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POLLINATION-INDUCED INHIBITION OF MAIZE SILK ELONGATION

A Thesis in

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by

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ABSTRACT

In response to pollination maize silks undergo an accelerated process of senescence that includes an inhibition of elongation. The inhibition of silk elongation became apparent beyond 12 h after pollination. However, the possibility that the reduction in elongation begins earlier than this can not be ruled out. Treatment with dead pollen indicated that the silk growth response requires interaction with viable pollen. Moreover, pollination of some silks on an ear did not have a long distance systemic effect on the elongation of un-pollinated silks on the same ear at 32 h post-pollination. Endogenous expansin activity, as evaluated by acid-induced extension and wall stress relaxation was not significantly reduced after pollination. Immunoblot analyses indicated that the abundance of either α- or β-expansin protein was not considerably decreased in response to pollination. In addition, extractable α-expansin activity was similar in control and pollinated silk walls. These findings suggest that the mechanism of pollination-induced inhibition of silk elongation is largely independent of changes in expansin abundance or activity. On the other hand, pollinated silk walls were less susceptible to extension by pollen β-expansin. Additionally, stress/strain analysis indicated a significant reduction in the wall plastic extensibility 6 h post-pollination. This reduction in the plastic extensibility only occurred after pollen tubes have traversed through a given region of silk. Numerous pollen tubes were initiated at the silk tip. This number gradually declined along the length of the silk and only 1-2 reached the ovary even after 24 h. Based on the timing of the reduction in wall extensibility and the slowing of silk elongation, these two phenomena may be causally related. In addition, it is possible that reduced wall extensibility is a mechanism to control the number of pollen tubes reaching the ovary.

Finally, compared to controls, pollinated silk walls had more carboxylic acid groups and phenolic substances. Wall polymer cross-linking by the coupling of feruloyl groups attached to glucuronoarabinoxylan or the ionic bridging of carboxylic acid groups in pectin may be the basis of the reduction in wall extensibility.

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Chapter 1

Introduction

1.1. Maize Reproductive System

Maize is a monoecious plant. The male and female florets are borne on different inflorescences which are located at physically separate parts of the plant. The male inflorescence, referred to as the tassel, arises from the shoot apical meristem. The tassel has a central axis (spike) with lateral branches. Paired spikelets are borne on the main axis and the lateral branches. Each spikelet has two florets (Cheng and Pareddy, 1994). There are 3 anthers in each floret and a single anther alone is reported to produce 2000-2500 pollen grains (Kieselbach, 1949; Miller, 1985). The ear or the female inflorescence originates from axillary buds (Cheng and Pareddy, 1994). It also has a main axis (spike) along which are produced spikelet pairs. Each spikelet primodium gives rise to two florets. However, one of them aborts during early development leaving only a single functional floret. Each functional floret has an ovary with an elongated silk (style). Along the length of the silks are produced structures called receptive trichomes. These are considered to be the main structures involved in pollen capture and germination (Miller, 1919; Kieselbach, 1949; Heslop-Harrison *et al*., 1984).

Initiation and Elongation of the Silk

During development the silk first appears as a ridge in the ovary wall of the aforementioned functional floret (Miller, 1919; Cheng and Pareddy, 1994). Spikelet differentiation along the main axis of the ear occurs in acropetal sequence. Consequently, silk initiation on spikelets at

different positions along the main axis is asynchronous (Carcova *et al*., 2003). The early elongation of the silk is accompanied by cell division in the basal region. Cell division progressively declines and thereafter elongation occurs exclusively through cell elongation (Heslop-Harrison *et al*. 1984). During early times after their initiation, silks elongate within husks. Silk emergence beyond the husks is referred to as silking.

Silk Elongation in the Absence of Pollination

If left un-pollinated after emergence, silks continue to elongate for several days, the duration being dependent on maize line and the growth conditions (for example field or greenhouse). Westgate and Boyer (1985) reported that cell elongation occurs along the entire length of the silk. Studies by Schoper and Martin (1989) and Bassetti and Westgate (1993) have indicated high rates of silk elongation immediately after emergence beyond husks. Afterwards the elongation rate declines over several days and about 8-10 days from emergence silks naturally start to senesce (Bassetti and Westgate, 1993). Factors affecting silk elongation during this phase have not been extensively studied. Schoper *et al* (1989) provided evidence that turgor and transpiration play key roles in silk elongation. According to Westgate and Boyer (1985), a water potential less than -0.75 MPa arrests silk elongation.

Silk Elongation after Pollination

Pollination inhibits silk elongation. This can be considered as part of an accelerated senescence process induced by pollination. Though this is a well known phenomenon which is used by farmers as an indicator of successful pollination, very little experimental work has been focused

on it. To my knowledge there is only one study that quantitatively evaluated this phenomenon. Carcova *et al.* (2003), who evaluated silk elongation with respect to thermal time, reported a dramatic decrease in elongation after pollination.

The above discussion indicates the limited nature of our knowledge about the physiological control of silk elongation. Moreover, to my knowledge nothing is known about the mechanism of the inhibition of silk elongation after pollination. Research described in this thesis was conducted to elucidate the mechanism underlying the silk growth response to pollination. I used the biophysical explanation of cell growth as a foundation for the design of my experiments.

1.2. Cell Growth: Biophysical Explanation

Growing plant cells have a turgor pressure of about 0.3 to 1 MPa. Such turgor pressures can generate tensile stresses in the range of 10 to 100 MPa in the cell wall. To withstand high tensile stress, the primary cell wall of the growing cells must be structurally strong. For cell growth to occur, the wall needs to enlarge without becoming structurally weak. This is achieved by a process termed **wall yielding,** i.e. a controlled and an irreversible expansion of the cell wall driven by turgor-generated tensile stress (Cosgrove, 2003).

Wall Yielding and Wall Loosening

Wall yielding is not a simple viscoelastic creep of wall polymers, i.e. it is not a mere structural deformation of the wall in response to turgor-generated wall stress. A large body of evidence suggests that wall yielding involves one or more **wall loosening** processes. **Wall loosening is**

defined as a modification of the cell wall that leads to its stress relaxation and irreversible expansion (Cosgrove, 2003). This is referred to as a chemorheological creep of wall polymers as opposed to simple viscoelastic creep. **Stress relaxation** is the decrease in wall stress without a change in wall dimensions. Since wall stress is the Newtonian counterforce of cell turgor, a reduction in wall stress results in a decrease in cell turgor and cell water potential. This generates the necessary water potential gradient for water uptake into the cell which leads to cell enlargement, i.e. growth (Cosgrove, 1993; Cosgrove, 2003). According to this biophysical explanation, the cell wall plays a key role in cell growth. Therefore, I postulated that the inhibition of silk elongation after pollination involves changes in silk cell walls. These putative wall changes can be broadly divided into two categories: **changes in wall loosening processe**s and **changes in wall structure**. Prior to further discussing these two aspects, it is pertinent to introduce the chemical and structural nature of the plant cell wall. Wall composition is described in more detail in Chapter 4.

1.3. The Primary Cell Wall: Chemical Composition and Structural Models

The cell wall of flowering plants is both compositionally and structurally complex. Cellulose microfibrils, which are composed of linear chains of (1-4)-β-glucose, constitute one of the major structural elements. Microfibrils are embedded in matrix polysaccharides which are categorized into two groups *viz.*, hemicelluloses and pectin. Xyloglucan and glucuroarabinoxylan are the major hemicelluloses described in flowering plants. A third group of hemicelluloses is (1-3), (1-4) β-D-glucan. Glucomannans, galactoglucomannan and galactomannan are other hemicelluloses, which are found in much less quantities. Pectins are a heterogeneous group of

polysaccharides with large and complex molecular structures. These are characteristically rich in galacturonic acid residues. Homogalacturonan and rhamnogalacturonan I are considered as fundamental constituents of pectin. Other complex pectin polysaccharides have also been described. In addition to the aforementioned polysaccharides, the cell wall also contains several groups of structural proteins *viz.*, hydroxyl-proline rich glycoprotein, proline-rich protein, glycine-rich protein, and arabinogalactan proteins (Carpita and McCann, 2000; Cosgrove 2002).

Structural Models of the Plant Cell Wall

Several structural models of the primary cell wall are described in the literature. One of the models proposes that cellulose-binding glycans such as xyloglucan tether the cellulose microfibrils, forming a xyloglucan-cellulose complex. In this scheme pectins and structural proteins fill the spaces in the xyloglucan-cellulose network. A second scheme suggests that xyloglucans are entrapped during the formation of the microfibrils. The untrapped portion of the xyloglucan can then attach to other microfibrils or matrix polymers. Yet another model posits that xyloglucans coat the cellulose microfibrils. In addition to coating the microfibrils, xyloglucan can attach to other matrix polysaccharides. In a fourth model xyloglucans are covalently linked to pectic polysaccharides forming a large supermolecule. Xyloglucans further adhere to the surface of cellulose microfibrils thereby embedding them in this supermolecule. It should be noted that depending on plant species, cell type and developmental stage the precise structural arrangements may change. Moreover, it is possible that some elements of each of the above models might be partially contributing to the overall architecture of the wall (Cosgrove, 2003; 2005).

1.4. Wall Extensibility

In general, wall extensibility can be defined as the ability of the wall to extend irreversibly (Cosgrove, 2003). However, it is more important to define **wall extensibility in the context of cell growth**. The Lockhart Model on cell growth can be used for this purpose. In this model,

$$GR = \varphi (P-Y) \qquad (1)$$

where **GR** is the **growth rate** (h^{-1}), **φ** is **specific cell wall extensibility** (h^{-1} MPa^{-1}), **P** is the cell **turgor pressure** (MPa) and **Y** is the **yield threshold** (MPa) or the turgor pressure at which growth ceases. Cosgrove (2003) considered both φ and Y as wall yielding properties because either an increase in φ or a decrease in Y results in a more extensible wall. Consequently, wall extensibility relevant for growth can be considered to encompass both φ and Y (Cosgrove, 2003). Wall extensibility described this way is affected by both wall loosening processes (Section 1.2) and structural features (Section 1.3) of the wall.

1.5. Measurement of Wall Extensibility

Cell wall extensibility can be measured using diverse methods. These methods are based on different principles and assumptions, thus giving rise to complications when relating the results to plant growth. Broadly, the methods can be categorized into two classes: *in-vivo* methods and *in-vitro* methods.

1.5.1. *In-Vivo* Techniques

One classical method of measuring wall extensibility involves the monitoring of changes in growth rate in response to changing turgor. Then, equation (1) can be used to estimate both φ and Y. Another technique used is *in-vivo* stress relaxation where an excised wall specimen is prevented from taking up water or losing water due to transpiration. Under these circumstances the relaxation of the wall (see Section 1.2) causes the cell turgor to decay to Y. A third method determines the extension of a wall sample in response to an applied force. This technique provides a measure of the viscoelastic (structural) properties of the wall. However, the viscoelastic parameters obtained can not be used to determine either φ or Y (Cosgrove, 1993; Cosgrove, 2003).

1.5.2. *In-Vitro* Techniques

These methods are referred to as *in-vitro* methods because tissue segments in which the protoplasm is no longer living are used. Protoplasm is disrupted either by a freeze/ thaw cycle or by boiling in methanol. These are effectively isolated wall samples where wall synthesis and some other biochemical processes are blocked and I will hereafter refer to them as isolated walls. A major limitation of using *in-vitro* methods is that the wall extensibility measured can not be directly related to the physiological parameters, φ and Y. Since much of the work described in this thesis involves *in-vitro* biophysical analyses of silk cell walls, I will describe the methods used in some detail. Descriptions are based on Cosgrove 1989 and 1993a.

Acid-induced Extension (Native Wall Creep)

This technique evaluates wall extensibility as the time-dependent extension of a wall sample under constant force. When segments of growing plant tissue are treated with acidic solutions, they undergo a rapid burst of growth. This is referred to as acid growth (Cleland *et al.*, 1987). Isolated walls, when held under tension, also exhibit this phenomenon. In this method a wall sample is clamped on an extensometer under constant load and the extension measured using a position transducer. The sample is initially bathed in neutral buffer. Then the bathing buffer is replaced by an acidic (pH < 6) solution. The extension is monitored for long periods of time (2.5 h or more). Though the wall extensibility determined using this method can not be directly related to φ and Y, it is suggested that this type of long-term wall creep is similar to the type of wall extension that occur during normal cell growth (Cleland *et al.*, 1987; Cosgrove, 1989). A variation of this method is to use heat-inactivated walls to test the ability of different agents to induce wall extension. I will refer to this as a **wall reconstitution assay**.

Wall Stress Relaxation

In this method an isolated wall sample is rapidly extended thereby generating a tension/ stress on the wall. Subsequently the sample is held at constant length. Molecular rearrangements of the wall polymers cause a decrease in the wall stress. This is measured by a force transducer. The change in force (dF) against the change in log time (dlogt) is plotted to generate stress relaxation spectra. This technique provides information on the rate of relaxation at different time-scales. For example, relaxation events that occur between 1-30 ms and those that occur between 100-200 ms presumably indicate different molecular rearrangements. Changes in wall extensibility

are determined as changes in the rates of stress relaxation. In some cases, the minimum relaxation time (To), defined as the relaxation time for the fastest relaxing event, has been used to express wall extensibility (Masuda, 1978)

Stress/strain (Instron) Analysis

Stress/strain analysis involves the extension of a wall sample by an applied force. The method directly measures the structural or viscoelastic properties of cell walls. Stress is the force divided by the area across which the force is applied. Strain is the relative change in length. In this technique a wall sample is extended in two cycles in between which it is brought back to original length. During the 1st extension, wall stress builds up in a curvilinear manner. The 2nd extension produces a different stress/ strain curve. Any further extensions using the same load produce stress/ strain curves similar to the second curve. The stress/ strain curve from the 1st extension represents two types of deformations: an elastic (or reversible) deformation and a plastic (or an irreversible) deformation. The 2nd stress/ strain curve only includes the elastic deformation. The slope of the 2nd extension is defined as the elastic extensibility. The difference between the slopes of the 1st and 2nd stress/ strain curves is the plastic extensibility. Plastic and elastic extensibilities are expressed as % extension per 100 g force.

1.6. Wall Loosening Agents

It was mentioned earlier that wall yielding associated with cell growth involves one or more wall loosening processes. Wall loosening increases cell wall extensibility. Several agents are being extensively evaluated as wall loosening agents.

Expansins

Expansins were first discovered as mediators of acid-induced extension of heat-inactivated cell walls (McQueen-Mason *et al.*, 1992). Based on amino acid sequence and phylogenetic analyses, expansins are described as a multigene superfamily divided into 4 families: α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Sampedro and Cosgrove, 2005). Candidate proteins from both α- and β-expansin families have been shown to have the ability to extend heat-inactivated cell walls. Moreover, these candidate proteins can enhance the stress relaxation of isolated walls (Li *et al.*, 1993; McQueen-Mason *et al.*, 1992 and 1995; Wu *et al.*, 1996; Li *et al.*, 2003). These findings among many others provide strong support to the idea that expansins are wall loosening proteins. The mechanism by which expansins loosens the cell wall is not well established. However it is proposed that they disrupt non-covalent bonds among wall polysaccharides (Cosgrove, 2005). Wall loosening is a biochemical characteristic of expansins and this can be utilized in multiple functional roles during plant growth and development. Many studies suggest the involvement of expansins in the growth of diverse plant organs (Cosgrove, 2005 and references therein). Expansins are also implicated in abscission and fruit ripening, thereby suggesting a functional role in wall disassembly (Rose *et al.*, 1997; Brummel *et al.*, 1999a, 1999b, and 2002; Harrison *et al.*, 2001; Mbeguie *et al.*, 2002). There is also evidence that expansins are involved in xylem cell differentiation (Im *et al.*, 2000).

Xyloglucan Endotransglucosylases/hydrolases, Endoglucanases and Hydroxyl Radicals

Xyloglucan endotransglucosylase/hydrolases (XTH) comprise a family of enzymes that restructure xyloglucan. Most members of this family exhibit strict xyloglucan endotransglucosylase (XET) activity, i.e. cleave and join xyloglucans. Some members are predominantly endohydrolases (Fry, 2004). XTHs have been implicated in various functions including wall loosening. XTH treatment has been reported to increase the wall extensibility measured by applied force methods similar to stress/ strain analyses (Kaku *et al.*, 2002). However, these enzymes have failed to extend isolated walls in native creep assays (Cosgrove, 2005; Saladie *et al*., 2006).

Hydroxyl radicals ($^{\cdot}OH$) also have the ability to extend isolated cell walls (Schoper *et al.*, 2002). However, these can only induce small amounts of wall extension. Moreover, unusually high concentrations of $^{\cdot}OH$ are needed. It is questionable whether such high concentrations are indeed found in plant cells (Cosgrove, 2005). Yuan *et al.* (2001) reported the ability of a fungal endoglucanase to induce the extension of isolated walls, thereby suggesting a possible role of plant endoglucanases in wall loosening.

1.7. Cell Wall Rigidification

As opposed to cell wall loosening processes which increase wall extensibility, reactions that rigidify the wall have the opposite effect. Diverse wall polymer cross-linking reactions are proposed to be involved in cell wall rigidification. These processes are discussed in more detail in Chapter 4 thus only a brief description is included here. Feruloyl groups attached to wall

polysaccharides can undergo oxidative phenolic coupling. Such a reaction is thought to cross-link wall polymers (Fry, 1986; Grabber *et al.*, 1995; Fry, 2004) and is generally considered as a major mechanism of wall polymer cross-linking in grasses (Carpita *et al*., 2000). Tyrosine residues in hydroxyproline-rich glycoproteins (HRGPs) can also be cross-linked to form isodityrosine (Brett and Waldron, 1996). Pectin de-esterification leads to the release of free carboxylic acid groups that can form ionic bonds with Ca^{2+}, resulting in a more rigid pectin gel (Yamaoka *et al.,* 1983; Goldberg, 1984; McCann *et al*., 1997). Other structural modifications, such as polymers becoming less branched, can also make the cell wall more rigid (Cosgrove, 1997, 2002).

1.8. Other Processes that Affect Cell Growth

The above discussion was confined to some of the cell wall changes (wall loosening and wall rigidification) that can potentially affect growth. One further cell wall related process is the regulation of wall pH. The cell wall pH of growing cells is typically maintained 4.5 to 6. Wall loosening processes such as those mediated by expansin is under pH regulation. Many agents, including auxin, regulate cell growth at least in part by changing wall pH (Cosgrove, 2005). Inhibition of maize root and leaf growth under water deficit was correlated with inhibited wall acidification (Fan and Neumann, 2004; Van Volkenburgh and Boyer, 1985; Bogoslavsky and Neumann, 1998). The importance of turgor maintenance for cell growth is clearly evident in the Lockhart Model (see equation (1)). This means that processes regulating water uptake into the cell are of utmost importance for growth. A case in point is the already mentioned inhibition of silk elongation at water potentials less than -0.75 MPa.

1.9. Rationale

The early development of silks and their elongation have been previously studied (Miller, 1919; Bonnet, 1966; Kieselbach, 1949; Heslop-Harrison *et al.,* 1984; Schoper *et al*., 1989; Bassetti and Westgate, 1993; Carcova *et al*., 2003). However, silk elongation after pollination has been paid very little attention. To my mind the pollination-induced inhibition of silk elongation is an interesting phenomenon. Moreover, this process is presumably important for the reproductive success of maize. In pre-pollination periods elongating silks are one of the major carbon sinks among maize female floral tissues (Eveland *et al*., 2006). It has been suggested that after pollination carbon is reallocated from elongating silks to the growing pollen tubes and the fertilized ovule (Xu *et al*., 1996). Therefore, the cessation of silk elongation has potential benefits in terms of resources. Valdivia *et al.* (2006) reported a possible link between delayed silk senescence after pollination and a higher incidence of fungal ear rot. This suggests that faster senescence might be advantageous for protection from pathogens. Consequently a detailed understanding of the mechanism(s) involved in the pollination-induced inhibition of silk elongation undoubtedly has significant implications on both basic research and applied agriculture.

