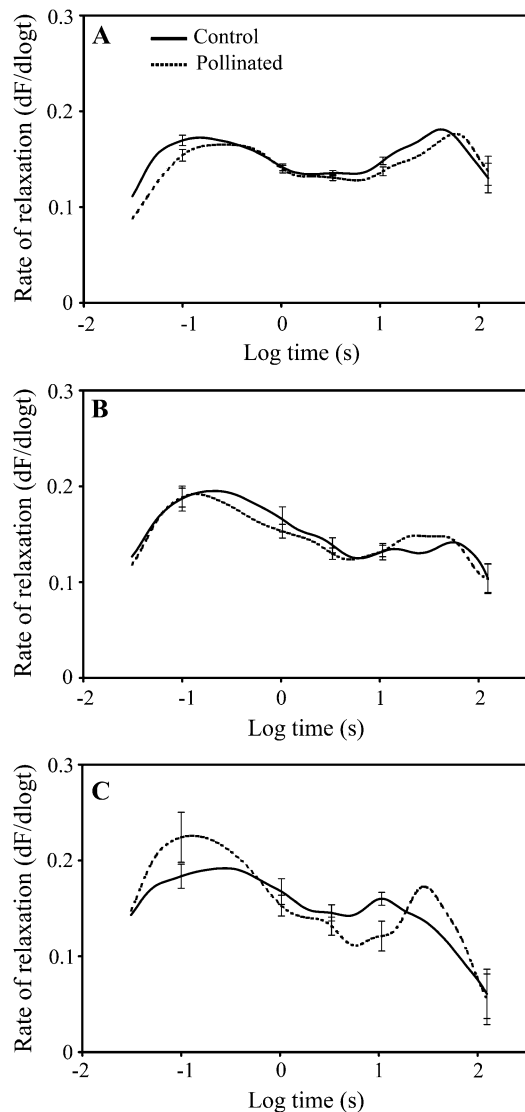


Fig. 7. Stress relaxation analysis of control and pollinated silks (mid silk). Silk samples were pre-treated in 50 mM sodium acetate (pH 4.5) for 20 min at room temperature before the stress relaxation measurements. (A), (B) and (C) Stress relaxation spectra of samples collected 12, 24, and 30 h after pollination, respectively. Data shown are means $\pm$ SE ($n=10-17$).

for the ~ 33 kDa bands: the lower molecular mass band underwent a 2-fold increase in intensity while the other increased in intensity by 12%. Two explanations can account for the increase in the intensity of immunoblot signals. The β -expansin Zea m1 is expressed at high levels in maize pollen and is thought to assist pollen tube growth through the silk (Valdivia *et al.*, 2007, 2009). It is likely that pollinated silks contain Zea m1 from pollen tubes. In fact both anti-ZmEXPB8 and anti-OsEXPB3 antibodies recognize Zea m1 (data not shown). A second possibility is that one or more silk β -expansins are up-regulated by pollination. Expansins are not exclusively involved in plant growth but serve multiple roles during plant development. For example, specific silk expansins may facilitate pollen tube growth by loosening the maternal cell walls.

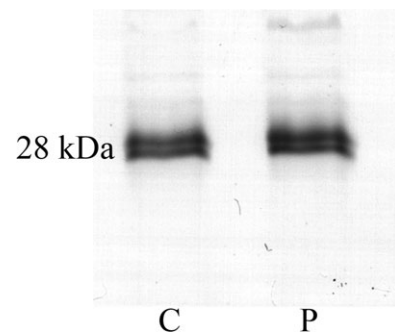


Fig. 8. Immunoblot analysis for α -expansin protein extractable from silk cell walls. Cell wall proteins were extracted from silks collected 24 h after pollination using buffer containing 1 M NaCl. C and P denote protein preparations from control and pollinated silks, respectively. A 6 μ g aliquot of crude total protein was loaded in each lane.

