

The Control of Single-Celled Cotton Fiber Elongation by Developmentally Reversible Gating of Plasmodesmata and Coordinated Expression of Sucrose and K⁺ Transporters and Expansin

Yong-Ling Ruan,¹ Danny J. Llewellyn, and Robert T. Furbank

CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia

Each cotton fiber is a single cell that elongates to 2.5 to 3.0 cm from the seed coat epidermis within ~16 days after anthesis (DAA). To elucidate the mechanisms controlling this rapid elongation, we studied the gating of fiber plasmodesmata and the expression of the cell wall-loosening gene expansin and plasma membrane transporters for sucrose and K⁺, the major osmotic solutes imported into fibers. Confocal imaging of the membrane-impermeant fluorescent solute carboxyfluorescein (CF) revealed that the fiber plasmodesmata were initially permeable to CF (0 to 9 DAA), but closed at ~10 DAA and re-opened at 16 DAA. A developmental switch from simple to branched plasmodesmata was also observed in fibers at 10 DAA. Coincident with the transient closure of the plasmodesmata, the sucrose and K⁺ transporter genes were expressed maximally in fibers at 10 DAA with sucrose transporter proteins predominately localized at the fiber base. Consequently, fiber osmotic and turgor potentials were elevated, driving the rapid phase of elongation. The level of expansin mRNA, however, was high at the early phase of elongation (6 to 8 DAA) and decreased rapidly afterwards. The fiber turgor was similar to the underlying seed coat cells at 6 to 10 DAA and after 16 DAA. These results suggest that fiber elongation is initially achieved largely by cell wall loosening and finally terminated by increased wall rigidity and loss of higher turgor. To our knowledge, this study provides an unprecedented demonstration that the gating of plasmodesmata in a given cell is developmentally reversible and is coordinated with the expression of solute transporters and the cell wall-loosening gene. This integration of plasmodesmatal gating and gene expression appears to control fiber cell elongation.

INTRODUCTION

A unique feature of cotton seed development is that ~30% of the ovule epidermal cells initiate into fibers from the outermost layer of integument at anthesis (See Figure 1A). Each cotton fiber is a single cell and elongates from 10 to 15 μm up to 2.5 to 3.0 cm by ~16 days after anthesis (DAA) before it switches to secondary cell wall cellulose synthesis (Basra and Malik, 1984; Tiwari and Wilkins, 1995). The rate of fiber elongation and the final length attained are well above that commonly seen for plant cells (Cosgrove, 1997) and render it perhaps the longest single cell in higher plants. Thus, the cotton fiber represents a unique system in which to study not only carbon partitioning to cellulose synthesis (Delmer and Amor, 1995; Ruan et al., 1997) but also the control of cell elongation without the complication of cell division and multicellular development. Apart from its significance in understanding basic cell biology, elucidating the cellular and

molecular basis of fiber elongation could also identify potential targets for genetic manipulation of fiber length, a key determinant of fiber yield and quality.

The rapid fiber elongation is believed to be driven by high turgor (Dhindsa et al., 1975; Ruan and Chourey, 1998; Smart et al., 1998) with a highly extendable primary cell wall (Ruan et al., 2000). Cell turgor in plants is achieved largely through the influx of water driven by a relatively high concentration of osmoticum within a cell (Cosgrove, 1997). The importance of turgor in fiber growth has been indicated in vitro by manipulation of the osmotic potential of culture media (Dhindsa et al., 1975). The accumulation of osmoticum into fibers may be coupled with the transmembrane proton gradient, because the plasma membrane H⁺-ATPase gene is expressed strongly during the rapid phase of fiber elongation (Smart et al., 1998). This H⁺ pump could also acidify the apoplast for cell wall loosening (Cosgrove, 1997). Pertinently, the expansin gene, of major importance in mediating cell wall extension (Cosgrove, 1997), is expressed in fibers, although its temporal expression pattern over the elongation period is not clear (Shimizu et al., 1997; Orford and Timmis, 1998).

¹To whom correspondence should be addressed. E-mail ruan@pi.csiro.au; fax 061-02-62465000.