In order to elucidate the mechanism of the pollination-induced inhibition of silk elongation, I investigated silk elongation kinetics in relation to changes in cell walls. With respect to wall loosening, the effects of pollination on expansins were evaluated. Experiments were also designed to examine possible structural changes in the wall induced by pollination. Moreover,

the progress of pollen tubes within the silk was also examined. The relationships among silk elongation, changes in the cell wall and pollen tube growth are discussed.

Chapter 2

Characterization of the Silk Elongation Response to Pollination

2.1. Introduction

2.1.1. Silk Elongation after Emergence Beyond Husks

Silks are the pollen receptive organs of maize female florets. They facilitate pollen capture and germination. To fulfill this function silks need to elongate and emerge beyond the husks which enclose them. This emergence is referred to as silking. Silks from the spikelets towards the base of an ear emerge beyond the husks first (Bassetti and Westgate, 1993; Carcova *et al.,* 2003). The rest continue to emerge over a few days (in some cases as long as 8 days) after the first appearance of silks (Bassetti and Westgate, 1993).

If left un-pollinated silks continue to elongate for several days after emergence. Final silk length is variety dependent. Heslop-Harrison *et al.* (1984) reported 65-70 cm maximum silk lengths. This extension in length proceeds through cell elongation (Kieselbach, 1949; Heslop-Harrison *et al.,* 1984; Westgate and Boyer, 1985), which according to Westgate and Boyer (1985), occurs along the entire length of the silk. High rates of silk elongation have been noted immediately after emergence (Schoper and Martin, 1989; Bassetti and Westgate, 1993). Thereafter the elongation rate decreases over several days. Silks naturally begin to senesce 8-10 days after first emergence (Bassetti and Westgate, 1993).

2.1.2. Pollination, Pollen Tube Growth and Silk Elongation

Along the length of the silk are receptive trichomes which function as the major structures of pollen-capture and germination (Miller, 1919; Kieselbach, 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. Transmitting tracts comprise specialized tissue that facilitates pollen tube growth towards the ovary (Miller, 1919; Kieselbach, 1949; Kroh *et al.,* 1979; Heslop-Harrison *et al*., 1984 and 1985) and they span the entire length of the silk to terminate in the upper ovary wall. Thereafter the pollen tubes progress towards the micropyle between the inner ovary wall and the inner integument (Heslop-Harrison *et al*., 1985). Usually only one pollen tube grows through the micropyle to participate in double fertilization (Miller, 1919; Heslop-Harrison *et al*., 1985).

Pollination inhibits silk elongation. From a qualitative perspective this is not a novel observation and indeed it is commonly used by farmers as an indicator of successful pollination. However, to my knowledge there is only one study that quantitatively monitored silk elongation after pollination. In a study that examined silk elongation of two hybrids in relation to flower development and pollination, Carcova *et al.* (2003) reported the silk elongation pattern with respect to thermal time. The authors showed a decrease in silk elongation after pollination. However, the measurements were made at daily rather than hourly intervals, thus limiting their utility in studies concerned with the mechanism underlying the silk elongation response to pollination.

2.1.3. Pollination Instigated Signaling in Silks

Diverse post-pollination signaling events have been reported in maize silks. Pollination induces ethylene synthesis. Moreover, elevated levels of auxin have been reported in pollinated silks (Mol *et al.,* 2004a). Interestingly, these changes in ethylene and auxin can be mimicked to some extent by treating silks with inert quartz sand particles. For example, though with different timing compared to true pollination, there was increased synthesis of ethylene in silks and ovaries. Quartz sand also caused IAA levels to rise. The increases in IAA were lower and slower when compared to true pollinations (Mol *et al*., 2004a). Moreover, Mol *et al*. (2004b) reported that both true pollination and sand treatment induce electrical signals that propagate through the silk to the nucellus. The maturation of egg cells in the ear was stimulated by pollination before actual fertilization, suggesting the existence of long distance communication between the silk and the ovary (Mol *et al*., 2000). Sand treatment can also cause egg cell maturation but to a much lower extent compared to true pollination (Mol *et al*., 2000).

These findings on signaling in silks raise interesting questions about the silk elongation response to pollination. The ability of both viable pollen and sand particles to induce egg cell maturation prompted me to ask whether silk elongation response is really specific to viable pollen, i.e. can it be triggered by mechanical stimulation. Moreover, according to the pattern of silk emergence under natural field conditions, during the early days of silking only some of the silks on an ear are pollinated while the others may still be elongating within the husks. Therefore, it is interesting to examine whether the pollination of some of the silks on an ear has a long distance effect on the un-pollinated silks (This will be referred to as a systemic effect of pollination).

2.1.4. Significance of the Inhibition of Silk Elongation after Pollination

Inhibition of silk elongation can be considered as part of an accelerated silk senescence process triggered by pollination. A large body of research conducted in diverse species support the notion that pollination accelerates floral senescence (O'Neil, 1997). Moreover, this senescence process is considered a measure to save resources (Evanhoe *et al.,* 2002). In pre-pollination periods elongating silks and fleshy floral structures around the ovary are thought to be the major carbon sinks among maize female floral tissues (Eveland *et al.,* 2006). Based on invertase gene expression, Xu *et al.* (1996) suggested that after pollination carbon is reallocated from elongating silks to the growing pollen tubes and the fertilized ovule. Yet another issue of crucial importance is the possible link between delayed silk senescence after pollination and a higher incidence of fungal ear rot (Valdivia *et al*., 2006). Therefore, the inhibition of silk elongation upon pollination is arguably advantageous to the reproductive success of the plant.

Despite its potential benefits to the plant, to date the mechanism(s) underlying the pollination-induced inhibition of silk elongation has not been studied. One of the important prerequisites of such analyses is the establishment of the timing of the phenomenon with respect to pollen deposition/pollination. This is one of the major goals of the experiments reported. In this chapter I describe the silk elongation kinetics in relation to pollen tube progression along the silk. The effect of dead pollen treatment (as a mechanical stimulation) and any systemic effects of pollination on silk elongation are also investigated.

2.2. Materials and Methods

2.2.1. 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, PA during the summer. For greenhouse experiments, plants were grown under standard conditions during the summer.

2.2.2. Controlled Pollinations

In both field and greenhouse experiments, ears were covered with glassine bags prior to silk emergence. Only the upper-most ear of a plant was used. 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: a) full-ear pollinations where all silks on an ear were pollinated using 200 µl of freshly collected pollen, and b) split-ear pollinations where approximately half the silks on an ear were pollinated with 100 µl of pollen; the rest of the silks were left as controls. In the case of a), a separate group of plants/ears were left as controls in which no silks were pollinated. In one set of experiments, dead pollen was used. This pollen had been stored at -80°C for about a year. Prior to use this pollen was heated at 100°C for 10 min. After pollination, ears were again covered with glassine bags to prevent further pollination. When photography was conducted (see Section 2.2.3), further pollination was prevented by bagging the tassels.

2.2.3. Silk Elongation Measurements

Two experimental strategies were adopted *viz.*, a) split-ear pollinated greenhouse experiment using time-lapse photography and b) full-ear pollinated field experiment with destructive sampling. In field experiments, control and pollinated silks were destructively sampled at given times after pollination. For simplicity these times will hereafter be referred to as time after pollination for both control and pollinated silks. Silks were harvested at spikelet position 24 from the base of the ear and their length measured with a ruler. From each ear 2-10 silks were measured.

In greenhouse experiments, split-pollinated ears were photographed at 15-30 min intervals and the length above the husks of the three longest control and pollinated silks was separately determined at given times after pollination. Spot-advanced software (Diagnostic Instruments) was used for length determination in digital images.

2.2.4. Determination of Pollen Tube Growth

Silks and ovaries were destructively sampled at 9, 12, 18, and 24 h 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. They were then serially rinsed with 100, 75, 50, 30 and 0% ethanol. Samples were left in each solution for approximately 15 min. Afterwards, they were cleared with 8 M NaOH for 40-45 min and washed 3 times with distilled water. After storing overnight in distilled water at 4°C, samples were stained with 0.05 % aniline blue in 0.033 M K_3PO_4 in the dark for approximately 20 min. Ovaries were bisected prior to

staining. Ovary and silk samples were observed by UV-fluorescence microscopy. The methods were based on personal communications with K. Carroll.

2.3. Results

2.3.1. Silk Elongation Kinetics

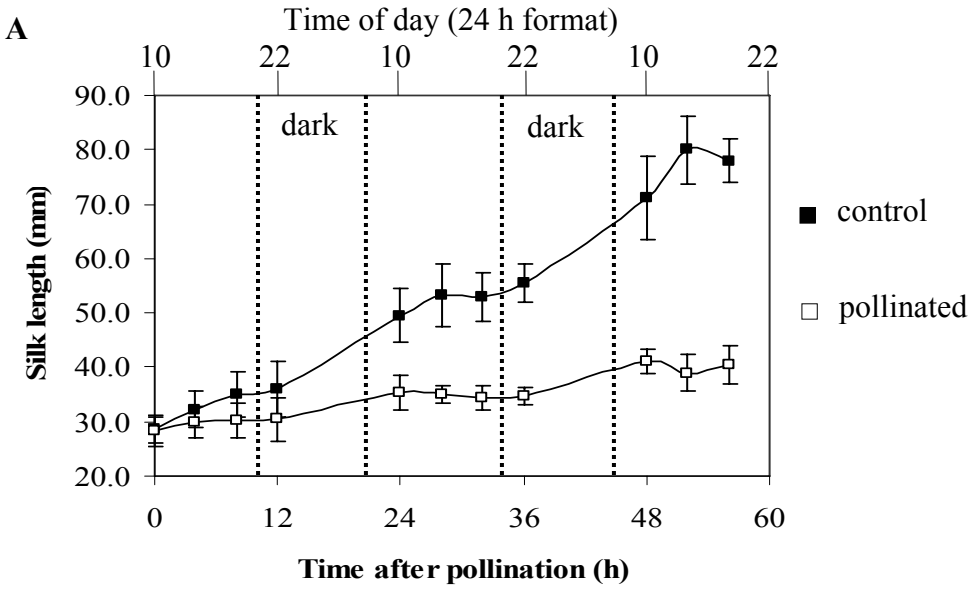
Figure 2.1 shows the phenomenon of focus of this thesis, i.e. silks cease their elongation and senesce after pollination. To determine the timing of the growth response, silk elongation kinetics was characterized both under greenhouse and field conditions.

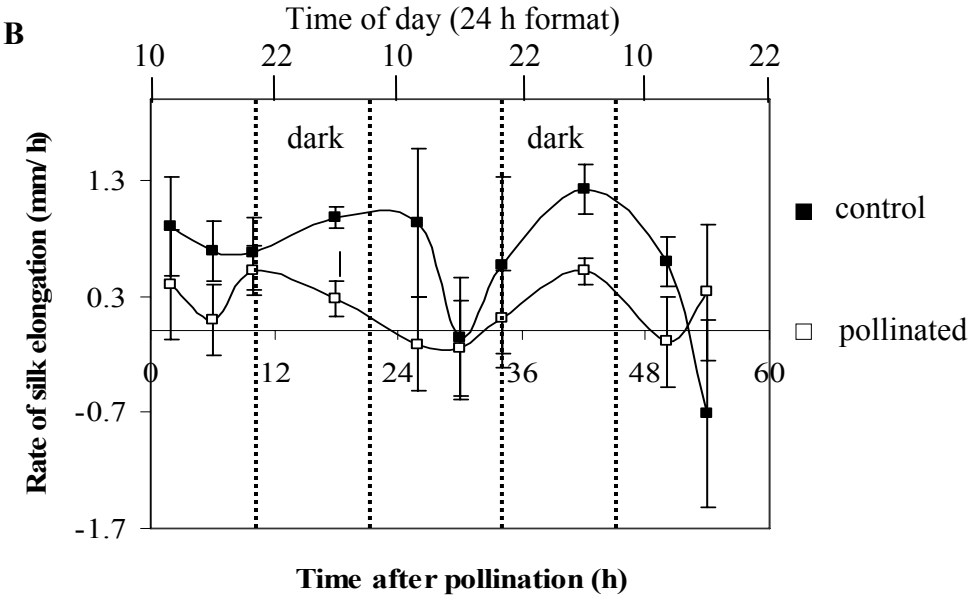
Figure 2.1. Silk senescence (including the inhibition of elongation) after pollination. **A:** Healthy and elongating un-pollinated (control) silks. **B:** Pollinated silks cease their elongation and senesce. **C:** A slit-pollinated ear showing the difference in length between pollinated and un-pollinated silks.

Results obtained from the split-ear greenhouse experiments are depicted in Figure 2.2A-C. Un-pollinated silks continued to elongate during the entire period of measurement (56 h). There was a noticeable increase in length (Figure 2.2A) and rate of elongation (Figure 2.2B) in the dark. In

general the highest rates of silk elongation were noted about 4 am. Compared to controls, the length of pollinated silks was considerably reduced beyond 12 h after pollination. Results also indicate a trend of decreasing silk length even before 12 h post-pollination. Statistically significant decreases in length were noted at 24 h after ($t_9 = 3.06$, $p<0.05$) and beyond. During the entire period of evaluation, pollinated silks only elongated 1.2 cm beyond husks in contrast to the 4.9 cm of the controls. Significant reductions of elongation rates in response to pollination were observed 18 ($t_9 = 3.96$, $p<0.05$) and 42 h ($t_9 = 2.92$, $p<0.05$) after pollination. At other time points, though elongation rates were numerically lower in pollinated silks, statistical significance could not be detected due to the high variability in the data.

Since part of a given silk is covered by husks, the photographic method used in the above experiments does not allow the determination of entire lengths of silks i.e. from the ovary-silk junction to the tip of the silk. Moreover, preliminary observations indicated that the length of silks from the middle and tip-most spikelet positions on an ear, when measured at a given time after first silk emergence, were different (data not shown). Therefore silk elongation was measured using destructive sampling in field experiments in which all silks were collected at spikelet position 24. The results gathered from this approach were similar to those from the split-ear/photography method (Figure 2.3A and B).


A
Time of day (24 h format)
10
22
10
22
10
22
dark
dark
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
Silk length (mm)
0
12
24
36
48
60
Time after pollination (h)
control
pollinated


B
Time of day (24 h format)
10
22
10
22
10
22
dark
dark
1.3
0.3
-0.7
-1.7
Rate of silk elongation (mm/ h)
0
12
24
36
48
60
Time after pollination (h)
control
pollinated

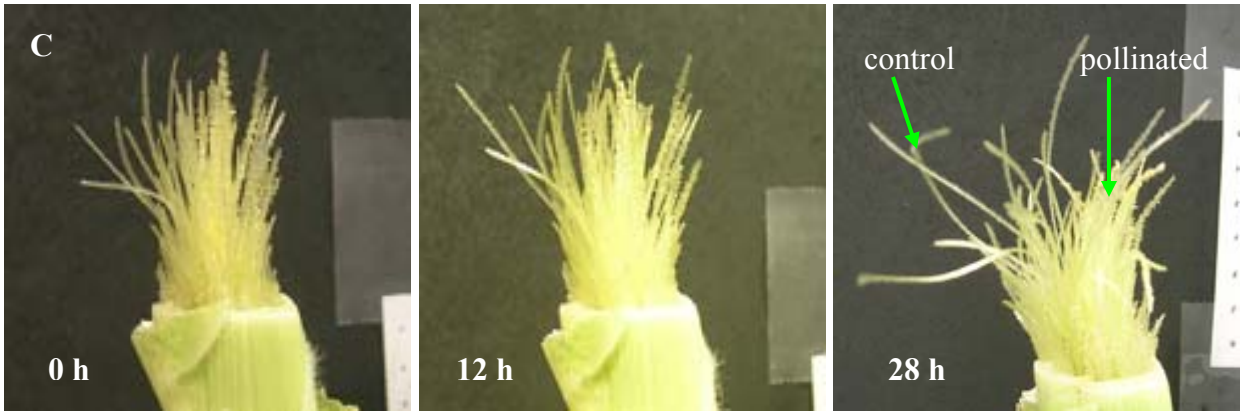
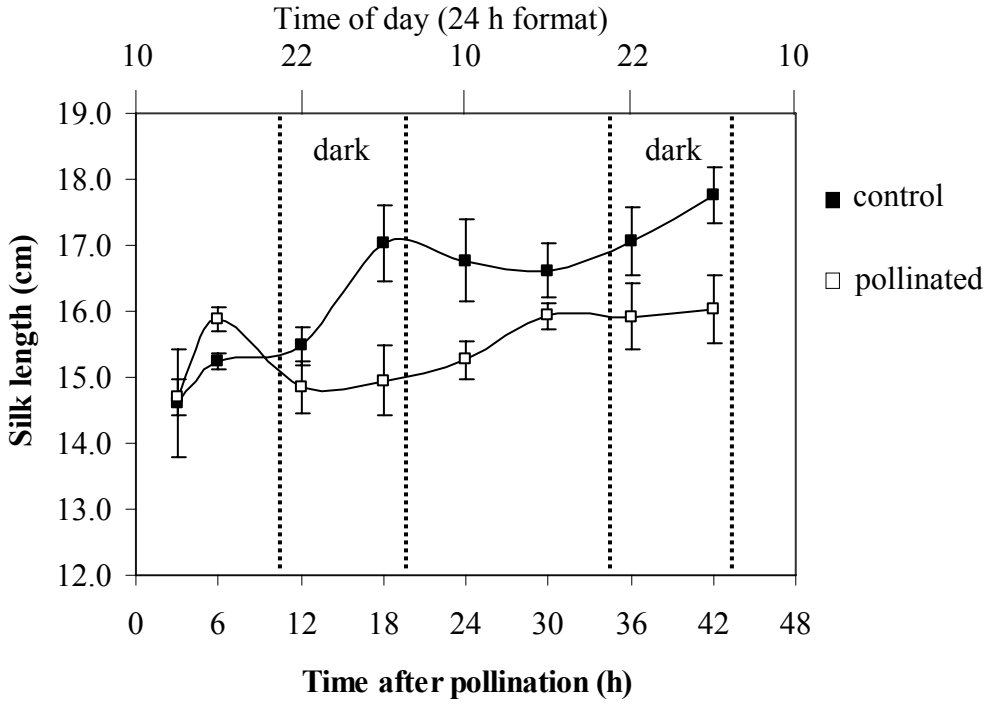


Figure 2.2. Effect of pollination on silk elongation (split-ear pollination experiments in the greenhouse). **A**: silk length beyond the husks; **B**: rate of silk elongation. **C:** images captured at different times after pollination. In each ear, approximately half of the silks were pollinated and the others left un-pollinated (controls). Then each ear was photographed at 15-30 min intervals. 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 and averaged. These averages for individual ears were used to calculate the means shown in **A**. Rate of elongation, **B** is the slope of **A**. Error bars are SEM values (n = 5 ears).

In the field experiment, during the course of 42 h, the pollinated silks elongated only 1.3 cm compared to the 3 cm of controls. Length of pollinated silks was clearly reduced beyond 12 h after pollination. However, significant reductions in length were only recorded at 18 ($t_9 = 2.65$, $p<0.05$) and 42 h ($t_9 = 2.54$, $p<0.05$) after pollination. One curious observation is the increase in length of the pollinated silks compared to controls at 6 h post-pollination. It is likely that this is an experimental artifact because the length decreased rather sharply by ~ 1 cm at 12 h post-pollination. Due to destructive sampling, it was not possible to perform statistical analyses on the rates of silk elongation. However, noticeable decreases in rates could be noted 9-18 h after pollination.