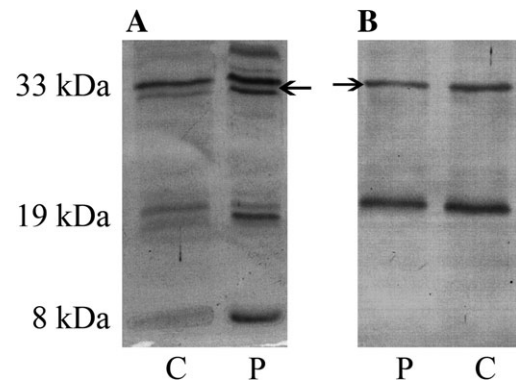


Fig. 9. Immunoblot analysis for β -expansin protein extractable from silk cell walls. NaCl- and SDS-extracted wall proteins were used in (A) and (B), respectively. For a given blot, an equal amount of total protein (12 μ g in A and 6 μ g in B) was loaded in each lane and separated by SDS-PAGE. Antibodies raised against ZmEXPB8 protein were utilized. C and P represent protein preparations from control and pollinated silks, respectively, collected 24 h after pollination. Bands indicated with arrows represent putative β -expansin protein.

Two protein bands of ~ 19 kDa and another of ~ 8 kDa were also identified in immunoblot analysis of NaCl extracts using anti-ZmEXPB8 antibodies (Fig. 9A). The intensity of one of the ~ 19 kDa bands was slightly down-regulated after pollination while the other was up-regulated 4-fold. Furthermore, there was a 4-fold increase in the intensity of the ~ 8 kDa band 24 h post-pollination. It is possible that the 8 kDa and 19 kDa bands represent proteolytic breakdown products of Zea m1 or silk β -expansin. In contrast to the numerous protein bands noted with NaCl extracts, only two bands were detected in immunoblot analyses with SDS extracts: one of ~ 33 kDa and another ~ 19 kDa. The intensities of these bands were not markedly affected by pollination (Fig. 9B). It is likely that the ~ 33 kDa band represents a β -expansin that binds tightly to

the cell wall and hence cannot be released by NaCl. The ~19 kDa band is probably due to a proteolytic degradation product of β -expansin.

Discussion

Silk elongation kinetics

Inhibition of silk elongation after pollination is a well known (Bassetti and Westgate, 1993a; Carcova *et al.*, 2003) but rarely studied phenomenon, and one of the main goals of the present study was to elucidate the mechanism involved in this growth response. Analysis of silk elongation kinetics under field and greenhouse conditions showed that silk elongation was considerably down-regulated beyond 12 h after pollination (Fig. 1).

Notably, unpollinated silks showed a marked increase in elongation during the night and early morning, and a decrease in elongation during the day time: 10:00 h to 16:00 h. Similar observations were made by Westgate and Boyer (1985). It is likely that turgor and transpiration play important roles in silk elongation. Schoper and Martin (1989) provided evidence that the rate of transpiration can largely explain the differences of turgor in two maize varieties: the hybrid with the lower rate of transpiration exhibited higher turgor and higher elongation. Moreover, silks have limited or no capacity for osmotic adjustment (Westgate and Boyer, 1985; Schoper *et al.*, 1987; Schoper and Martin, 1989). In maize, transpiration rates in general are the highest during late day time and the lowest during pre-dawn (Westgate and Boyer, 1984). Based on such a pattern of transpiration, silks can be expected to have higher turgor and consequently increased elongation growth during the night and early morning. During the day time the reverse would be true.

Effect of pollination on cell wall structure: cell wall rigidification and inhibition of silk elongation

Since the plant cell wall is a key regulator of cell enlargement, it was postulated that one or more mechanisms involved in down-regulating wall loosening are responsible for the inhibition of silk elongation. Stress/strain analysis, a technique which provides direct evidence of wall structural alterations (Cosgrove, 1993), and wall susceptibility (in terms of wall extension) to exogenously added pollen β -expansin provided evidence that pollination induces a rigidification of silk cell walls (Figs 2, 3). For example, wall plastic compliance was significantly reduced by 6 h post-pollination. The fact that wall rigidification precedes the major slowing of silk elongation suggests that the wall changes and the growth phenomenon may be causally related. The conclusive establishment of cause and effect requires further work on the molecular basis of the wall rigidification process.