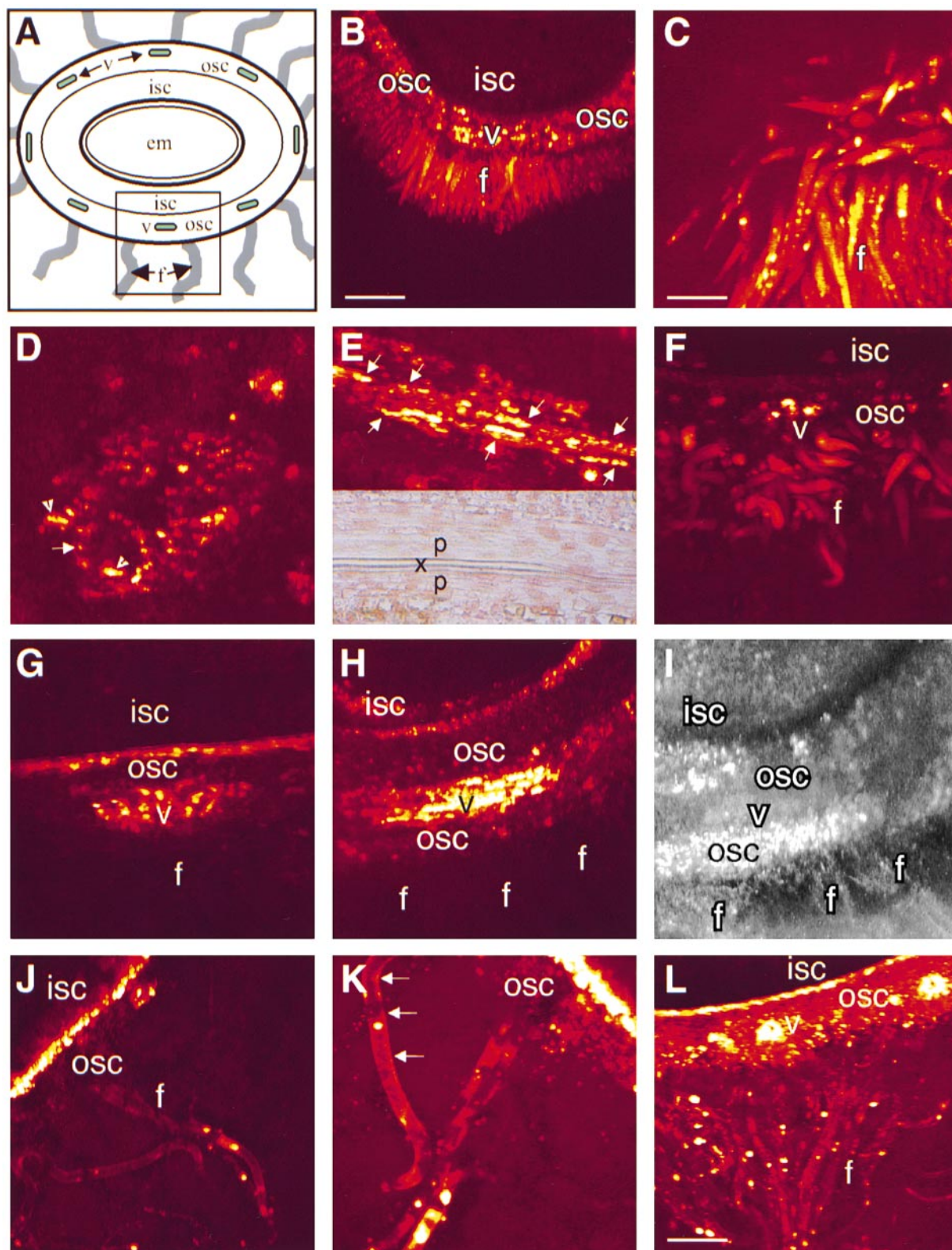


Figure 1. Confocal Imaging of CF Transport from Phloem in Seed Coat into Elongating Cotton Fibers at 2, 6, 10, and 16 DAA.

Despite this progress, critical questions remain to be answered regarding the control of fiber elongation. The notion that the elongation is driven by high turgor is largely based on circumstantial evidence (Dhindsa et al., 1975; Smart et al., 1998). It is unknown whether the turgor is indeed higher in fibers than in the underlying seed coat cells. It is even more intriguing how the turgor in the single-cell fiber is maintained and regulated. The fiber cells elongate 1600- to 3000-fold in 16 days, accompanied by only 5- to 10-fold expansion of interconnecting seed coat cells, indicating a high degree of cell autonomy in fibers. One mechanism for such a discrepancy in cell growth and function is symplastic isolation between the respective cell/tissue types through closure of plasmodesmata, the intercellular cytoplasmic connections that act as gates controlling molecular trafficking from cell to cell (McClean et al., 1997; Oparka and Turgeon, 1999). It is therefore important to explore whether or when the fiber plasmodesmata (Ryser, 1992) close at their basal connection to the seed coat during the elongation period. The major osmotically active solutes in elongating fibers are soluble sugars, K^+ , and malate, which together account for ~80% of the fiber sap osmolality (Dhindsa et al., 1975; Ruan et al., 1997). Whereas malate is synthesized locally by refixing CO_2 through phosphoenolpyruvate carboxylase in the fiber cytoplasm (Basra and Malik, 1984; Smart et al., 1998), sugar and K^+ are imported externally from the phloem in the seed coat (van Iersel and Oosterhuis, 1996; Ruan et al., 1997). Previous studies suggest that sucrose is the major form of photoassimilate imported into developing cotton fiber (Buchala, 1987; Ruan et al., 1997). Very little is known, however, about the pathway of sucrose and K^+ movement into fibers and whether it occurs by passive diffusion or is