In summary, both experimental methods used to characterize the phenomenon suggest that silk elongation is considerably reduced beyond 12 h after pollination.

A


Time of day (24 h format)
10
22
10
22
10
dark
dark
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
Silk length (cm)
0
6
12
18
24
30
36
42
48
Time after pollination (h)
control
pollinated

B

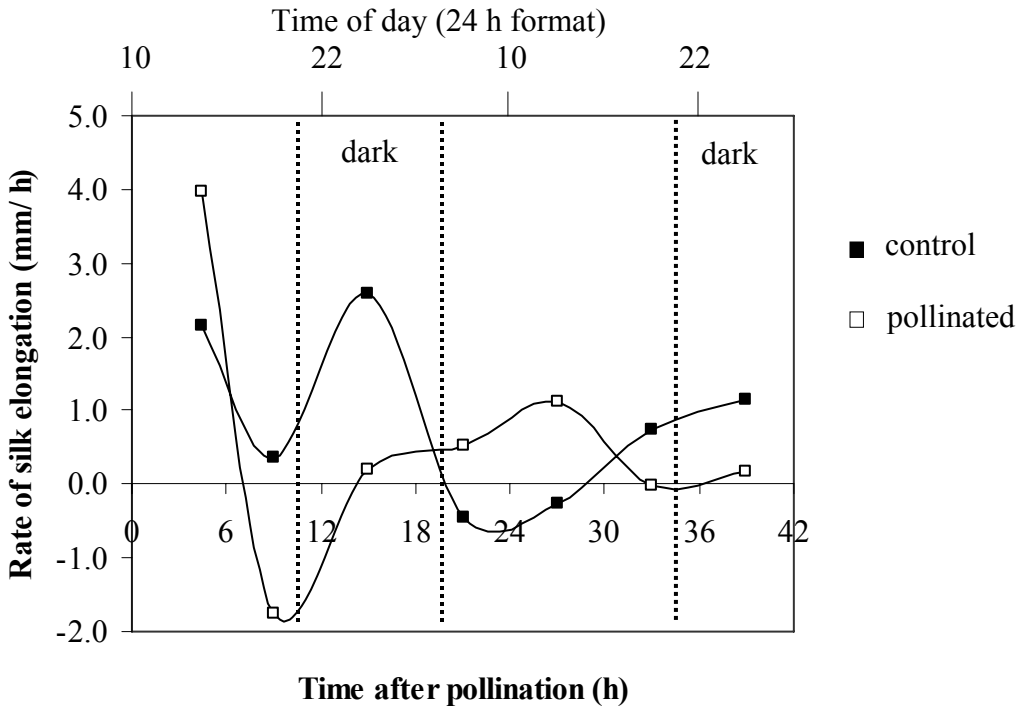

Time of day (24 h format)
10
22
10
22
dark
dark
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
Rate of silk elongation (mm/ h)
0
6
12
18
24
30
36
42
Time after pollination (h)
control
pollinated

Figure 2.3. Effect of pollination on silk elongation (full-ear pollination experiments in the field). Control and pollinated silks were on separate ears. **A**: silk length; **B**: rate of silk elongation. At each time point silks were destructively sampled from spikelet position 24 (counting from the base of the ear) and the length measured. Data in **A** are means +/- SEM (n = 5 ears. In each ear 2-10 silks were measured and the lengths averaged. These average values were used to calculate the mean and SEM for the 5 ears). The rate of elongation, **B** is the slope of **A**.

2.3.2. Progress of the Pollen Tubes along the Silk

To map the progress of the pollen tubes, silks and ovaries from spikelet position 24 were sampled at different times after pollination and stained with aniline blue for UV fluorescence microcopy.

As can be seen in Figure 2.4A, pollen tubes can reach the ovary 9 h after pollination indicating a pollen tube growth rate of ~ 1.6 cm h^{-1} (The silks used were ~ 14.5 cm long.). At all the time points examined plots of the number of pollen tubes along the length of the silk showed a reverse J shape. By 24 h after pollination many of the pollen tubes initiated on a silk had failed to reach the ovary. There seem to be 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 2-2.5 cm proximal to the silk-ovary junction. In between these two regions there was a more gradual decrease in the number of pollen tubes (Figure 2.4B and C). By 18 and 24 h after pollination pollen tubes have progressed well within the ovary.

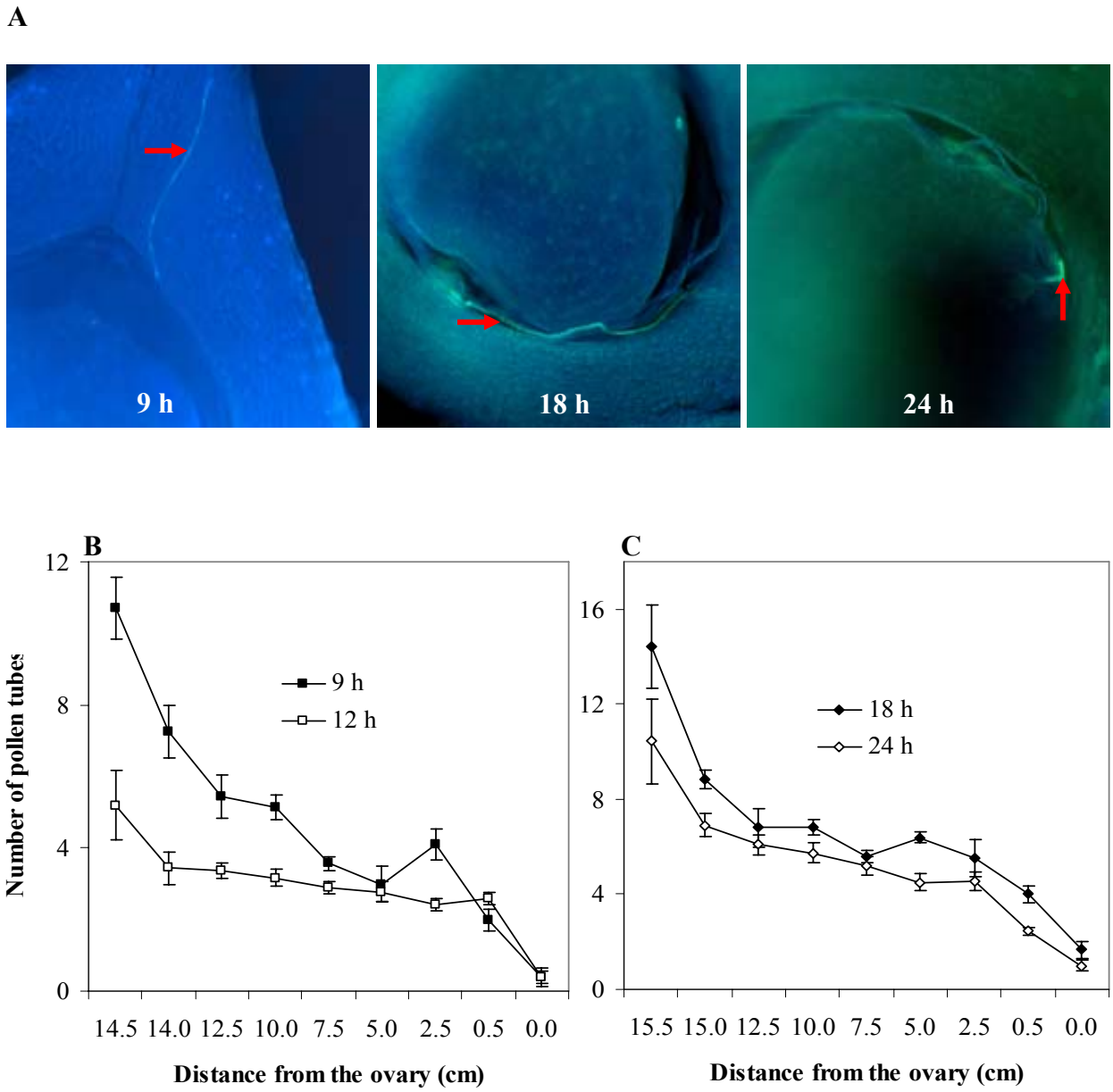


Figure 2.4. The progression of the pollen tubes along the length of the silks. At 9, 12, 18, and 24 h after pollination, silks and ovaries were destructively sampled from ovary position 24. Due to destructive sampling different sets of plants (3 for each time point) were used at different times. Silks were divided into serial segments along their entire length. Ovaries were bisected. Pollen tubes inside silks and ovaries were observed by aniline blue staining followed by UV fluorescence microscopy. **A:** aniline blue stained ovaries 9, 18, and 24 h post-pollination (Arrows indicate pollen tubes.). **B and C:** number of pollen tubes at different distances from the ovary at 9 through 24 h after pollination. Zero distance indicates the inside of the ovary. Data are means +/- SEM (n = 5-12).

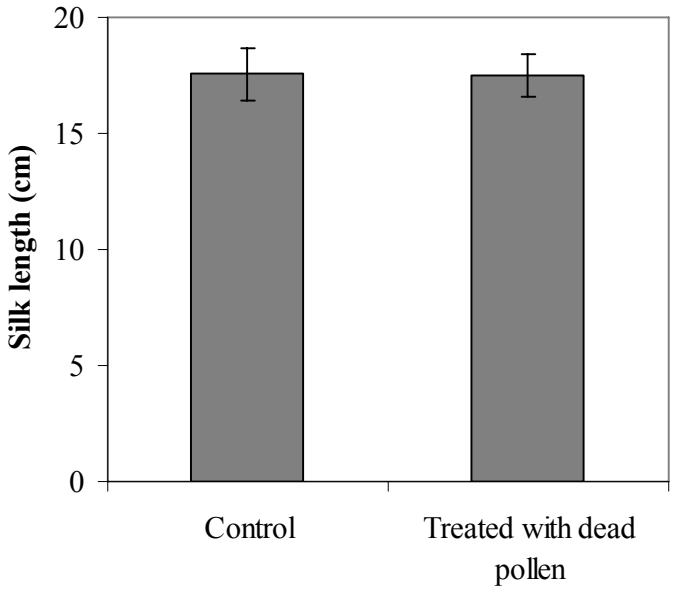
2.3.3. Effect of Dead Pollen on Silk Elongation

The effect of dead pollen was examined using both split-ear treatment and full ear treatments. Dead pollen did not cause a significant reduction ($t_9 = 0.051$, $p>0.05$) in silk elongation by 27 h after pollination (figure 2.5A and B). Inability of these pollen grains to participate in successful pollination was evident by the lack of tissue collapse at the silk-ovary junction. The tissue collapse is a characteristic feature of successful pollen tube growth or fertilization. In addition, during the course of the experiment it was apparent that unlike viable pollen, dead pollen did not attach well to the silks.

2.3.4. Systemic Effect of Pollination on Silk Elongation

To investigate whether the pollination of some silks on an ear has a systemic effect on the elongation of un-pollinated silks, field grown maize plants were given three different treatments a) full-ear pollinations, b) split-ear pollinations and c) full-ear controls. The results are depicted in Figure 2.6. As expected, 32 h after treatment, the pollinated silks were significantly shorter than any type of control ($p<0.01$). However, there was no significant difference in the lengths of the two types of controls at this time ($t_{14} = 0.19$, $p>0.05$). Therefore at least by 32 h afterwards, pollination did not have a systemic effect on silk elongation.

A



B

Figure 2.5. Effect of dead pollen treatment on silk elongation (27 h post-treatment). **A:** Length of un-pollinated (control) silks compared to the length of silks treated with dead pollen. Data shown are means +/- SEM (n = 5 ears). **B:** a split-ear experiment where only about half the silks on an ear were treated with dead pollen. Both untreated controls and silks treated with dead pollen showed similar elongation.

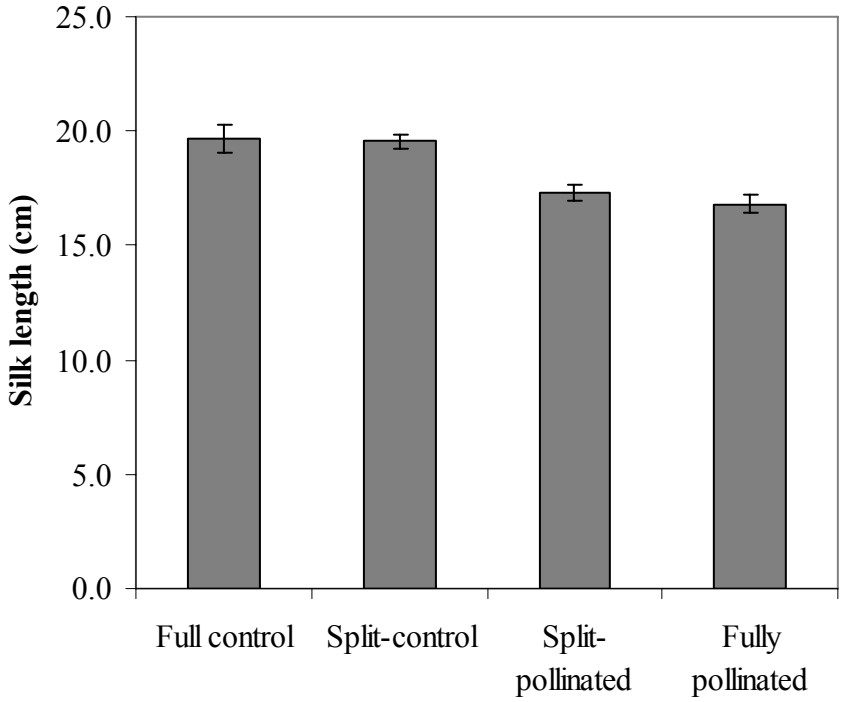


Figure 2.6. Effect of pollinating some of the silks on the elongation of the remaining (un-pollinated) silks on the same ear (Systemic effect of pollination). **Full control:** no silks were pollinated; **split control and split pollinated:** approximately half the silks pollinated and the others left un-pollinated; **fully pollinated:** all silks on the ear pollinated. Thirty two hours after pollination silks were destructively sampled from spikelet position 24 and the length measured. Data are means +/- SEM (n = 5-8 ears. In each ear 5 silks were measured and averaged to calculate the mean and SEM shown.).

2.4. Discussion

2.4.1. Silk Elongation Kinetics

One of the major goals of the experiments described in this chapter was to determine the timing of the inhibition of silk elongation in response to pollination. As shown in Figures 2.2 and 2.3, silk elongation considerably slows down beyond 12 h after pollination. The fact that two different experimental approaches performed under different environmental conditions produced very similar results strengthens the conclusions.

It is not possible to pin-point the exact time at which the silks begin to slow their elongation in response to pollination. The results shown in Figure 2.2A suggest that the slowing of silk elongation possibly **begins** earlier than 12 h post-pollination. However, further experiments are necessary to clarify this issue. The photography method (see Section 2.2.3 and 2.3.1) has several limitations. During the time period when elongation was monitored, husks and the main axis of the ear continue to elongate (data not shown). Moreover, the ovaries become enlarged. This means that "the length beyond the husks" measured here could be slightly different from the absolute length of a given silk. The method is useful to see relative differences in the length of pollinated and control silks, but not for the determination of absolute silk lengths. Consequently, the determination of the exact time of the beginning of the silk growth response would best be done by destructively measuring silk length more frequently prior to 12 h post-pollination. It would also be of interest examine any spatial differences in the elongation rate along the length of the silk in response to pollination.

Interestingly, un-pollinated silks showed a noticeable increase in elongation rate during the night and early morning. In general the highest rates of silk elongation were observed at 1-4 am clock time (Figures 2.2. and 2.3.). As opposed to this, a decrease in silk elongation rate was noted during day time: 10 am – 4 pm clock time. Similar results have been published 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 they examined. 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 leaves transpiration rates in general are the highest from ~ 10 am – 6 pm and the lowest from ~ 12 am – 6 am (Miller *et al.* 1918). Assuming a similar pattern of transpiration, silks can be expected to have higher turgor during the night and early morning thereby increasing their elongation growth. During day time the reverse would be true. Curiously in pollinated silks this presumed response to turgor is almost completely lost.

2.4.2. The Progress of the Pollen Tubes

The pollen tubes reached the ovary 9 h after pollination (Figure 2.4). The rate of pollen tube growth inside silks based on my observations (~ 1.6 cm h^{-1}) agrees with published rates of ~ 1 - 1.6 cm h^{-1} (Bedinger, 1992; Mol *et al.*, 2004b). The timing of pollen tube entry into the ovary suggests that the major slowing of silk elongation probably occurs after the pollen tubes have reached the ovary. Whether these two events are causally related remain to be examined. Once a pollen tube has reached the ovary the silk has served its purpose. Therefore, it is interesting to investigate whether a signal originated at the ovary triggers the inhibition of silk elongation. This will be further discussed in Chapter 5. Based on my observations it is difficult to estimate the exact time of fertilization. Since ovaries were only bisected (not serially sectioned) for aniline blue staining, the entry of a pollen tube into the embryo sac can be easily missed. Also, it is not possible to detect the cellular events associated with fertilization with the methods used.

As shown in Figure 2.4., there were numerous pollen tubes initiated 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. The observation that many pollen tubes initiate on the silk tip but a few enter the ovary has been previously discussed (Heslop-Harrison *et al*., 1985, Mol *et al.,* 2004a). 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. I observed a sharp decrease in the number of pollen tubes at the tip-most 0-0.5 cm of silks suggesting that the aforementioned competition plays a significant role. Heslop-Harrison *et al.* (1985) also described that a constricted zone of the transmitting tracts in the upper ovary wall also contribute to the reduction in the number of pollen tubes reaching the micropyle. This may be one of the reasons for my observation that the number of pollen tubes is reduced somewhat sharply at the basal-most 2-2.5 cm of the silk. However, it should be noted that while it is quite easy to visualize and count the number of pollen tubes in the silks, it is much harder to visualize all pollen tubes within the ovaries.

2.4.3. Effect of Mechanical Stimulation with Dead Pollen on Silk Elongation

In the literature pollination-induced signals are divided into two categories *viz.*, a) primary signals and b) secondary signals. The primary signal may result from the physical contact between pollen and the stigma, from the penetration of the pollen tube into the stigmatic tissue or from pollen-borne chemicals (O'Neill, 1997). I used silk treatment with dead pollen to test whether silk elongation response to true pollination could be mimicked just by comparable (to that of viable pollen) physical contact. The mechanical stimulation by dead pollen does not have an effect on silk elongation, at least until 27 h (Figure 2.5). This finding is reinforced to some extent by the fact that trimming (see Section 2.2.2) of the silks prior to pollination does not cause

silk elongation to cease (This, however, is only a qualitative observation and a quantitative analysis is required to draw definitive conclusions.). My findings suggest that inhibition of silk elongation probably involves signaling mechanism(s) which specifically requires the participation of viable pollen. This is in agreement with the results of several previous studies. For example, mock pollination of orchid flowers using latex beads did not induce ethylene synthesis, which is characterized as an early response to pollination in several species (Zhang *et al.,* 1993). In maize some signaling events as well as developmental processes such as the maturation of the egg cells can be induced both by pollination and treatment with inert quartz sand (Mol *et al*., 2000, 2004a and 2004b). However, the authors noted marked differences between true and mock pollinations.