Several studies have documented that a reduction in wall extensibility (as measured by stress/strain and equivalent applied force methods) is involved with some other plant growth cessations (Kutschera, 1996; Fan *et al.*, 2006). On

the other hand up-regulation of plant growth is sometimes associated with a more pliant wall. Promotion of cucumber hypocotyl growth by gibberellic acid was closely linked to an increase in the plastic compliance (Taylor and Cosgrove, 1989). Increases in rice coleoptile elongation were correlated with higher wall extensibility (Tan *et al.*, 1991). The present results are also consistent with documented trends concerning wall susceptibility to expansin and plant growth. Cosgrove and Li (1993) reported that oat coleoptile walls from non-growing regions were significantly less susceptible to expansin-induced extension. Experiments in non-growing regions of maize root have also produced similar results (Wu *et al.*, 1996). These results support the idea that pollination-induced cell wall rigidification plays a key role in silk growth inhibition. The molecular mechanism of the wall response to pollination is yet to be elucidated. Cell wall rigidification may occur by a number of mechanisms including the coupling of feruloyl side chains attached to wall polysaccharides (Fry, 2004), formation of isodityrosine links (Prasad and Cline, 1987), and the strengthening of pectin-calcium networks (McCann *et al.*, 1994).

Signalling involved in wall rigidification and silk growth cessation

Comparative analyses of pollen tube location and wall extensibility indicated that the decrease in wall plastic compliance moves towards the ovary along the silk and only occurs after a pollen tube has traversed a given region of silk (Fig. 5). This indicates that the signalling behind cell wall alterations does not originate in the ovary due to fertilization but is locally initiated by the presence and/or the movement of the pollen tube(s). Moreover, once a pollen tube has traversed a region of silk, it takes time for the wall rigidification to become apparent. These findings suggest that at least some of the signals involved in the silk elongation response to pollination may be locally generated within silks. The nature of signalling involved in wall rigidification and silk growth cessation indeed opens up an interesting area of future research.

Effect of pollination on expansins

Since a large body of research has established that expansins are involved in plant growth stimulation, it was hypothesized that silk growth inhibition is associated with a down-regulation of expansin abundance and/or activity. However, three lines of experimental evidence contradicted this proposition. First, although silk elongation was considerably down-regulated beyond 12 h post-pollination, the rates of acid-induced extension were not significantly reduced even as late as 44 h post-pollination (Fig. 6). Secondly, pollination did not cause a significant decrease in wall stress relaxation throughout the entire time scale (5 min) examined (Fig. 7). A limitation of the latter method is that any relaxation event(s) that might occur outside the time scale of detection are not recorded. This is important because different expansins have been shown to enhance

stress relaxation at different time scales. For example, two different α -expansins from cucumber enhance stress relaxation at 1–30 s and >100 s time scales (McQueen-Mason and Cosgrove, 1995). So, the possibility that the method used was not able to detect changes in the stress relaxation spectra which in theory could be related to the activity of hitherto untested expansins cannot be ruled out. Despite this limitation, the results obtained by acid-induced extension and wall stress relaxation collectively suggest that pollination does not cause a significant reduction in endogenous expansin activity.

The third line of evidence for a lack of expansin down-regulation by pollination stems from experiments on protein abundance. These experiments indicated that at least two putative α -expansins (~28 kDa) and at least two putative β -expansins (~33 kDa) can be extracted from silk walls using NaCl. At least one putative β -expansin (~33 kDa) was also detected in SDS extracts. According to immunoblot analyses, none of these proteins decreased considerably compared with controls by 24 h post-pollination, a time point well beyond the slowing of silk elongation (Figs 8, 9). Similar observations of growth inhibition without a concomitant decrease in expansin abundance have been reported. Inhibition of cell elongation in basal root segments in maize under water stress did not involve a reduction in the abundance of α -expansins (Wu *et al.*, 1996). The abundance of expansin protein as detected by anti-LeEXPA1 antibodies was similar in light- and dark-grown tomato hypocotyls even though in the dark the elongation rate was six times higher than that in the light (Caderas *et al.*, 2000). The general conclusion of expansin activity and protein abundance measurements is that the mechanism of pollination-induced inhibition of silk elongation is largely independent of a down-regulation of expansin protein abundance. It should, however, be cautioned that the present methods did not allow evaluation of changes in either the abundance or the activity of individual expansins.