mediated by their respective transporters, as shown in other plant systems (Kim et al., 1998; Lalonde et al., 1999). Finally, the most important issue is perhaps how the symplastic connection between fibers and the seed coat, the import of sucrose and K^+ , and the dynamic of cell wall loosening might be coordinately regulated to achieve and also to terminate fiber elongation.

In this study, we investigate the gating of fiber plasmodesmata and possible expression of the plasma membrane sucrose and K^+ transporters and expansin in elongating cotton fibers at selected stages of development. The results provide a remarkable example of how the gating of plasmodesmata and the expression of genes for solute import and cell wall loosening are developmentally coordinated to potentially control single-cell elongation.

RESULTS

The Gating of Fiber Plasmodesmata Is Developmentally Reversible

To examine the gating status of fiber plasmodesmata, the phloem-mobile fluorescent probe carboxyfluorescein (CF) was ester-loaded into shoots through their cut ends. The subsequent unloading pattern of CF from the phloem of the outer seed coat to the fiber cells was monitored in situ using confocal laser scanning microscopy. The xylem discontinuity in the peduncle of developing cotton fruit (van Iersel and Oosterhuis, 1996) ensures phloem-specific transport of CF into fruits and seeds from the shoots.

Figure 1. (continued).

- (A) A schematic representation of a developing cotton seed. The boxed area corresponds to the following confocal images of CF movement from the vascular bundle in the outer seed coat into fibers.
- (B) Optical cross-section of seed at 2 DAA from shoot fed with CF for 24 hr, showing CF movement from the vascular bundle into fibers.
- (C) Imaging of the surface of the intact seed shown in (B). Note strong CF signals in fibers.
- (D) Cross-section of a major vascular bundle from seed at 6 DAA after feeding CF for 16 hr. Note that CF signals were initially detected in the sieve element (arrow) and sieve element-companion cell complexes (arrowhead), but not in xylem between them.
- (E) Longitudinal section of a vascular bundle at 6 DAA, showing CF fluorescence in phloem (arrows), flanked by nonfluorescence xylem, which is shown in the inset. Note the thicker cell wall of the xylem in the inset.
- (F) Preferential transport and accumulation of CF from unloading area to fibers at 6 DAA after 24-hr feeding.
- (G) Blockage of CF movement into fibers at 10 DAA after 24-hr feeding. Note the stronger and wider spread CF signals in the vascular region than that at 6 DAA (F), suggesting that sufficient CF has been unloaded. Also, the dye preferentially accumulated at the inner side of the outer seed coat, in contrast to that at 6 DAA (F).
- (H) Optical section at 10 DAA after extended feeding of CF for 48 hr. The dye spread throughout the outer seed coat but was not present in fibers.
- (I) Autofluorescence image of (H) at 514 nm, showing the position of fiber and other tissues.
- (J) CF signals were detected again in fibers at 16 DAA after 24-hr feeding.
- (K) Enlarged view of fiber shown in (J). Note CF signals in cytosol lining to plasma membranes (arrows) and appeared patchy in some areas.
- (L) A montaged image of seed coat at 16 DAA after extended feeding for 48 hr. CF moved extensively into fibers.
- f, fiber; isc, inner seed coat; osc, outer seed coat; p, phloem; v, vascular bundle; x, xylem. Bar in (B) = 500 μm for (B) and (F) to (J); bar in (C) = 200 μm for (C) to (E) and (K); bar in (L) = 1200 μm .