2.4.4. Systemic Effects of Pollination on Silk Elogation

Some of the developmental changes induced by pollination are initiated in organs distal to the stigma suggesting the existence of inter-organ communication. Transmissible secondary pollination signals are thought to be responsible for this inter-organ communication (O'Neill, 1997). This raised the question whether there is a long-distance systemic effect of pollination on the elongation of un-pollinated silks of an ear. Moreover, it was necessary to validate the split-ear pollination approach used to characterize the silk elongation kinetics. The results obtained indicated that by 32 h after pollination, there is no systemic effect on silk elongation (Figure 2.6). In contrast, Valdivia *et al.* (2006) observed an inhibitory systemic effect on silk elongation 96 h post-pollination. About 28 h after pollination the tissue at the silk-ovary junction collapses. By 48 h after pollination, this tissue collapse leads to the complete detachment of the silk from the

ovary. Consequently, 96 h after pollination can be thought to represent a very different developmental and physiological status compared to 32 h. This could be one of the reasons why my results are different from those of Valdivia *et al.* (2006).

In summary, the results discussed in this chapter indicate that silk elongation is considerably inhibited beyond 12 h after pollination. Interaction with viable pollen, as opposed to dead pollen, is necessary for the inhibition of silk elongation to occur: at the very least dead pollen can not induce the inhibition of silk elongation with similar kinetics to that caused by viable pollen. Furthermore, no evidence for the existence of a systemic effect of pollination on silk elongation was found by 32 h post-pollination.

Chapter 3

The Effect of Pollination on Expansins

3.1. Introduction

3.1. 1. The Cell Wall and Cell Growth

Walls of rapidly growing plant cells are under high tensile stress (in the order of 100 MPa) due to the turgor pressure of the protoplast. As a result, the primary cell wall needs to be strong to withstand this stress. This necessitates the loosening of the wall as a prerequisite for cell enlargement. As a consequence of wall loosening processes, the wall yields irreversibly to turgor with a simultaneous reduction in turgor pressure and the cell water potential. This leads to water uptake into the cell, causing extension of the wall and increase in cell volume (Cosgrove, 1993; Cosgrove, 1997; Cosgrove, 2003; Cosgrove, 2005). Cosgrove (2003) defined **wall loosening** as a modification that leads to the stress relaxation (reduction in wall stress without a change in wall dimensions) and the irreversible expansion of the cell wall. Based on this biophysical explanation the plant cell wall plays a key role in cell growth. Therefore it is possible that the cessation of silk elongation after pollination described in Chapter 2 involves changes in silk cell walls.

Several aspects of the plant cell wall are of relevance in the control of cell enlargement. First is the synthesis and activity of cell wall loosening agents. Some of the wall loosening agents so far being studied are: a) expansins (Cosgrove, 2000; 2003; and 2005), b) wall hydrolases (Yuan *et al*., 2001), including xyloglucan endotransglucosylase/hydrolase (XTH), and c) hydroxyl radicals

(Schopfer, 2001). The second aspect is the compositional and structural features of the cell wall itself which is discussed elsewhere. Other relevant processes include cell wall pH regulation and wall synthesis.

3..1.2. Expansins and Cell Wall Loosening

Expansins are one of the best characterized cell wall loosening agents. Low pH treatment stimulates the extension of the walls from elongating cells, a phenomenon referred to as **acid-induced extension or native wall creep** (Rayle and Cleland, 1970; Cleland *et al*., 1987; Rayle and Cleland, 1992; Cosgrove, 2000; 2003) and this can be inactivated by heat treatment. Expansins were fist discovered based on their ability to extend heat-inactivated cell walls (McQueen-Mason *et al*., 1992). This analysis using heat-inactivated cell walls is referred to as a **wall reconstitution assay** (McQueen-Mason et al., 1992). Expansins also enhance the rates of **wall stress relaxation,** which is measured by holding a wall segment under stress at constant length and monitoring the decay of force on the sample (McQueen-Mason *et al*., 1995). Both acid-induced extension and wall stress relaxation are considered, at least for the time being, hallmark features of expansin activity (Cosgrove, 2003; Li *et al.*, 2003).

Based on amino acid sequence and phylogenetic analyses, expansins are described as a multigene superfamily divided into four families *viz.*, α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Sampedro and Cosgrove, 2005). Candidate proteins tested to date from both α- and β-expansin families have been shown to have the ability to extend heat-inactivated walls (McQueen-Mason *et al*., 1992; Li *et al*., 1993; Wu *et*

al., 1996; Cosgrove *et al*., 1997; Li *et al*., 2003; Cosgrove, 2005). In terms of this activity, α-expansins tested to date are more effective on dicot cell walls as compared to grass cell walls (McQueen-Mason et al., 1992). Li *et al.* (2003) showed that Zea m1 (a maize pollen β-expansin) induced the extension of grass cell walls but not dicot walls. It is important to note that dicot and grass cell walls have distinct compositional differences and are thus described as type I and type II walls respectively (see Chapter 4). Both α- and β-expansins (proteins so far tested) have been shown to enhance cell wall stress relaxation as well (McQueen-Mason *et al.*, 1995; Li *et al.*, 2003).

In the literature two groups of β-expansins have been described: β-expansins from pollen and the β-expansins found in other organs. The later have been generally referred to as **vegetative β-expansins** (Li *et al.*, 2003). Vegetative β-expansins are yet to be biochemically characterized. Attempts to address this issue by producing biologically active recombinant ZmEXPB8 protein have thus far failed (Pers. Comm. L-C. Li). Recently, Lee and Choi (2005) reported that OsEXPB3 is localized to the cell wall. However, whether vegetative β-expansins also have a wall loosening function in plant growth and development is yet to be established (Lee and Choi, 2005).

3.1.3. Expansins in Maize

A total of thirteen expansins have been so far characterized in maize: five α-expansins and eight β-expansins (Wu *et al.*, 2001a). These expansins are expressed in diverse organs and developmental stages. For example, *ZmEXPB1* is highly expressed in maize pollen. One α-

expansin (*ZmEXPA1*) and four β-expansins (*ZmEXPB2*, *ZmEXPB7*, *ZmEXPB8* and *ZmEXPB4* at very low level) are expressed in maize silks.

3.1.4. Expansins and Plant Growth

Expansins are implicated in diverse plant developmental processes including growth, fruit ripening, abscission, and pollen tube penetration into maternal tissue (Cosgrove, 1997a). A considerable body of evidence support the notion that expansins are involved in plant cell enlargement and hence growth stimulation. Firstly, acid-induced extension of isolated cell walls is, in general, considered to be correlated with plant growth rate (Cosgrove, 1989). Expansins have the ability to mediate this process. In addition, growing cells typically have a wall pH between 4.5 and 6 which corresponds well with the pH optima of expansin activity as measured by acid-induced extension and wall stress relaxation analyses (McQueen-Mason *et al*., 1992; Li *et al*., 2003; Cosgrove, 2005). Secondly, addition of expansins to live cells stimulated their enlargement (Link and Cosgrove, 1998; Fleming *et al.*, 1997 and 1999). Thirdly, in many cases, over-expression and suppression of expansins have been demonstrated to respectively stimulate and down-regulate plant growth (Cosgrove, 2005 and references therein). A forth line of evidence is that the temporal and spatial patterns of *EXPANSIN* gene expression are, in most instances**,** correlated with plant growth. For instance, Wu *et al.* (2001b) reported that *ZmEXPA1*, *ZmEXPA5*, *ZmEXPB2* and *ZmEXPB8* expressed in the apical elongating zone of the maize primary roots are up-regulated by water-stress. The expression pattern correlated well with the maintenance of elongation at the apical-most region of the roots under water-stress. Kam *et al.* (2005) also provided evidence that *ZmEXPB2* is involved in root elongation.

Immunolocalization studies on gravistimulated maize roots have also indicated the possible involvement of expansins in cell elongation (Zhang *et al.,* 2000). In addition to these examples from maize, a large number of studies in diverse species support a growth stimulatory effect of expansins.

Based on the foregoing discussion on the involvement of expansins in plant growth, it can be postulated that the pollination induced inhibition of maize silk elongation involves a down-regulation of expansins. To test this possibility, I evaluated the aforementioned biophysical analyses (acid-induced extension and wall stress relaxation) as indicators of expansin activity, expansin protein levels, and extractable expansin activity in maize silks.

3.2. Materials and Methods

3.2.1. Plant material

Field grown Pioneer hybrid 34M94 plants were used in all the experiments. Controlled pollinations (full-ear) were done as described in Chapter 2. For acid-induced extension and wall stress relaxation analyses mid-silk samples were collected from spikelet position 24 (see Figure 3.1). Samples were collected at different times after pollination. At equivalent times control samples were also collected in parallel. For protein extraction, silks were sampled from spikelet positions 15 through 24 at 24 and 36 h post-pollination. In this case, entire length of silks except the segment protruding beyond the husks was collected. Upon harvest, silks were quick-frozen in liquid nitrogen and stored at -80°C until used.

3.2.2. Acid-induced Extension (Native wall creep)

Acid-induced extension analyses were performed as previously described (Cosgrove, 1989). Frozen mid-silk samples were directly transferred into ice cold water to prevent browning. They were then abraded using a slurry of carborandum and pressed between glass slides under weight to remove cell sap. Approximately 5 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. The extension of the wall segment was measured by a position transducer attached to the lower clamp of the extensometer. Once the rate of extension was stabilized at neutral pH, the buffer bathing the sample was changed to 50 mM sodium acetate, pH 4.5. Extension was monitored for a total of 150 min.

3.2.3. Wall Stress Relaxation Analysis

The methodology as described by Cosgrove (1989 and 1997b) was followed. In brief, frozen mid-silk samples were thawed, abraded and the cell sap removed as described above in acid-induced extension. They were treated with 50 mM sodium actetate, pH 4.5 for 20 min at room temperature and then kept on ice until the analysis was complete. Silk samples (5 mm) were clamped in an Instron-style instrument and rapidly extended. The wall sample is held at constant length and the decay of force on the sample monitored using a computer. The change in force (dF) against the change in log time (dlogt) is plotted to generate stress relaxation spectra.

3.2.4. 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. Briefly, silks were homogenized in buffer containing 25 mM HEPES, 3 mM sodium metabisulfite and 2 mM EDTA (pH 7.0). 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 DTT (pH 4.5) using Amicon Ultra centrifugal devices (Millipore), proteins were quantified using the Coomassie plus protein assay reagent (Pierce). Cell wall material first extracted with 1 M NaCl was washed well with Millipore water to remove salt and homogenized in 1% SDS using motar and pestle. The homogenized suspension was then heated at 100°C for 5 min and the filtered using nylon mesh (Pers. Comm. D. Choi). Total protein in the filtrate was quantified using the BCA protein assay kit (Pierce) following the manufacturers instructions.

3.2.5. Immunoblot Analysis

Protein samples were separated by SDS-PAGE on a 13% (w/v) polyacrylamide gel using a minigel apparatus (Bio-Rad Laboratories, CA) following the method of Laemmli (1970). Equal amounts of total protein were loaded in each lane. Prior to loading, samples were heated in SDS sample buffer at 95°C for 5 min. After SDS-PAGE, proteins were transferred on to Protran BA nitrocellulose membrane (Scleicher and Schull, NH) using a semidry blot apparatus (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad Laboratories, CA) in a solution of 192 mM

Gly, 25 mM Tris and 20% (v/v) methanol at 25 V for 100 min. Blots 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 blots were incubated with the rabbit I^{ry} antibody (1:1000x dilution for anti-CuEXPA1 and 1:5000 dilution for anti-ZmEXPB8 and anti-OsEXPB3 antibodies) for 1 h and washed twice (5 min each) with PBS and twice (5 min each) with tris-buffered saline (TBS). Subsequently the blots were incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (1: 8000) in TBS with 0.05% (v/v) Tween 20 and 5 mM sodium azide. After washing 4 times (5 min each) with TBS, the blots were developed with 0.1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate and 0.2 mg ml^{-1} nitroblue tetrazolium (Sigma-Aldrich) in the substrate buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$).

3.2.6. Extractable expansin Activity (Wall Reconstitution Assay)

The methodology as described by McQueen-Mason *et al.* (1992) and Cosgrove (1989) was followed. In brief, frozen, thawed cucumber hypocotyls segments (apical 12 mm) were abraded using a carborandum slurry and washed with water. They were pressed between glass slides under weight to express cell sap and boiled in water for 15 s. Hypocotyl segments were then clamped in a custom extensometer as described in Section 3.2.2 under a constant tension of 20 g force. Initially the samples were bathed in 50 mM sodium acetate pH 4.5 and once the rates of extension were stabilized the bathing buffer was replaced by a solution of silk cell wall proteins extracted with 1 M NaCl and buffer- exchanged with 50 mM sodium acetate pH 4.5.

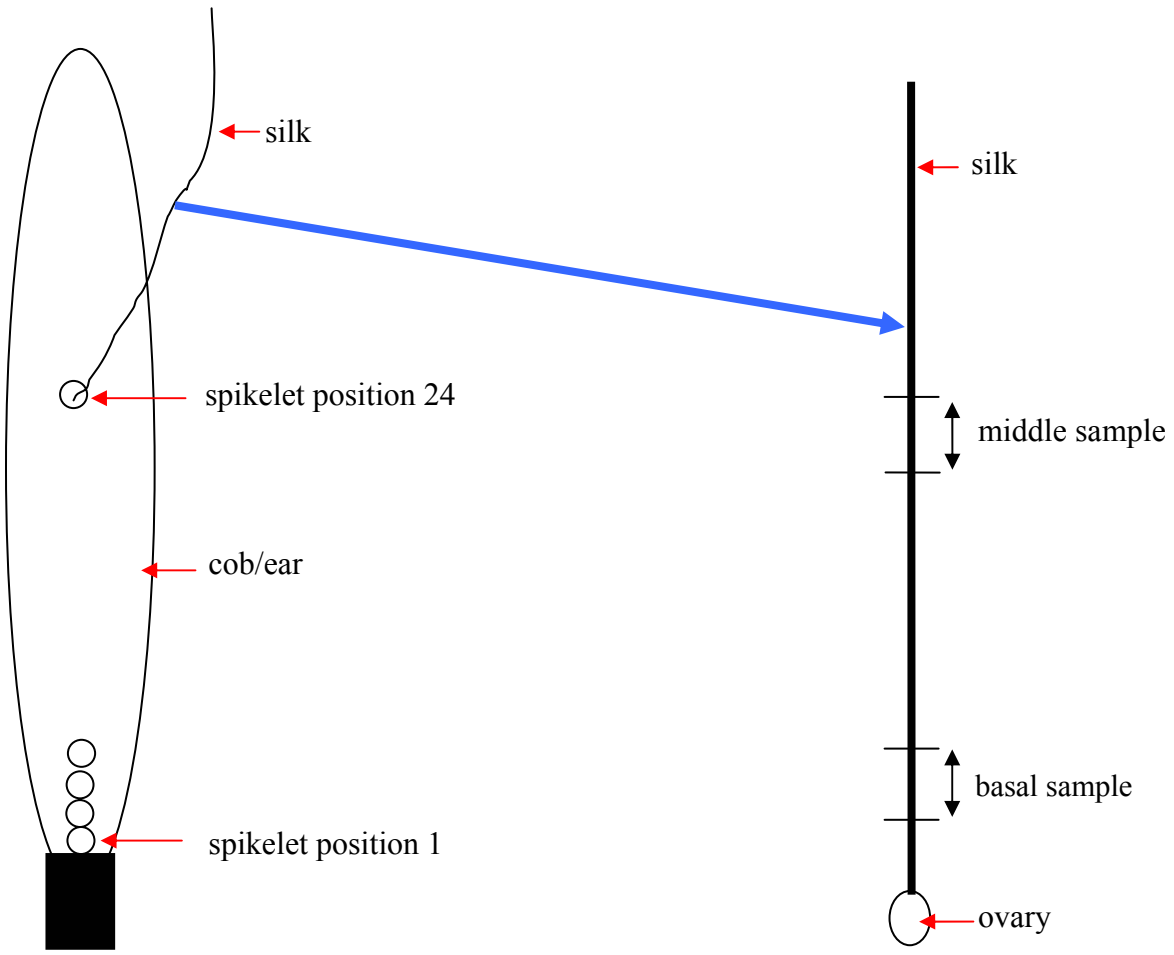


Figure 3.1. Schematic diagram of the silk sampling strategy (not drawn to scale). Silks were collected from spikelet position 24 (counting from the base of the ear). For cell wall biophysical analyses (described in Chapters 3 and 4) middle and basal silk segments were selected as shown in the diagram. In the case of protein analyses entire length (except the part protruding beyond husks at the time of sampling) of silks collected from spikelet positions 15-24 were used to accumulate sufficient wall material.

3.3. Results

3.3.1. Biophysical Indicators of Expansin Activity

3.3.1.1. Acid-induced extension (Native Wall Creep) of Maize Silk Cell Walls

Figure 3.2 depicts the results of a time-course analysis of the acid-induced extension of isolated maize silk cell walls. Representative traces of the rate of extension over time (150 min) from control and pollinated silks are shown in Figure 3.2A. Figure 3.2B shows the average rate of extension during the period 50 to 70 min after the low pH treatment. At all the times tested the pollinated walls had lower rates of native wall creep compared to controls. The reduction ranged from 13 to 27% depending on the post-pollination time point. This reduction in the rate was not statistically significant ($p > 0.05$) at any of the individual time points. However an ANOVA performed by pooling samples across all post-pollination times indicated a significant decrease ($p<0.05$) in the extension rates of pollinated silks. It is interesting that even as late as 44 h after pollination there was no striking drop in the native wall creep of silk walls. By 44 h post-pollination silk elongation is considerably down regulated (see Chapter 2) and they are starting to detach from the ovary due to tissue collapse at the silk-ovary junction.