It is interesting to note that despite significant decreases in wall compliance (plastic and elastic) and susceptibility to maize pollen β -expansin, the reduction in rates of acid-induced extension was much smaller. According to the immunoblot signals, β -expansin abundance increased markedly after pollination (Fig. 9A), probably due to Zea m1 from pollen tubes or the up-regulation of the expression of silk β -expansin(s) by pollination. During the *in vitro* measurement of native wall creep of isolated walls, increased expansin abundance may compensate for the decreased plastic compliance.

Cell wall rigidification, polyspermy, and disease resistance

Prevention of polyspermy (fertilization of a female gamete by more than one sperm) is essential for the reproductive success of many species. In maize, at least two types of barriers to polyspermy have been discussed in the literature. One barrier involves measures to reduce the number of pollen tubes entering the embryo sac. The second concerns

the short-range guidance of a pollen tube through the micropyle by Egg Apparatus 1, a protein specifically expressed in the maize egg apparatus (Marton *et al.*, 2005). In addition, indirect experimental evidence in support of the existence of polyspermy block on the egg itself has been presented (Spielman and Scott, 2008). As shown in Fig. 4, numerous pollen tubes are present at the tip region of the silk. This number gradually declined towards the base of the silk and only 1–2 pollen tubes entered the ovary. According to Heslop-Harrison *et al.* (1985), several mechanisms help reduce the number of pollen tubes entering the ovary. There is competition among pollen tubes to enter the receptive trichomes and transmitting tracts. The significance of this competition is illustrated by the sharp decrease in the number of pollen tubes at the tip-most 0.5 cm of silks. Heslop-Harrison *et al.* (1985) also stated that a constricted zone of the transmitting tracts in the upper ovary wall contributes to the reduction in the number of pollen tubes reaching the micropyle.

The present results support the idea that silk cell wall rigidification is yet another mechanism to reduce the number of pollen tubes reaching the ovary. With regard to this, two points merit discussion. First, the decrease in wall extensibility occurred only after pollen tubes traversed a given region of silk. Secondly, biochemical and functional analysis of Zea m1 has provided evidence that wall loosening is required for successful pollen tube growth within silks (Cosgrove *et al.*, 1997; Li *et al.*, 2003; Valdivia *et al.*, 2007, 2009). Therefore, one might expect that a rigidification of the wall would have the opposite effect. Consequently, the rigidification of silk walls would potentially serve as a strategy to select for the fastest growing pollen tubes.

Valdivia *et al.* (2006) provided evidence that delayed silk senescence due to slower pollen tube growth puts maize ears at higher risk of fungal infection. Snetelaar *et al.* (2001) reported that pollination reduces the risk of *Ustilago maydis* infections of maize ovaries, probably due to tissue collapse at the silk-ovary junction. Pollination-induced cell wall rigidification observed in the present experiments may also play a protective role against fungal pathogens. Reinforcement of cell walls is a well documented physical defence response against invading pathogens (Showalter *et al.*, 1985; Facchini *et al.*, 1999). Pollen tubes enter the receptive trichomes by pushing between the cells (Kroh *et al.*, 1979), a process that would create multiple openings in the silk. Such a situation can potentially put the plant at risk of pathogen infection. Therefore, it would be advantageous for the silk walls to become rigidified, thereby hindering both fungal penetration into and hyphal growth within the silk. In the mutant line used by Valdivia *et al.* (2006), cell wall rigidification would presumably have been delayed (along with senescence) due to slower pollen tube growth. It should be noted that this kind of pollination-induced defence response, if it truly occurs, is a precautionary mechanism. Indeed, examples of such events involving very different elicitors have previously been reported (Russo *et al.*, 1997; Hatcher and Paul, 2000; Heil and Bostock, 2002).

Conclusions

The results of this study enable a model to be proposed describing the fine-tuning of some of the biochemical and physiological processes which accompany silk elongation and pollen tube growth. It can be suggested that increased abundance of β -expansin protein, probably due to *Zea m1* from pollen tubes or local expression of silk β -expansin, loosens the silk cell walls, thereby enabling the growth of pollen tube(s) through a given region of transmitting tract. However, once the fastest growing pollen tube(s) have grown through a silk segment, the cell walls become rigidified, impeding the progression of their slower growing counterparts. In addition to the effect on pollen tube growth, wall rigidification also contributes to the slowing of silk elongation. In this proposed model, wall rigidification is part of a complex process that controls pollen tube growth, silk elongation, and probably resistance to disease.

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