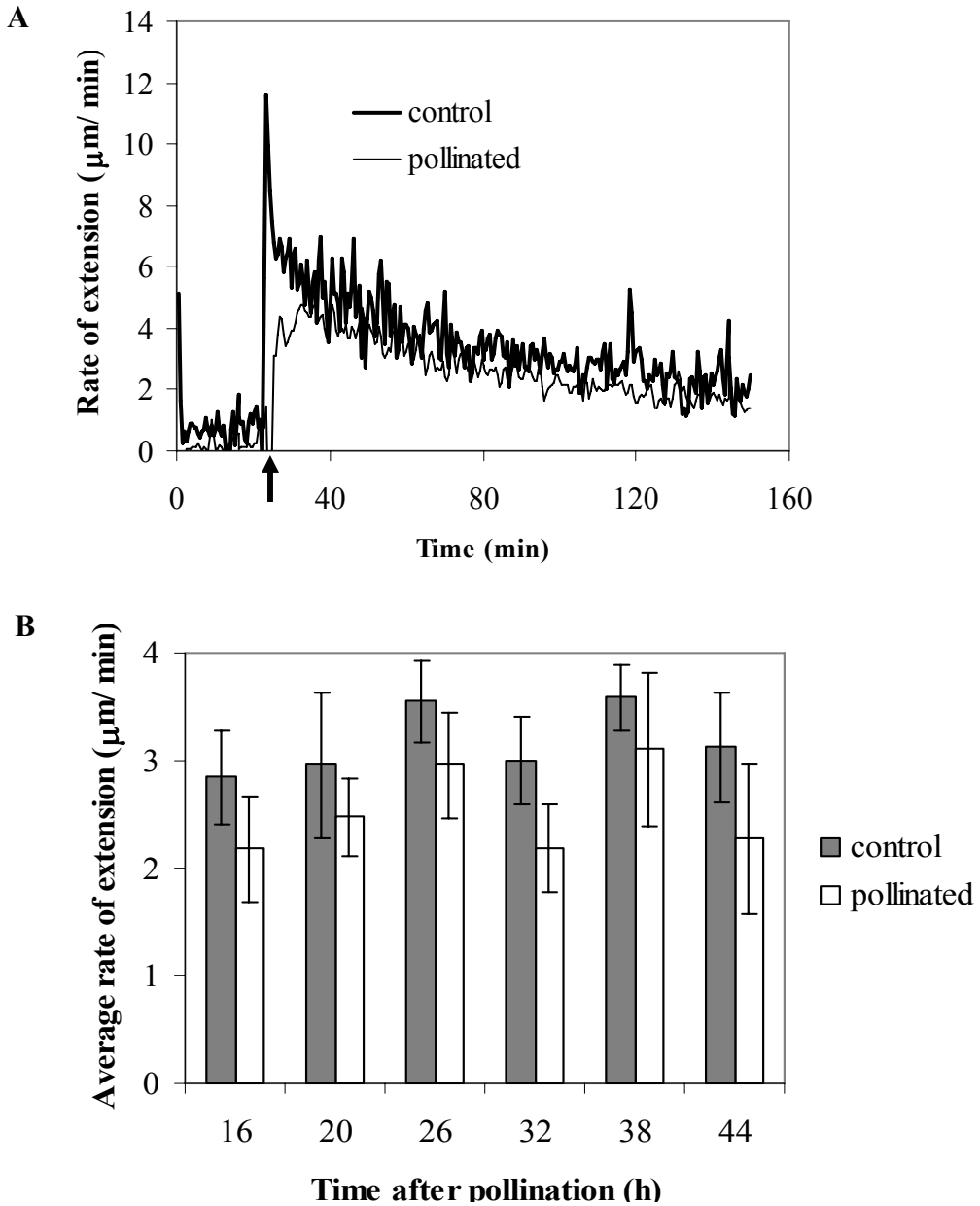


Figure 3.2. Acid-induced extension (native wall creep) of control and pollinated silk cell walls. Samples were initially bathed in neutral buffer (50 mM HEPES pH 6.8). Once the rates of extension were stabilized (~ 20 min, arrow) low pH treatment was administered by changing the bathing buffer to 50 mM sodium acetate pH 4.5. A typical experiment proceeds for 150 min. **A:** representative extension rate profiles obtained for control and pollinated silks collected 16 h post-pollination. **B:** mean rates of extension at different times after pollination. 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 different post-pollination times indicated. Data shown are means +/- SEM (n = 5-11).

3.3.1.2. Wall Stress Relaxation Analysis

For stress relaxation analysis a wall sample is rapidly extended and then held at constant length. The subsequent decay of the force on the sample is monitored for 5 min. The results are presented as the derivative of the decay in force against logarithmic time and referred to as stress relaxation spectra. This technique enables the evaluation of wall stress relaxation at different time-scales during the course of the experiment, i.e. 5 min (Cosgrove, 1993a). Rates of relaxation at different timescales provide insight into the nature of molecular rearrangements in the wall. For example, compared to larger molecular rearrangements smaller changes occur faster and appear earlier in the stress relaxation spectra (Masuda, 1978; Cosgrove, 1989). Data collected at 12, 24, and 30 h post-pollination are shown in Figure 3.3A-C. As can be seen there were no significant differences (at $p < 0.05$) in the rates of relaxation of control and pollinated silk walls (over the entire timescale of measurements) at any of the times tested (12, 24, 30 h after pollination). The time-course analysis proceeds well beyond the timing of silk elongation inhibition after pollination. Silk growth response is apparent beyond 12 h post-pollination.

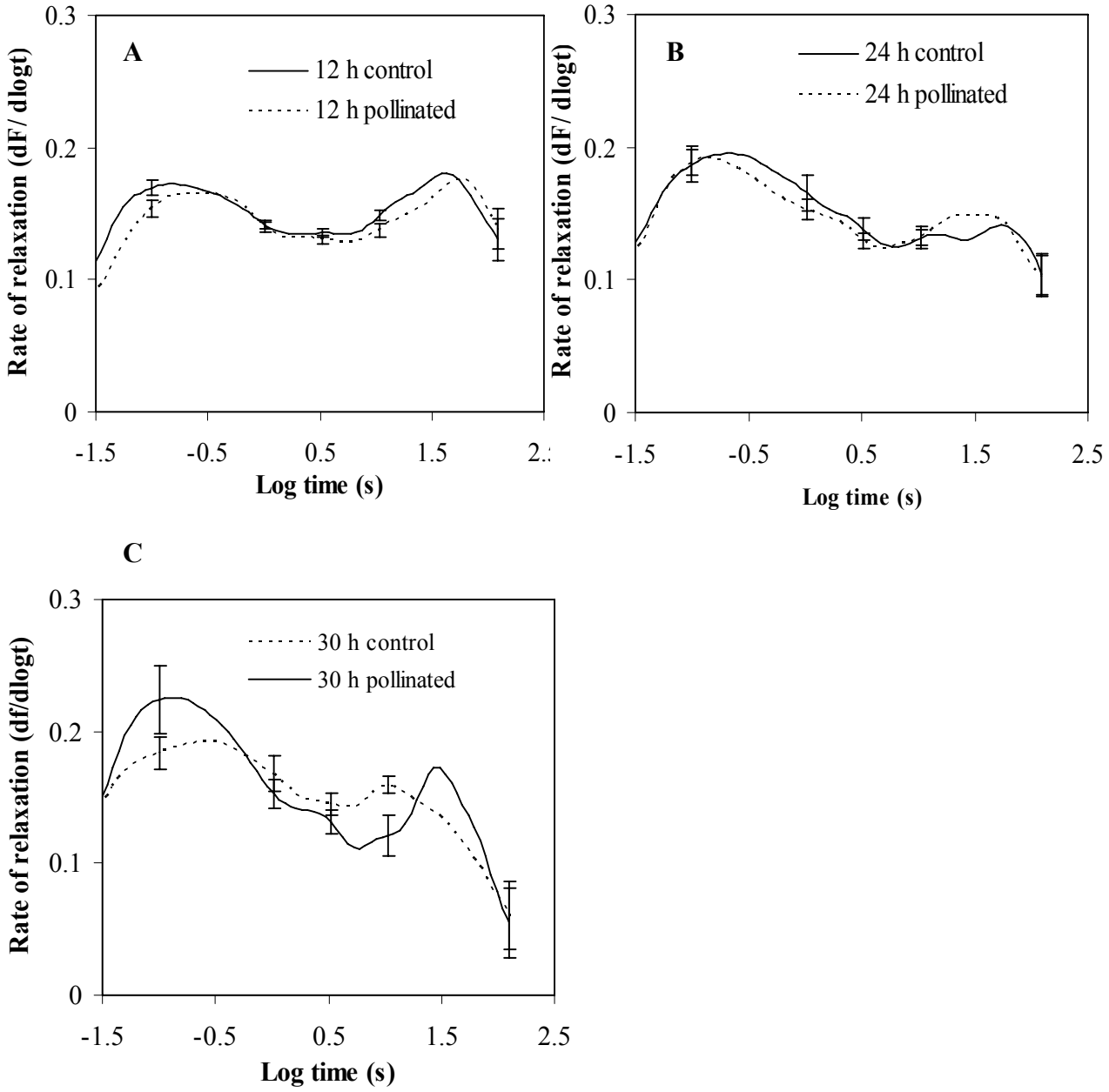


Figure 3.3. Stress relaxation analysis of control and pollinated silks. Silk samples were pre-treated in 50 mM sodium acetate (pH 4.5) for 20 min at room temperature before the stress relaxation measurements. Subsequently all samples were kept on ice until the analysis was complete. **A**, **B**, and **C** correspond to samples collected 12, 24 and 30 h after pollination respectively. Data shown are means +/- SEM (n = 10-17)

3.3. 2. Immunoblot Analysis of Expansins

Immunoblot analyses were performed to see whether pollination causes a change in α- and β-expansin protein levels.

3.3.2.1. Effect of Pollination on Putative α-expansin

Polyclonal antibodies raised against CsEXPA1 (Li *et al.*, 1993) were used to detect putative α-expansins. Silk cell wall proteins were extracted with buffer containing 1 M NaCl. Results shown in Figure 3.4 indicate that two isoforms antigenically related to cucumber α-expansins are expressed in maize silk. These isoforms have a molecular mass of approximately 28 kDa. The size estimate is similar to previously described α-expansins (McQueen-Mason *et al.*, 1992; Wu *et al.*, 1996). These need to be considered as putative α-expansins until definitive identification of proteins has been done. The abundance of the two isoforms did not change significantly in response to pollination. Specifically there was no dramatic reduction but a small increase (6 and 13% in the two isoforms) in the protein levels.

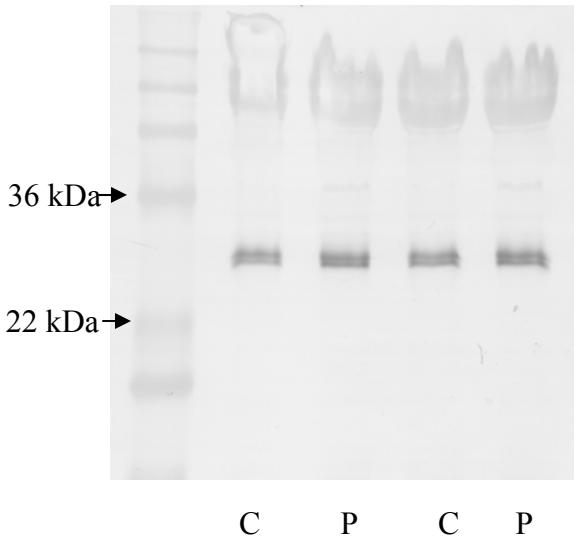


Figure 3.4. Immunoblot analysis of putative α-expansin protein in silk cell walls. Cell wall proteins were extracted from silks collected 24 h after pollination using buffer containing 1 M NaCl. Protein samples were separated by SDS-PAGE (13% gel) and probed with antibodies raised against CsEXPA1 protein. C and P denote protein preparations from control and pollinated silks (Two samples per extract were loaded in the same gel.) respectively. The same amount of crude total protein (6 μg) was loaded in each lane.

3.3.2.2. Effect of Pollination on Putative β-expansins

Two antibodies raised against β-expansins were used to evaluate two different types of maize silk cell wall protein preparations *viz*., NaCl extracts and 1% SDS extracts. The two antibodies were raised against ZmEXPB8 and OsEXPB3 proteins respectively. Results of immunoblot analyses using NaCl and SDS extracts are shown in Figure 3.5. In general, the signals obtained were weak. For a given type of extract, both antibodies showed a more or less similar banding pattern.

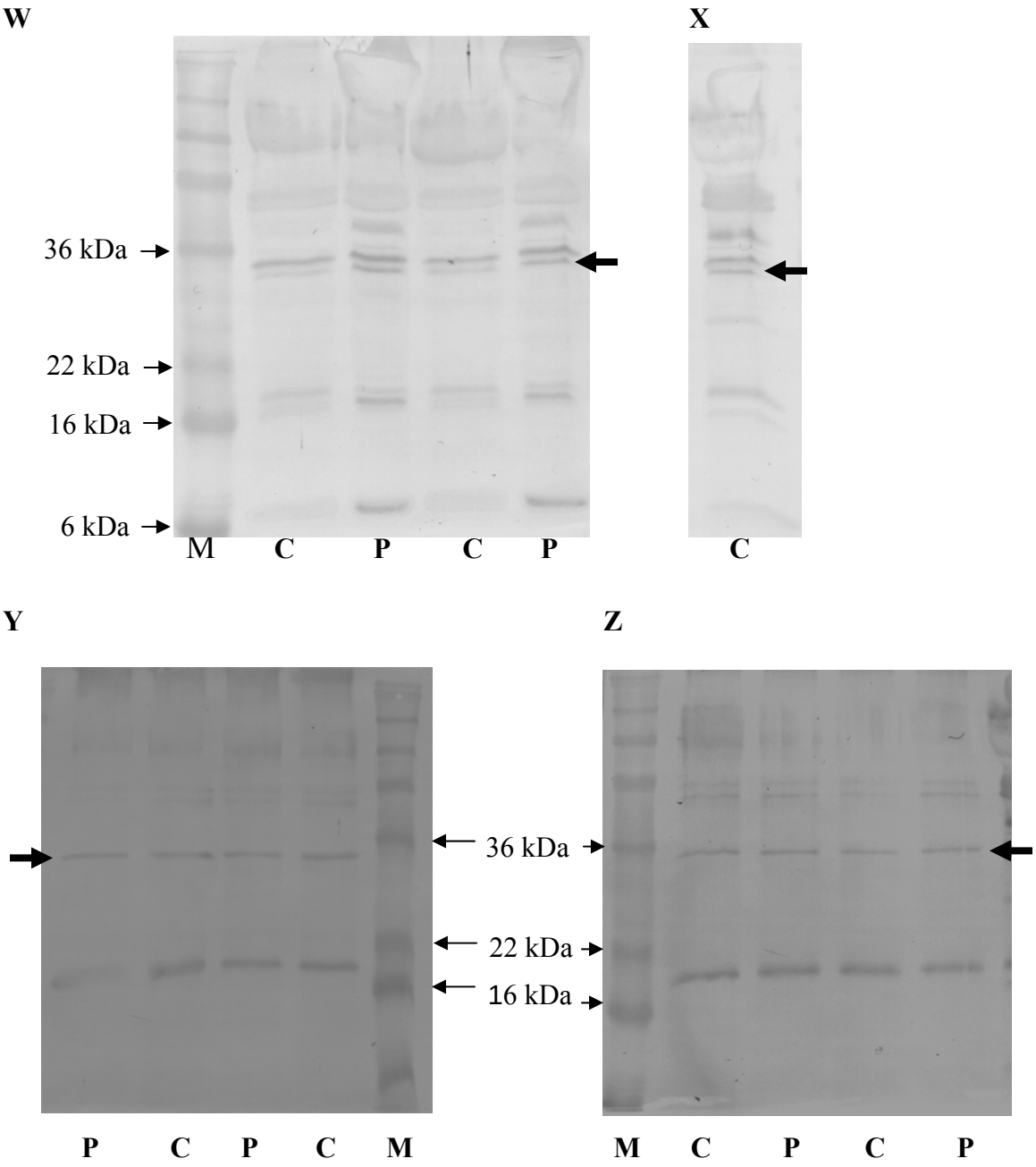


Figure 3.5. Immunoblot analysis of β-expansin protein in silk cell walls. NaCl extracted wall proteins were used in **W** and **X**. In **Y** and **Z** wall proteins extracted with 1% SDS were used. On a given blot, an equal amount of total protein (12, 6 and 8 µg in W, Y, and Z respectively) was loaded in each lane and separated by SDS-PAGE. Antibodies raised against ZmEXPB8 (panels **W** and **Y**) and OsEXPB3 (panels **X** and **Z**) proteins were utilized. **C** and **P** represent protein preparations from control and pollinated silks collected 24 h after pollination. M is the molecular weight standard. Except on **X**, each sample was run twice in the same gel. Bands indicated with bold arrows may represent β-expansin protein.

As shown in Figure 3.5, antibodies against ZmEXP8 and OsEXPB3 recognized a ~ 33 kDa protein in both NaCl and SDS extracts. Based on this antigenic relationship this protein may be a β-expansin. The fact that two different antibodies recognize a band of similar molecular weight in two types of protein preparations adds to the confidence. Lee and Choi (2005) also described a protein of similar molecular weight recognized by anti- OsEXPB3 antibodies in rice coleoptiles. Based on the *ZmEXPB8* nucleotide sequence the encoded protein is approximately 26 kDa (excluding the signal peptide) and has two predicted N-glycosylation sites (Wu *et al.*, 2001). However, this information is inadequate either to accept or refute the possibility that the ~ 33 kDa protein is encoded by ZmEXPB8. Moreover, the antibodies are polyclonal and exhibit non-specific binding (Figure 3.4). Consequently, further experiments are necessary to see whether the ~ 33 kDa protein is indeed a β-expansin. There was no major reduction (reduction was ~ 7%) in the abundance of this putative β-expansin 24 h after pollination.

The two antibodies recognized a second band of about 19 kDa in both NaCl and SDS extracts. Yet another protein of about 8 kDa was also detected in NaCl extracts. Moreover, there was an increase in the intensity of two bands (~ 18-19 kDa and ~ 8 kDa) in pollinated samples extracted with NaCl (Figure 3.5 P).

3.3.3. Extractable α-expansin Activity (Wall Reconstitution Assay)

To examine whether pollination causes a change in extractable expansin activity, I tested the ability of silk wall protein preparations (NaCl extracts) to extend heat-inactivated cucumber hypocotyls. Since α-expansins are more effective in extending dicot (type I) walls, cucumber

walls were used to specifically compare extractable α-expansin activity from control and pollinated maize silk walls.

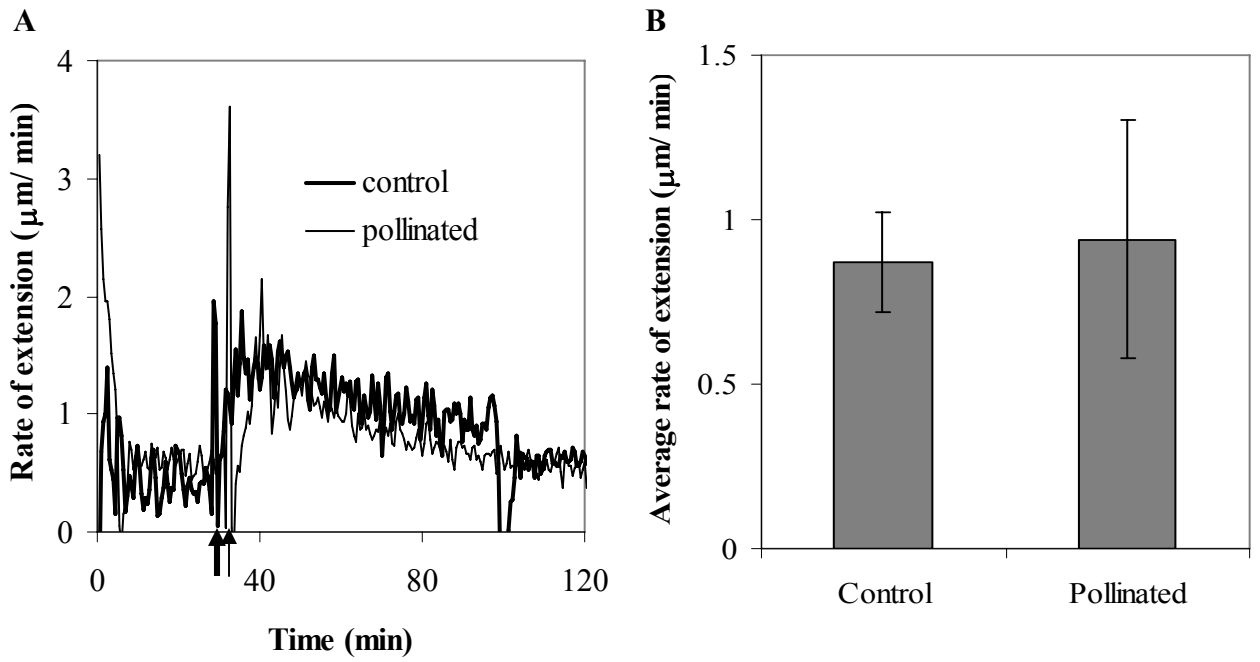


Figure 3.6. Extractable α-expansin activity from silk cell walls. Samples collected 36 h after pollination were used to extract cell wall proteins using 1 M NaCl. The activity of the protein preparations was tested on heat-inactivated cucumber hypocotyls in a wall reconstitution assay. **A:** Traces of wall extension rates obtained by protein preparations from control and pollinated walls (Arrows indicate the addition of protein preparations.). **B:** Average rate of extension during the period 20-40 min from the protein treatment (per 180 μg of total protein). Data shown are means +/- SEM (n = 2-3).

The results obtained are depicted in Figure 3.6. As can be seen, I was able to detect only low levels of activity in the protein preparations from silk cell walls. Nonetheless it can be seen that pollination did not cause a significant reduction ($t_4 = 0.23$, $p>0.05$) in the extractable α-expansin activity (as measured by wall extension in the reconstitution assay) by 36 h after pollination. It

should be noted that this activity is expressed as specific activity per equal amount of total protein.

3.4. Discussion

3.4.1. Biophysical Indicators of Expansin Activity

Increased acid-induced extension and enhanced wall stress relaxation are, at least to date, considered characteristic features of expansins (Cosgrove, 2005). Therefore these *in vitro* analyses using isolated cell walls were performed as biophysical indicators of possible changes in expansin activity after pollination.

Compared to controls, the rates of acid-induced extension of pollinated silk walls exhibited only a small reduction at all the post-pollination times tested: even as late as 44 h (Figure 3.2). Since silk elongation was considerably down regulated beyond 12 h after pollination (see Chapter 2), these observations suggest that the silk elongation response is largely independent of any major effects on the wall extension response to low pH.

Pollination did not cause significant changes in wall stress relaxation throughout the entire timescale examined. This was true for 12, 24, and 30 h post-pollination (Figure 3.3) thereby indicating that the down-regulation of silk elongation does not involve changes in wall stress relaxation as measured by the *in-vitro* method used. A limitation of the method is that any relaxation event(s) that might occur outside the timescale of detection are not recorded. This is important because different expansins, when added exogenously to heat-inactivated walls, have

been shown to enhance stress relaxation at different timescales. For example, two different α-expansins from cucumber enhance stress relaxation 1-30 s and > 100 s (McQueen-Mason *et al.*, 1995). So, the possibility that I was unable to detect changes in the stress relaxation spectra which in theory could be related to the activity of hitherto un-tested expansins can not be ruled out.

The results obtained in the above biophysical analyses collectively suggest that pollination does not cause a significant reduction in the endogenous expansin activity.

3.4.2. Expansin Protein Abundance

To determine whether the lack of reduction in expansin activity indicated by the biophysical analyses is reflected in protein abundance, both α- and β expansin protein levels were investigated by immunoblot analyses. These experiments recognized two putative α-expansins (~ 28 kDa) and one putative β-expansin (~ 33 kDa). As measured by immunoblot analyses, none of these proteins decrease considerably compared to controls by 24 h after pollination (Figures 3.4 and 3.5). These results suggest that the silk elongation response to pollination does not involve a major reduction in expansin protein abundance. However, it should be reiterated that this conclusion relies on at least 5 assumptions: a) the ~ 28 kDa proteins and ~ 33 kDa protein are indeed α- and β-expansins respectively, b) the extraction methods released the expansin protein (with respect to the bands recognized) equivalently from both control and pollinated silk cell wall material, c) that the antibodies used here recognized all possible expansin proteins involved in silk elongation, d) any contribution of pollen tubes growing within the silks to

expansin levels is negligible, and e) the putative expansins detected here are indeed involved in silk elongation (Expansins are known to be involved in many plant developmental processes). Consequently, at least as a first step, it is important to identify the putative expansins observed in these immunoblot experiments.

Irrespective of the problems/alternative explanations, 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 putative 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).

Immunoblot analysis with NaCl extracts using antibodies against ZmEXPB8 and OsEXPB3 showed two other proteins which were specifically up-regulated in pollinated silk walls. Molecular weights of these are ~ 19 and ~ 8 kDa. In the case of SDS extracts an approximately 18 kDa band was recognized in both control and pollinated silk walls. It would be worthwhile to identify these proteins and investigate whether they are related to expansins. It is interesting that a putative β-expansin was recognized in NaCl extracts of silk walls. Since NaCl does not inactivate expansins (as measured by wall reconstitution assays), provided sufficient amounts of protein can be purified using this method it would enable the biochemical characterization of this

protein. This is important because to date only β-expansins from pollen have been biochemically characterized.

3.4.3. Extractable Expansin Activity

Wall reconstitution analysis using cucumber hypocotyl walls and NaCl extracted silk cell wall proteins showed miniscule wall extension activity (Figure 3.6). One reason for little activity could be the low amount of total protein (180 μg or 0.6 mg ml^{-1}) added to each hypocotyl sample. It should be noted that these were unpurified protein preparations. When using unpurified protein, typically ~ 1 mg or more of total protein is added per wall sample in these experiments (McQueen-Mason *et al.*, 1992). I was only able to extract less than 1 mg total protein per 100 g silk fresh weight. The fresh weight of a 1 cm silk segment is ~ 1 mg. Since I only sampled silks from spikelet positions 15 through 24 for protein extraction, collection of material to extract large amounts of protein was time consuming and laborious. This made it difficult to use large amounts of protein or a higher number of replicates in activity analyses. Irrespective of these technical flaws it is clear that NaCl extracts extended heat-inactivated cucumber cell walls. When protein preparations from control and pollinated silks were compared at 36 h post-pollination there was no significant difference in the extractable expansin activity (specific activity with reference to total protein) as measured here. α-expansins are more efficient in extending dicot walls (type I walls) in wall reconstitution assays. The reverse is true for the maize pollen β-expansin Zea m1. So, based on our current understanding about the biochemical features of expansins, the results obtained indicate that the extractable α-expansin activity was not reduced in silks in response to pollination: even by 36 h afterwards.

Consequently the slowing of silk elongation does not seem to involve a decrease in the extractable α-expansin activity. This conclusion again is based on at least 4 assumptions: a) all α-expansin protein was equally released from both control and pollinated walls by NaCl, b) the wall extension activity detected is not due to any other agent present in the unpurified protein preparations, c) the activity as measured here is relevant to elongation, and d) any contribution from pollen tubes is minimal. Wu *et al.* (1996) and Caderas *et al.* (2000) also reported instances where growth inhibition did not accompany a reduction in extractable α-expansin activity. Moreover, irrespective of a large difference in oat coleoptile growth under light and dark conditions, extractable expansin activity was not significantly changed (Cosgrove and Li, 1993b).

I have discussed 3 lines of evidence that suggest expansins are not down regulated by pollination in a timely manner that may have an effect on the inhibition of silk elongation. Firstly, biophysical indicators (extension of heat-inactivated cell walls and wall stress relaxation) did not suggest major decreases in expansin activity. Secondly, the abundance of either putative α- or β-expansin protein was not significantly reduced after pollination. Thirdly, extractable α-expansin activity did not decrease as a result of pollination. Collectively these findings indicate that the pollination-induced inhibition of silk elongation proceeds through some other mechanism that does not involve large decreases in either expansin protein abundance or activity.

Chapter 4

The Effect of Pollination on Cell Wall Structure

4.1. Introduction

The role of the cell wall in cell growth was discussed in Chapters 1 and 3. In this Chapter, I will specifically focus on the relationship between cell wall structural changes and plant growth and how it can be linked to the pollination-induced inhibition of maize silk elongation.

4.1.1. The Primary Cell Wall

The primary wall of plant cells is a complex polymeric structure with cellulose microfibrils embedded in matrix polysaccharides. Two major classes of matrix polysaccharides are described in the literature *viz.*, hemicelluloses and pectin. In flowering plants the major hemicelluloses described are xyloglucan and glucuroarabinoxylan. Xyloglucan has a backbone of (1-4) β-D glucose and side chains containing xylose, galactose and often fucose. In glucuroarabinoxylan the backbone consists of xylose units to which are attached arabinose and glucuronic acid side chains. This however is only a generalized description of the chemical nature of hemicelluloses: depending on the cell type and plant species there can be other chemical groups (Carpita and McCann, 2000; Cosgrove, 2002). Pectins have large and complex molecular structures and comprise polysaccharides containing both acidic and neutral sugars. There are also several classes of structural proteins in the cell wall: hydroxyproline rich glycoprotein (HRGP), proline-rich protein (PRP), and glycine-rich protein (GRP) (Cosgrove, 2002).

Based on the wall chemical composition, the higher plants are described as having two types of cell walls. All dicots and the commelinoid monocts are said to have type I primary cell walls. Type I walls characteristically have xyloglucan as the hemicellulose fraction. On the other hand, type II walls which are found in the order Poales (includes grasses and cereals such as maize) contain glucuronoarabinoxylan (Carpita and Gibeaut, 1993). Compared to the ~ 35 % pectin (based on dry weight) in type I walls, grass cell walls have little pectin: ~ 10 % (Cosgrove, 2000). Type II walls are also characterized by a network of phenolic substances (Carpita and Gibeaut, 1993; Carpita 1996).

4.1.2. Cell Wall Rigidification and Plant Growth

According to the biophysical explanation of cell growth discussed in Chapter 3, it can be postulated that structural changes in the wall, i.e. a rigidification of the wall by polymer cross-linking, may be involved in the cessation of plant growth. Several ways of wall rigidification have been discussed in the literature. One proposed mechanism is the oxidative phenolic coupling of feruloyl side-chains attached to wall polysaccharides by a peroxidase or laccase catalyzed reaction. Such a reaction, which produces dimers and trimers of ferulate, is thought to cross-link wall polymers (Fry, 1986; Grabber et al., 1995; Fry, 2004). This process is generally considered as a major mechanism of wall polymer cross-linking in grasses (Carpita and McCann, 2000).

Many studies performed in diverse species have indicated increases of diferulates in the wall during a reduction in plant growth (MacAdam *et al*., 1992a and b; Sanchez *et al*., 1996; De

Souza *et al.,* 1998; Zarra *et al*., 1999; MacAdam *et al.,* 2002; Tabuchi *et al*., 2001). Suppression of cell wall bound ferulic and diferulic acid has been noted during increased growth (Wakabayashi *et al.*, 1997). Additionally, accumulation of diferulates has been shown to correlate with decreases in cell wall extensibility determined by *in-vitro* biophysical assays (Kamisaka *et al*., 1990; Tan *et al*., 1991).

Tyrosine residues in hydroxyproline-rich glycoproteins (HRGPs) can also be cross-linked via a peroxidase catalyzed reaction to form isodityrosine (Brett and Waldron, 1996). This type of cross-linking has been implicated in the cessation of shoot elongation in *Pharbitis* (Prasad *et al.,* 1987). Pectin de-esterification can also cause wall rigidification by strengthening the pectin-calcium networks. This process is also reported to be associated with growth cessation (Yamaoka *et al.,* 1983; Goldberg, 1984; McCann *et al*., 1997). It has also been shown that arabinoxylan of maize coleoptiles becomes less branched during maturation (Carpita, 1984). Decreased branching may facilitate increased hydrogen bonding of hemicellulose to other wall polymers (Cosgrove, 1997b).

The foregoing discussion supports the idea that pollination may inhibit silk elongation by rigidifying the silk cell walls. Here I describe experiments performed to test this possibility. Silk cell walls were biophysically assayed using 2 methods: wall susceptibility to exogenously added expansin (wall reconstitution) and stress/strain (Instron) analysis. Possible changes in wall chemical composition were examined by Fourier-transform Infrared Spectroscopy (FT-IR) and chemical analysis.

4.2. Materials and Methods

4.2.1. Plant material

Field or greenhouse-grown Pioneer hybrid 34M94 plants were used. For wall susceptibility to expansin and stress/strain analyses, mid- and basal silk samples were collected from spikelet position 24 at different times after pollination. FT-IR experiments were conducted using basal silk samples collected 36 h post-pollination from spikelet position 24. For ferulic acid and diferulic acid quantification, mid-silk segments from spikelet positions 20-24 sampled 24 h after pollination were used.

4.2.2. Protein Extraction from Maize Pollen

Protein extraction from maize pollen was done as essentially described by Li *et al.* (2003). Approximately 5 g of maize pollen was extracted with 20 ml of 50 mM sodium acetate pH 4.5 for 1 h at 4°C. The suspension was centrifuged at 15 000 g at 4°C and the supernatant loaded onto a CM-Sepharose Fast Flow (Pharmacia Biotech) column equilibrated in 20 mM sodium acetate pH 4.5. The column was then washed with the same buffer until the A_{260} returned to baseline. Proteins were eluted with a linear gradient of NaCl (0-500 mM in 5 h). All Zea m1 isoforms (a β-expansin) and group II pollen allergens (see Li *et al.* 2003 and Valdivia 2005) were collected. After desalting and buffer exchange with 50 mM sodium acetate pH 4.5, the protein preparation was used in wall susceptibility analysis described below.

4.2.3. Wall Susceptibility to Expansin (Wall Reconstitution)

Methodology as described by Cosgrove *et al.* (1997) was followed. Briefly, frozen silk samples were thawed and abraded using a carborandum slurry. Samples were then pressed between glass slides to express cell sap. Afterwards, silk segments were microwaved in 100 ml of distilled water for 90 s and clamped (5 mm silk segment between clamps) on a custom made extensometer under a constant tension of 7.5 g. Initially the samples were bathed in 50 mM sodium acetate pH 4.5 buffer and once the rates of extension were stabilized the bathing buffer was replaced by a protein extract of maize pollen.

4.2.4. Stress/strain Analysis

Stress/strain analyses were conducted as described by Yuan *et al.* (2001). Frozen, thawed silk samples were pressed between two glass slides under weight to express cell sap. Subsequently, they were kept on ice, bathed in 50 mM sodium acetate 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 (mid-silk) or 5.0 g (basal silk) and immediately returned to original length. In the case of each extension, a second-degree polynomial was fitted to the stress/strain data and the slope at the end of the cycle was calculated. Plastic and elastic extensibilities are expressed as % extension per 100 g force.

4.2.5. FT-IR Analysis of Silks

Silk samples were squashed on barium fluoride windows using a miniature rolling pin. The barium fluoride windows were then supported on the stage of a Nicolet Continuum series

microscope accessory to a 670 IR spectrophotometer equipped with a liquid-nitrogen cooled mercury cadmium telluride detector (Nicolet Instrument Corporation). To improve the signal-to-noise ratio, one hundred and twenty eight interferograms were collected from a 100 μm x 100 μm area at 8 cm^{-1} resolution and co-added. Baseline-corrected and area normalized spectra were subsequently used in multivariate data analysis. Principle component analysis of the spectra in the region 1800-800 cm^{-1} was performed using WIN-DAS software (K. Kemsley, Institute of Food Research, Norwich, UK).

4.2.5. Quantification of Wall-bound Ferulate and Diferulate

The methodology as described by Tan *et al.* (1991) and Tabuchi et al (2001) were adopted. Silk samples were boiled in methanol for 5 min and washed 3x with 95 % methanol. Samples were re-hydrated by washing 3x with ice-cold Millipore water and subsequently homogenized in a minimum volume of Millipore water. The homogenate was centrifuged twice at 2000 rpm at 4°C for 10 min. After each centrifugation the supernatant was discarded. The recovered pellet was sequentially washed with ice-cold water, acetone, methanol : chloroform (1:1 v/v), and 95 % ethanol 3x in each solution. The residue was dried overnight at 55°C and the dry weight measured. To remove starch, the dried residue was treated with a 10 units ml^{-1} solution of α-amylase in 20 mM sodium acetate pH 6.5 for 3 h at 37°C. After centrifugation (4000 rpm for 10 min at 4°C) the supernatant was discarded and the pellet washed 3x with ice-cold water. Pectin in the residue was extracted 3x with 50 mM EDTA pH 6.8 (each extraction 15 min at 90°C). After each extraction the samples were centrifuged (4500 rpm for 10 min at room temperature) and the supernatant collected. The residue after the removal of pectin was used to extract

ferulate and diferulate. The residue was extracted 3x with 0.1 M NaOH. The first extraction was immediate: residue vortexed in 0.1 M NaOH, centrifuged and the supernatant collected. The second and third extractions were overnight and 1 h respectively. The three supernatants were pooled and acidified (to ~ pH 4.0) using 0.5 M HCl. Afterwards, ferulate and diferulate released by 0.1 M NaOH were extracted with ethyl acetate 2x (for 2 h each). The ethyl acetate extracts were pooled and dried under continuous air-flow at 40°C.

The dried residues were re-suspended in equal volumes of pyridine and 0.1 mM sodium acetate pH 4.0 to quantify ferulate and diferulate by reverse phase HPLC. Ferulates and diferulates were analyzed at 40°C with a linear gradient of acetonitrile (10-50%) in 0.05 M sodium acetate pH 4.0 using a Breeze HPLC system (Waters). A YMC ODS-AQ 5C18 column (Waters) was used. Ferulic acid (4-hydroxy-3.methoxy-cinnamic acid, Sigma) and 5-5'-coupled diferulic acid were used as standards. The later was a kind gift from Dr. K. Wakabayashi at Osaka City University.

4.3. Results

4.3.1. Biophysical Indicators of Wall Structural Changes

Two biophysical analyses that indicate structural modifications in the cell walls were performed using pollinated and un-pollinated maize silks. Unless otherwise specified mid-silk samples from spikelet position 24 were used (see Figure 3.1, Chapter 3 for a diagram of the sampling strategy).

4.3.1.1. Wall Susceptibility to Exogenously Added Expansins (Wall Reconstitution)

A time-course analysis of the extension response of heat-inactivated silk walls to maize pollen extract containing β-expansin protein is shown in Figure 4.1. Compared to un-pollinated, the extension response of pollinated walls was significantly reduced at 26 ($t_{15} = 3.51$, $p<0.05$) and 38 h ($t_{12} = 3.37$, $p < 0.05$) post-pollination. At 16 h, though the pollinated walls were less susceptible, there was no statistical support ($t_{13} = 1.49$, $p > 0.05$). The reductions in the extension response, with respect to controls, were 36, 60 and 63 % respectively at 16, 26 and 38 h post-pollination.

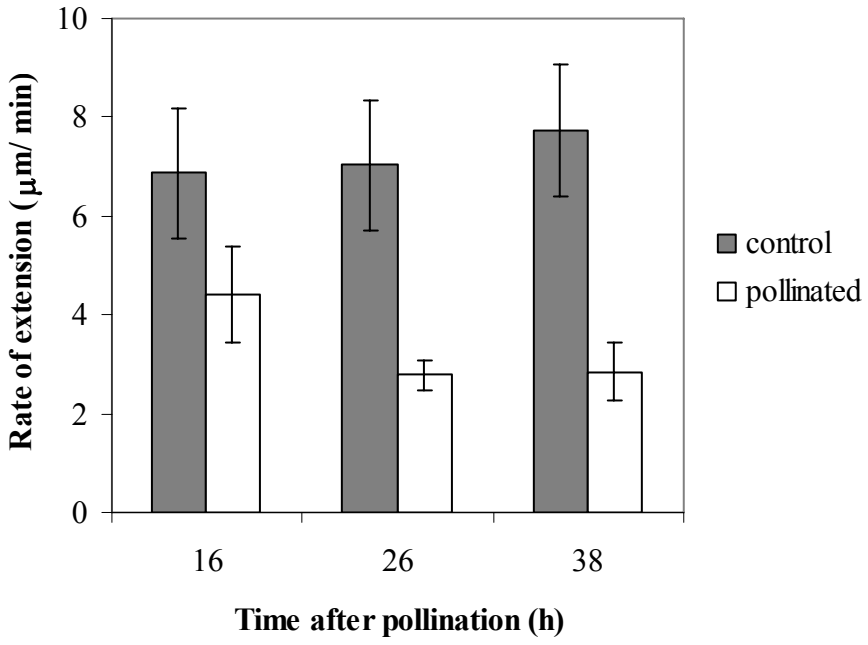


Figure 4.1. Extension response of heat-inactivated silk walls to maize pollen extract containing β-expansin protein. The protein extract was added at a concentration of 0.34 mg/ ml, i.e. 120 µg total protein per sample. For each sample the average rate of extension during the time period 40-60 min after 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. Data are means +/- SEM (n = 7-8 silks).

4.3.1.2. Stress/strain (Instron) Analysis

When a wall sample is stretched by applied force the total deformation has two components. Part of the deformation is reversible. This is called the elastic component. The second component is not reversible and therefore called the plastic component. In stress/strain analysis, walls are extended in two cycles and each of the two components is expressed as % change in length (dL) per force equivalent of 100 g (hence the units % dL/ 100 g). The two components are referred to as elastic and plastic extensibilities.

Compared to controls, the plastic extensibility of pollinated silks (**mid-silk samples**) was significantly reduced 6 h ($t_{17} = 3.14$, $p<0.01$) after pollination and beyond ($p<0.001$ at 18 h post-pollination and beyond) (Figure 4.2A). The reduction ranged from 33-41 % at different time points. However, this phenomenon was not observed at 3 h after pollination. There was a small (~ 5 %) but significant reduction in the elastic extensibility at 12 h ($t_{18} = 3.34$, $p<0.01$) after pollination and beyond ($p<0.01$-0.05) (Figure 4.2B). These findings suggest that pollination induces structural change(s) in the cell wall which occur between 3 and 6 h after pollen deposition.

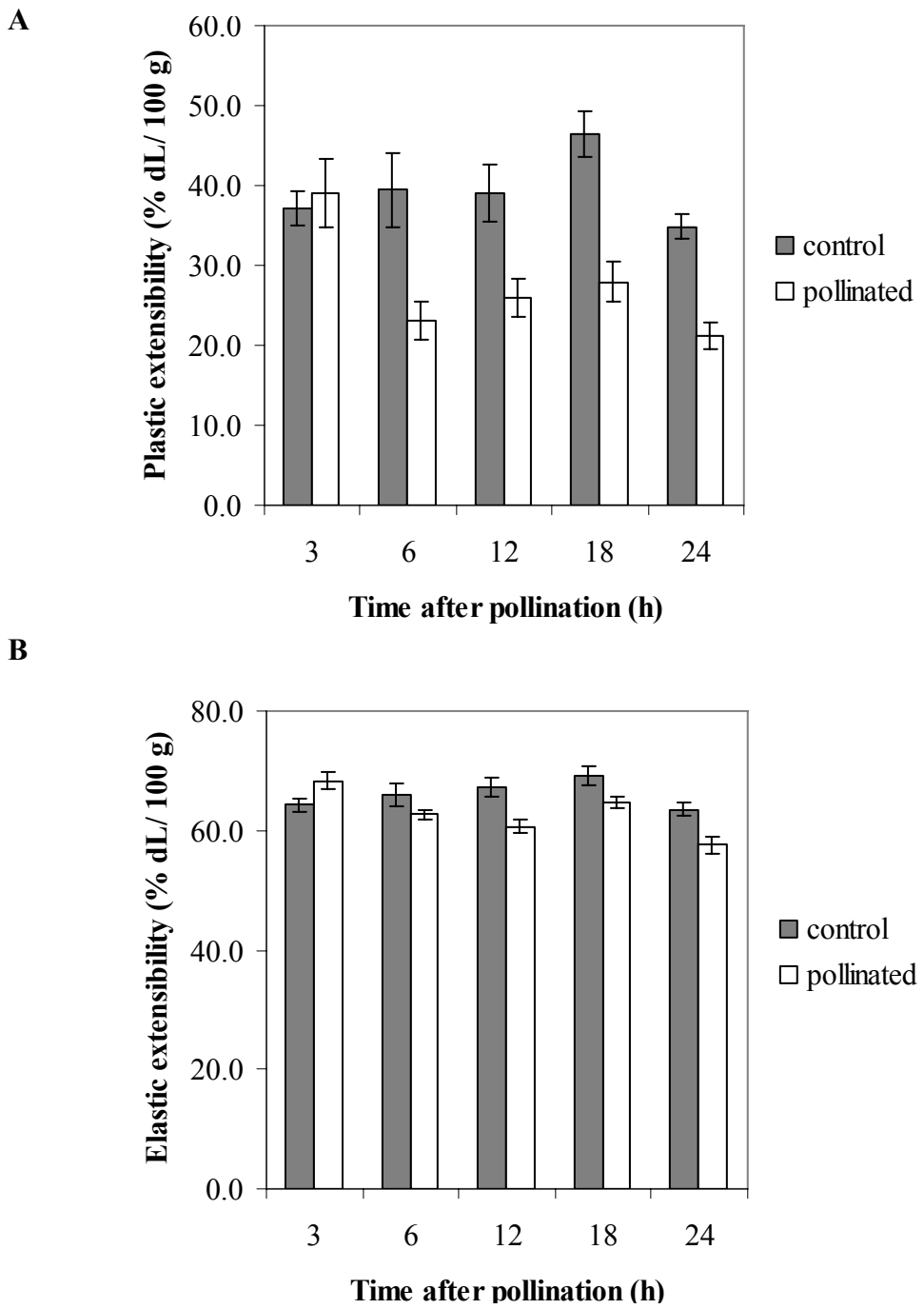
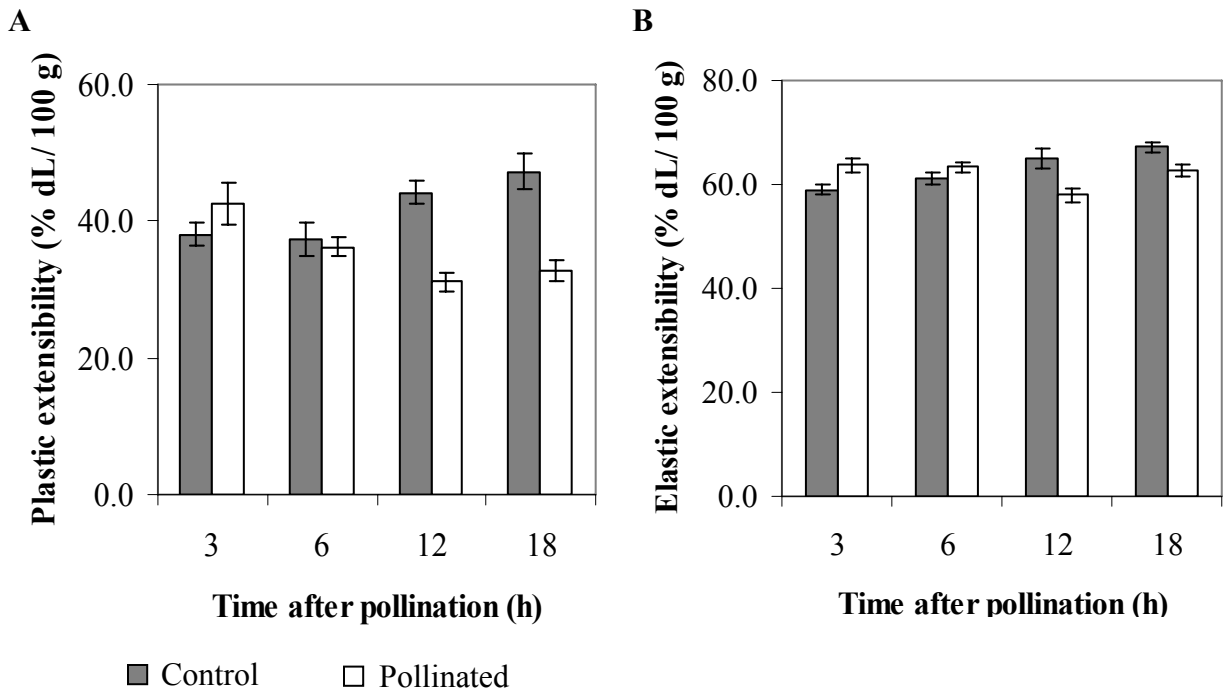


Figure 4.2. Stress/strain analysis of wall samples collected from the **mid-silk** region. **A**: plastic extensibility; **B**: elastic extensibility. Frozen, thawed samples were pressed between 2 glass slides under weight to express cell sap. Subsequently, they were kept on ice bathed in 50 mM HEPES buffer (pH 6.8) until the analysis was complete. A load limit of 4.0 g was used. Data are means +/- SEM (n = 9-21).

Stress/strain Results and Pollen Tube Growth

The results of the stress/strain analysis raised the question whether the reduction in wall extensibilities measured here is related to pollen tube movement along the silk. To address this issue, analysis was extended to the basal silk segments.

As can be seen in Figure 4.3A and 4.3B, the plastic and elastic extensibilities of **basal silk samples** were significantly reduced 12 h (plastic: $t_{19} = 5.71$, $p<0.001$; elastic: $t_{19} = 3.11$, $p<0.01$) post-pollination and beyond. However, the extensibilities of basal samples collected 6 h after pollination did not decrease significantly (plastic: $t_{29} = 0.33$, $p>0.05$; elastic $t_{29} = -1.17$, $p<0.05$). Aniline blue staining of basal silk samples showed the presence of pollen tubes in both middle and basal silk samples 6 h post-pollination (Figure 4.3C). This shows that the changes in the wall extensibilities occur only after pollen tubes have traversed a given region of silk.



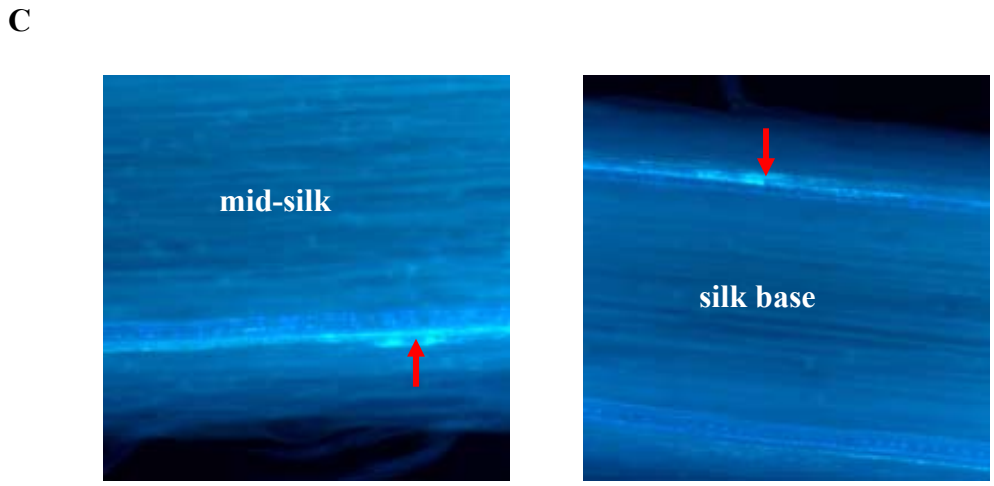


Figure 4.3. Stress/strain analysis and pollen tube growth. **Basal silk samples** collected from spikelet position 24 were used in stress/strain analysis. **A:** Plastic extensibility; **B:** Elastic extensibility; **C:** Pollen tubes (arrows) stained with aniline blue in mid- and basal silk samples collected 6 h post-pollination.

4.3.2. FT-IR Spectroscopy of Silks

Since the biophysical assays indicated that pollen tube growth may have caused structural changes in the wall, silk cell walls were analyzed by FT-IR spectroscopy to investigate possible compositional changes. In this technique the absorbance of infra-red radiation (over a range of wavelengths) by cell wall samples is measured. The analyses were conducted by Dr. J. Tewari and Dr. M. C. McCann at Purdue University.

Initial experiments were performed using silk samples collected 36 h post-pollination from spikelet position 24. Principle component analysis showed significant differences in the FTIR spectra gathered from control and pollinated silks (Figure 4.4A). In Figure 4.4B is depicted the difference spectrum generated by digitally subtracting the average spectrum of pollinated silk walls from that of the control walls. The chemical groups represented by peaks 1737 cm^{-1}, 1598 cm^{-1}, 1416 cm^{-1} and 1246 cm^{-1} are more abundant in pollinated silk walls. Peaks at 1598 cm^{-1} and 1416 cm^{-1} can be assigned to carboxylic acid stretches (McCann *et al.*, 1997; Sene *et al.*, 1994). The peak at 1737 cm^{-1} may correspond to alkyl or phenolic esters (Sene *et al*., 1994; McCann *et al*., 1997; Fan *et al*., 2006) while the peak at 1246 cm^{-1} is indicative of phenolic and ester groups (Sene *et al*., 1994; Fan *et al*., 2006). These results suggest that carboxylic acid groups, esters and phenolics are increased in pollinated silk walls. The difference spectrum shows other differences between control and pollinated walls which are hard to interpret due to the lack of published data for use as reference. FT-IR analysis of silk samples collected 24 h post-pollination is currently being conducted.

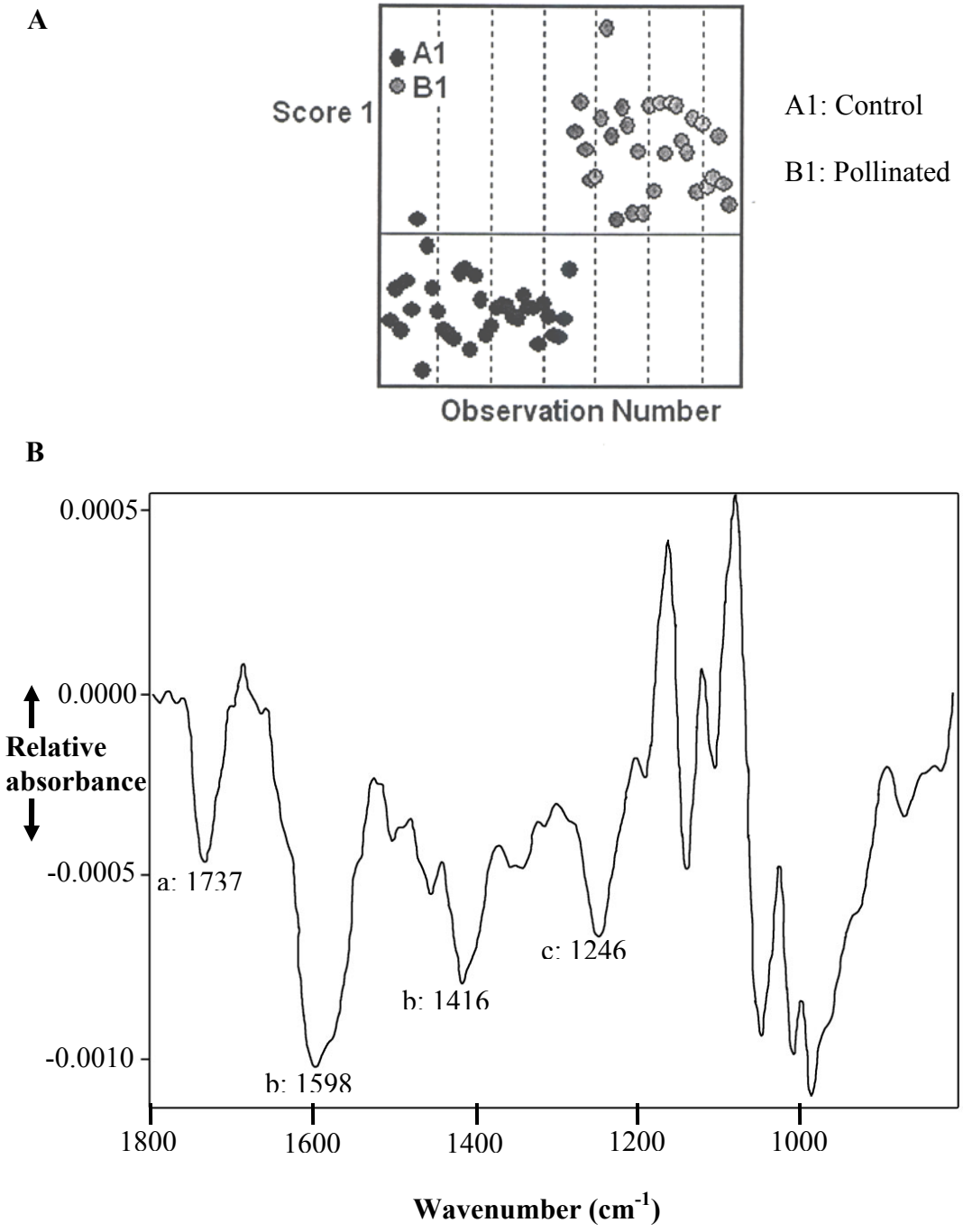
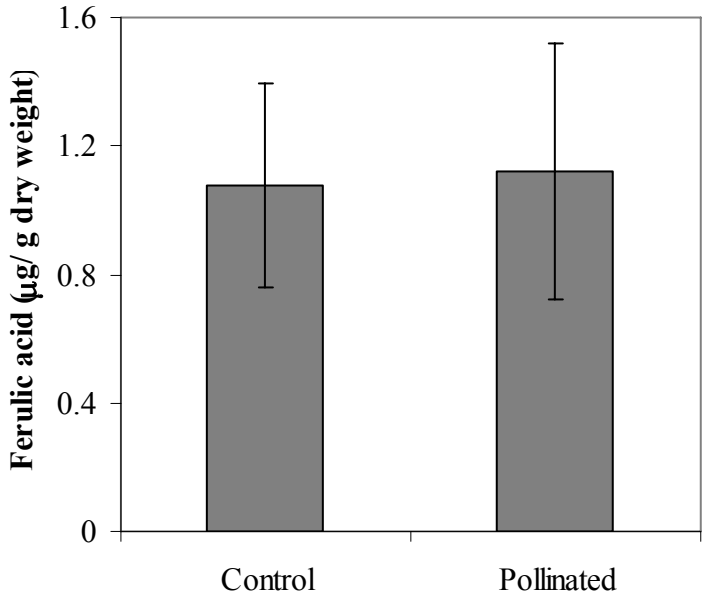


Figure 4.4. FT-IR spectroscopy of silk cell walls. **A:** Principal component analysis of control and pollinated walls; **B:** Difference spectrum generated by digitally subtracting the average spectrum of pollinated samples from that of the controls. Peaks of interest are labeled: **a:** alkyl or phenolic esters; **b:** carboxylic acid groups; **c:** phenolic and methyl ester groups.

4.3.3. Chemical Analysis of Cell Wall-bound Ferulate and Diferulate in Silks

The oxidative coupling of ferulic acid leads to the formation of its dimer, diferulic acid. This reaction may lead to the cross-linking of wall polymers. Moreover, the results of FT-IR analyses suggested possible increases in phenolic substances in pollinated silk walls as compared to controls. To directly examine whether pollination induces changes in the abundance of wall-bound ferulate and diferulate, they were chemically extracted and quantified by reverse-phase HPLC. Though several dimers of ferulic acid have been described in plants, only the abundance of 5-5'-coupled diferulic acid was determined due to the availability of the standards. Results are shown in Figure 4.5. Compared to 5-5' diferulic acid, there was a higher abundance of ferulic aicd. After 24 h, the control and pollinated silks had similar levels of ferulic acid. There was an increase (~ 60%) of diferulic acid in the walls of pollinated silks. However, this increase was not statistically significant ($t_5 = -1.003$, $p>0.05$) due to the high variability in the data.

A



B

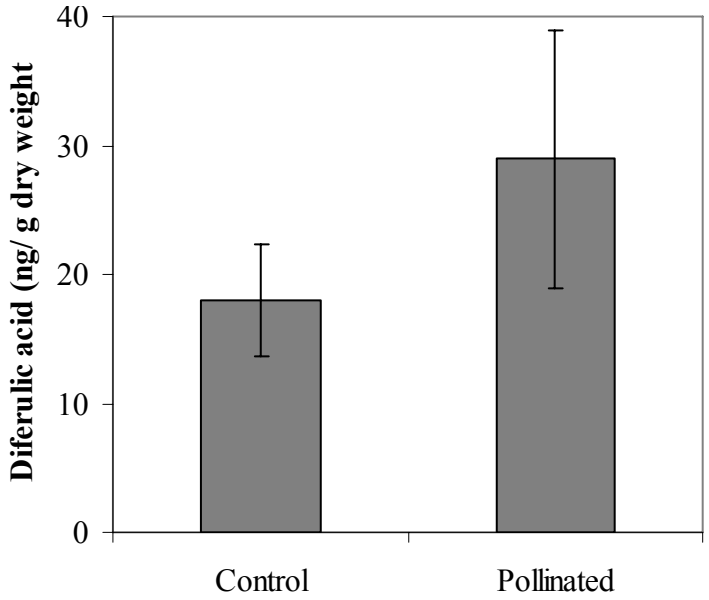


Figure 4.5. Abundance of ferulic acid (**A**) and 5-5'-coupled diferulic acid (**B**) in control and pollinated silk walls. Mid-silk samples collected 24 h post-pollination from spikelet positions 20-24 were used. Data shown are means +/- SEM (n = 3 ears)

4.4. Discussion

4.4.1. Biophysical Analyses

4.4.1.1. Wall Susceptibility to Exogenous Expansin

Susceptibility (in terms of wall extension) to exogenously added expansin has been previously utilized as an indirect measure of wall structural modifications (Cosgrove and Li, 1993b; Wu *et al.*, 1996). Since frozen, thawed and heat-inactivated walls are used, any endogenous biochemical processes are eliminated. I used this approach to investigate possible wall changes incurred by pollination in silks. Within 16 h after pollination, silk walls underwent a 36 % reduction in the extension response to added pollen β-expansin (Figure 4.1). The results suggest that the major slowing of silk elongation and the loss of susceptibility to expansin may be concomitant. It is possible that the response to expansin changes even before the major slowing of silk elongation. However, the time-course of the experiment is inadequate to support such a temporal relationship. Cosgrove *et al.* (1993a) also reported that oat coleoptile walls from non-growing regions were significantly less susceptible to expansin-induced extension. Similar results were obtained by Wu *et al.* (1996) with reference to maize root growth under water deficit.

The mechanism behind this loss of susceptibility to expansin is not yet established. Changes in wall polymer cross-linking, structural changes in polymers, or alterations in the charge density or porosity of the walls have been discussed as possible explanations (Cosgrove and Li, 1993a; Wu et al., 1996). One interesting aspect of my findings is that even though the pollinated walls were much less susceptible to exogenous expansin, their extension response to low pH treatment

(acid-induced extension, Chapter 3 Figure 3.2) was not significantly reduced. One explanation of these observations is that pollination changes the wall in a way that makes it more difficult for exogenously added expansins to reach their target substrates or sites of activity. This might occur by any of the aforementioned alterations discussed by Wu *et al.* (1996) and Cosgrove *et al.* (1993a).

4.4.1.2. Stress/strain (Instron) Analysis of Silk Cell Walls

To further investigate the possible wall modifications indicated by wall susceptibility assays to added expansin, stress/strain analyses were conducted. This technique is thought to provide information about any structural alterations in the cell walls (Cosgrove, 1993). Isolated walls are used and additionally the duration of the assay per sample is short, thereby eliminating interference from on-going biochemical process.

Beyond 6 h post-pollination, the plastic extensibility of mid-silk cell walls was significantly reduced (Figure 4.2). Elastic extensibility also decreased significantly even though the change was numerically small. Inhibition in silk elongation was apparent beyond 12 h post-pollination. It is possible that the slowing of elongation begins before 12 h. Therefore, the reduction in wall extensibility (especially the plastic extensibility) probably precedes the **major slowing** of silk elongation. However, it is also possible that the wall changes and the changes in elongation are concomitant.

My findings are in agreement with numerous studies that have suggested a positive correlation between wall extensibility (as measured by stress/strain and equivalent applied force methods) and plant growth. For example, growth promotion of cucumber hypocotyls by gibberellic acid was closely linked to an increase in the plastic extensibility (Taylor and Cosgrove, 1989). Increases in rice coleoptile elongation were correlated with higher wall extensibility (Tan *et al.*, 1991). According to Kutschera (1996), the cessation of cell elongation in rye coleoptiles involves a loss of plastic extensibility. Recently it has been reported that the growth inhibition of the sub-apical regions of maize root under water stress accompanies a decrease in the cell wall extensibility (Fan *et al*., 2006).

One interesting observation is that the considerable decrease in the plastic extensibility after pollination apparently did not have a major effect on acid-induced extension (native wall creep). Acid-induced extension is thought to involve some type of wall polymer creep where the load-bearing polymers undergo a shearing or slipping movement (Cosgrove, 1998; Marga *et al*., 2005). The reduction in plastic extensibility is probably caused by some type(s) of wall polymer cross-linking. Therefore, a possible explanation of the discrepancy between the changes in plastic extensibility and the native wall creep is that any cross-linking reaction caused by pollination does not change the polymer interactions controlling wall creep (Pers. Comm. D. Cosgrove). Stress/strain analysis measures a short-term extension of the wall. In contrast native wall creep entails a long-term extension. Therefore it is possible that these two processes involve the deformation of different wall components (Cosgrove, 1993; Cutillas-Iturralde *et al.,* 1997).

Does a Reduction in Wall Extensibility Cause the Slowing of Silk Elongation?

Both biophysical analyses suggest that pollination induces some type(s) of wall rigidification. Based on the timing of the reduction in wall extensibility (especially the plastic extensibility) and the slowing of silk elongation, these two phenomena may be causally related. However, a strong conclusion can not be drawn due to several reasons. Plastic extensibility has not been satisfactorily linked to either specific wall extensibility (φ) or the yield threshold (Y) of the Lockhart model of cell growth discussed in Chapter 1 (Cosgrove, 1993 and 2003). A further complication is the already discussed discrepancy between the effect of pollination on the plastic extensibility and the long-term native wall creep. The latter is thought to be similar to the wall extension that occurs during plant cell growth (Cosgrove, 1989 and 1993). Moreover, examples where changes in plant growth were not reflected in changes in wall extensibility have been reported in several plant species (Cosgrove, 1993 and references therein).

What Signals the Reduction in the Wall Extensibility Measured in Stress/strain Method?

In order to examine whether the presence and/or movement of pollen tubes signal the reduction in wall extensibility basal silk samples were assayed by the stress/strain method. Moreover, comparable samples were stained with aniline blue to visualize pollen tubes. The results indicated that the decrease in wall extensibility only occurs after a pollen tube(s) has traversed a given region of silk (Figure 4.3). This suggests that the signaling behind the reduction in wall extensibility does not originate in the ovary but is locally initiated by the presence and/or the movement of the pollen tube(s). Assuming that the decrease in wall extensibility plays a role in the inhibition of silk elongation then it can be proposed that at least some of the signals involved

in the silk elongation response to pollination are locally generated. Interestingly this idea is consistent with experiments which indicated that interaction with viable pollen is necessary for the inhibition of silk elongation (Chapter 2 Figure 2.5).

4.4.3. Effect of Pollination on Silk Cell Wall Chemical Composition

FT-IR spectroscopy was performed to identify potential chemical changes that may be involved in the reduction in silk wall extensibility discussed above. One of the more reliable findings of the FTIR analyses is that the pollinated silk cell walls are significantly different from control walls in terms of chemical composition. The method is less useful in the definitive identification of the specific chemical groups and the nature of any cross-linking that may occur. The digital subtraction of the spectra from the two groups of samples indicated that pollination probably causes increases in phenolic substances in silk cell walls (Figure 4.4). It should however be noted that FT-IR spectra are known to be not diagnostic for phenolic groups (Sene *et al*., 1994). Chemical analysis of silk walls indicated an increase in the wall-bound 5-5'-coupled diferulic acid in pollinated walls compared to controls. Therefore a pollination-induced dimerization of wall-bound ferulic acid is a possibility. However, the increase in diferulic acid was not statistically significant ($p>0.05$) due to the high variability in the data (Figure 4.5). Therefore this experiment is inconclusive as of now and will be repeated with a larger number of replicates.

An interesting finding of the FT-IR spectroscopy is that pollination seems to induce changes in silk wall pectins. The free carboxylic acid groups have evidently increased in pollinated samples compared to controls. Even though the proportion of pectin in grass walls is known to be low

(~ 10 % according to Cosgrove, 2000), increased levels of carboxlic acid can potentially rigidify the wall by ionically binding to Ca^{2+}. One excellent case regarding this is discussed by McCann *et al.* (1997). Methyl de-esterification of pectin produces free carboxylic acid functional groups. In the walls of tobacco cells grown in suspension culture, the carboxylic acid groups were increased with a concomitant decrease in esters upon the cessation of cell elongation. The reverse was true during maximal cell elongation. These findings suggest a link between pectin de-esterification in the wall and growth cessation of tobacco cells under the experimental conditions used. In FT-IR analyses of silk walls, such a trend is not readily seen because both esters and carboxylic acid groups seem to be more abundant in pollinated silk walls compared to controls. However, it is also possible that synthesis of esters is up regulated after pollination.

A problem concerning both FT-IR and chemical analyses is that even though they can show changes in the abundance of certain substances in the wall, they fail to provide insight into the nature of any cross-linking of wall polymers. For example, even if increases in diferulates were noted in pollinated walls, that does not necessarily mean the existence of inter-polymeric cross-links. Moreover, to conclusively link a wall cross-linking reaction(s) to growth inhibition, extensive pharmacological and genetic experiments are needed.

In summary, the experiments discussed in this Chapter shows that the silk cell wall extensibility as measured by stress/strain analysis and the susceptibility to exogenous expansin was reduced after pollination. The timing of this phenomenon supports the idea that the reduction in wall extensibility may be causally related to the inhibition of silk elongation. However, such an

interpretation is fraught with serious questions. The reduction in wall extensibility is probably initiated by local signals generated by the presence and/or movement of pollen tubes. Pollination seemingly induces changes the silk cell wall chemical composition: probably pectic and phenolic substances.

Chapter 5

Summary and General Discussion

The experiments discussed in this thesis were conducted in order to answer a deceptively simple question: how does silk elongation cease after pollination? This is a very interesting phenomenon which serves an important functional role in the reproductive success of maize. However, to my knowledge nothing is known about the physiological mechanisms underlying this growth response. Extension in silk length occurs through cell elongation (Kieselbach, 1949; Heslop-Harrison *et al.,* 1984; Westgate and Boyer, 1985). Since the cell wall plays a key role in cell elongation I postulated that pollination induces certain changes in the wall which leads to the inhibition of silk elongation. To test this idea the effects of pollination on silk cell wall structure and the wall loosening agent expansin were investigated in relation to silk elongation kinetics.

5.1. Overall Results and Conclusions

Experiments performed under both greenhouse and field conditions showed that **silk elongation undergoes a major down-regulation beyond 12 h after pollination** (Chapter 2). Dead pollen failed to inhibit silk elongation, suggesting that the growth response requires the interaction with viable pollen. Moreover, pollination of some silks on an ear did not have a systemic (long distance) inhibitory effect on the elongation of un-pollinated silks on the same ear.

Pollination did not cause dramatic changes (specifically reductions) in either α- or β-expansin protein abundance. Moreover, neither endogenous expansin activity as evaluated by biophysical

indicators nor extractable α-expansin activity was decreased in response to pollination (Chapter 3). **These observations lead me to propose that the mechanism of pollination-induced inhibition of silk elongation is largely independent of changes in expansin.**

There was **a significant reduction in silk cell wall extensibility** as measured by stress/strain analysis and wall susceptibility to added β-expansin after pollination (Chapter 4). This can be considered as a process of **wall rigidification**. The decrease in wall extensibility either precedes the slowing of silk elongation or these two processes are concomitant. Therefore these two phenomena may be causally related. The molecular nature of the reduction in wall extensibility is uncertain at this time. Experiments conducted so far suggest that carboxylic acid groups, ester and phenolic substances probably become more abundant in pollinated walls (Chapter 4). Both carboxylic acid groups and phenolic substances have been implicated in cell wall cross-linking reactions that may reduce wall extensibility (Carpita, 1996; McCann *et al.*, 1997; Fry, 2004).

The reduction in wall extensibility measured here occurred only after pollen tubes have traversed through a given region of silk (Chapter 4). This suggests that local signals (rather than signals from the ovary) in response to the presence or movement of pollen tubes may be involved in the change in wall extensibility. If we assume that the reduced wall extensibility is indeed a major cause of the slowing of silk elongation, then it can be suggested that the growth response is initiated by local signals due to pollen tubes.

5.2. Limitations and Further Experiments

One major limitation of my work is that the **experiments conducted did not unambiguously reveal the mechanism behind the pollination-induced inhibition of silk elongation**. The reduction in wall extensibility might play a role. Problems associated with such an interpretation were discussed in Chapter 4. Further experiments are necessary to conclusively show that the decrease in wall extensibility indeed causes the slowing of silk elongation. As a first step any wall polymer cross-linking reaction(s) should be precisely identified. Thereafter, extensive pharmacological and genetic experiments can be performed to demonstrate a link between the wall changes and silk elongation.

I confined my investigations to changes in expansin and cell wall structure as potential mechanisms underlying the inhibition of silk elongation. However, many other mechanisms that can cause growth changes have been discussed in the literature. Water potentials below -0.75 MPa completely arrest maize silk elongation (Westgate and Boyer, 1985). Down-regulation of maize root growth by water deficit was correlated with an inhibition of cell wall acidification (Fan *et al.* 2004). Similar trends have been noted with regard to maize leaf growth under water stress (Van Volkenburgh and Boyer, 1985; Bogoslavsky and Neumann, 1998). This is of particular importance because even though decreases in expansin activity were not detected after pollination an inhibition of cell wall acidification would down-regulate expansin mediated wall loosening.

There are numerous studies that suggest a correlation between xyloglucan endotransglycosylase (XET) activity and plant growth (Wu *et al.,* 1994; Fry, 2004 and references therein). According to Rodriguez *et al.* (2002) reactive oxygen species are necessary for maize leaf growth. These findings suggest that a comprehensive understanding of the mechanism(s) involved in the inhibition of silk elongation after pollination requires careful examination of diverse processes. My findings on the timing of the silk growth response would be invaluable in such investigations.

The observations reported in this thesis open further avenues of research. One very interesting issue is the nature of the signaling involved in the reduction in wall extensibility. The experiments performed have identified the timing and the location of the response, thus providing the necessary basic foundation. The initial signal may be triggered by different means. Firstly, pollen tube-borne chemical messengers may be involved. Secondly, wounding of the silk by pollen tube penetration and growth can be speculated to initiate a signaling cascade. Though not investigated in maize silk, pollen tube growth has been shown to cause extensive cellular damage in the transmitting tissue of other species (Cheung *et al*., 1996; Wang *et al*., 1996). These prospective studies on the changes in cell wall can be coupled to examine signaling involved in the inhibition of silk elongation. Such experiments may indeed help dissect the relationship (if any) between wall extensibility and silk elongation.

5.3. Further Speculations

The reduction in wall extensibility (or wall rigidification) discussed above may fulfill at least two other functional roles. It was shown in Chapter 2 that the number of pollen tubes decreases from the tip of the silk to the ovary (Figure 2.4). In addition, not all the pollen tubes initiated at the tip (site of pollen deposition) reach the ovary even after 24 h. As mentioned in Chapter 2, Heslop-Harrison *et al*. (1985) discussed several mechanisms that prevent multiple pollen tubes from entering the ovary. It is possible that the decrease in wall extensibility might be involved in blocking the progress of slow growing pollen tubes once the faster ones have proceeded thorough a given region of silk. This idea is supported by the observations of Valdivia *et al*. (2006) who studied a maize line having a Mutator (*Mu*) transposon insertion in the pollen β-expansin *ZmEXPB1*. The authors reported a significant decrease in the *in vivo* growth rate of the pollen tubes carrying the *expb1::mu* allele compared to the wild type. However, the *in vitro* growth of the pollen tubes was not affected by the mutation. The protein encoded by *ZmEXPB1* (Zea m1) is therefore proposed to have an *in vivo* wall loosening function which facilitates pollen tube penetration and growth through the silks (Cosgrove, 2000; Li et al., 2003). Collectively these findings suggest that wall loosening is required for successful pollen tube growth. Therefore one might expect that a rigidification of the wall would have the opposite effect.

Valdivia *et al.* (2006) reported that the aforementioned transposon mutant line showed delayed silk senescence. Moreover there was a dramatic increase in fungal ear rot in the mutant compared to the wild type. These observations led the authors to propose that delayed silk senescence due to slower pollen tube growth makes the mutant plants at risk of fungal infection.

The authors attributed this to prolonged feeding on un-senesced silks by corn rootworm beetles which are vectors for fungi causing ear rot and/or the additional time made available for the fungi to grow on/through the silks to the ovaries. The cell wall rigidification observed in my experiments may play a protective role against fungal pathogens. Reinforcement of cell walls is a well documented physical defense 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). This process would create multiple openings in the silk. Such a situation can potentially make the plant at risk of pathogen infection. Therefore, it would be advantageous for the silk walls to become rigidified thereby hindering both fungal penetration and hyphal growth. 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 defense response, if it truly occurs, is a precautionary mechanism. Indeed examples of such events involving very different elicitors have previously been reported (Heil *et al*., 2002). According to Hatcher *et al.* (2000) beetle grazing induced resistance against fungal infections in *Rumex obtusifolius*. Thrip- and aphid-feeding has been shown to reduce the infection of water melon by *Colletotrichum orbiculare* (Russo *et al*., 1997).

5.4. Impacts

Very little is known about the mechanisms of cell wall rigidification. As discussed in Chapters 1 and 4, several chemical reactions that can cross-link wall polymers thereby rigidifying it have been described in the literature. However, whether these reactions indeed lead to polymer cross-

linking has not been conclusively demonstrated. To my mind the reduction in silk wall extensibility by pollination provides an excellent system to study wall rigidification reactions. Silks are elongated structures which makes it convenient to use them in wall biophysical assays. Pollination is a simple treatment that does not require elaborate experimental procedures. Moreover the decrease in extensibility occurs by 6 h after pollination. Another interesting issue is the discrepancy seen between acid-induced extension and wall plastic extensibility measurements of pollinated silk walls (see Chapter 4). The precise nature of the molecular rearrangements and polymer creep that occur during acid-induced extension is not well established. It might be possible to utilize the silk-pollination system to glean some clues about these molecular underpinnings. Such findings would have important impacts on cell wall structural models.

It is too early to speculate about any direct applications of my findings in agriculture. However, the reduction in silk cell wall extensibility may in the long run prove to be important. Firstly, the changes in the cell wall may indeed be causally related to the inhibition of silk elongation, which can be considered as part of an accelerated senescence process triggered by pollination. In terms of energy costs, faster senescence arguably would be beneficial to the reproductive success of the maize plant (see Chapters 1 and 2). Secondly, the decrease in wall extensibility may provide defense against pathogens. Both these issues would undoubtedly have large impacts on maize yield. Further work with genetic and molecular tools might lead to successful maize breeding programs based on changes in silk cell walls.

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