Figure 1A represents a diagram of cotton seed. It is similar to other dicotyledonous seed except that part of the outer seed coat epidermis becomes fiber cells. The boxed area in Figure 1A corresponds to the orientation of the images on CF movement between the outer seed coat and fibers shown in Figures 1 and 2. Confocal imaging of optical cross-sections at 2 DAA revealed that CF readily moved from the vascular bundle of the outer seed coat into the fibers after 24-hr shoot feeding (Figure 1B). Imaging on the surface of the same intact seed showed strong fluorescent signals of CF in the fibers (Figure 1C). No fluorescence was detected in fibers from shoots fed with water only (data not shown). The phloem origin of CF was confirmed by imaging vascular bundles of seed at 6 DAA after a 16-hr feeding. As shown in a cross-section view, the CF signals were first apparent in the sieve element-companion cell complex (Figure 1D). The movement of CF in the phloem, flanked by nonfluorescent xylem, was further demonstrated in an optical longitudinal section (Figure 1E). The position of the xylem was shown in a paraffin-embedded section from the same tissue

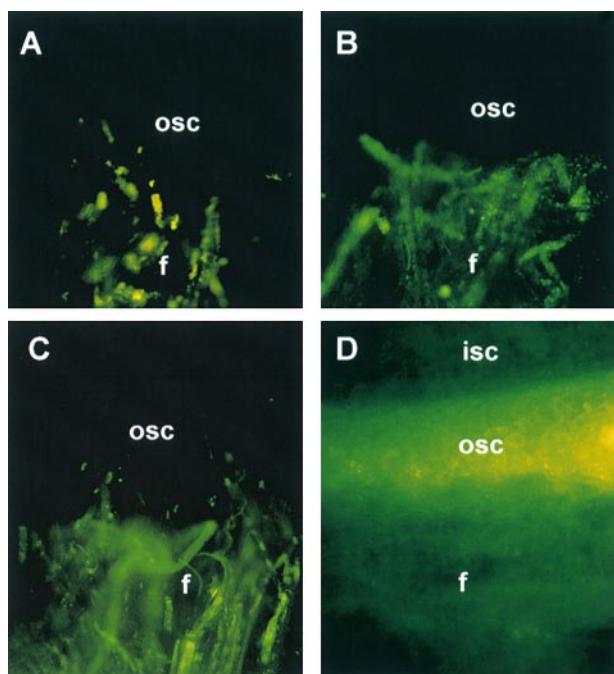


Figure 2. Light-Fluorescent Micrographs of CF in Hand-Cut Transverse Sections of Developing Cotton Seed Coats at 10 ([A] and [B]) and 16 ([C] and [D]) DAA.

(A) and (C) Fluorescent signals were confined in fibers after 20-min local loading into fibers from an attached fruit.

(B) and (D) After 2-hr incubation in buffer, CF failed to move into the outer seed coat from fiber at 10 DAA (B). In contrast, at 16 DAA, the dye readily moved into the seed coat (D).

f, fiber; isc, inner seed coat; osc, outer seed coat.

(Figure 1E, insert). After unloading from phloem, the CF preferentially moved into fibers with much lower signals in other regions of the seed coat at 6 DAA (Figure 1F). Significantly, at ~ 9 to 10 DAA, the CF failed to enter the fibers from the vascular region (Figure 1G). Here, it is important to note that the CF signal in the vascular region in Figure 1G (10 DAA) was stronger and spread more extensively than that in Figure 1F (6 DAA), when the dye readily moved into fibers. This indicates that the failure of CF transport into fibers at 10 DAA is not due to insufficient unloading of the dye. To confirm this further, we extended CF feeding to 48 hr on shoots bearing 10-day-old fruit. This led to intense CF signals in the vascular region and widespread movement of the dye throughout the outer seed coat (Figure 1H). However, the CF again failed to move into fibers (Figure 1H). The same image was viewed at a wavelength of 514 nm to show the position of fibers and other parts of the seed coat revealed by the autofluorescence of phenolic compound in those tissues (Figure 1I). The restriction of CF import into fibers, however, lasted for only ~ 5 days. As the fibers elongated to ~ 16 DAA, the end of the elongation period, the phloem-unloaded CF again moved readily to fibers (Figure 1J) and became concentrated in the peripheral region of the cytoplasm (Figure 1K). The montage of a series of images of 16-day-old seed after a 48-hr feeding provides an overview of CF movement into fibers (Figure 1L).

A feature of the CF fluorescent signals in fiber cells is that they are punctate and patchy in some areas of the cells at 16 DAA (Figure 1K). Although the exact cause is unknown, we postulate vacuolar sequestration as the likely basis for this phenomenon. It is possible that a small percentage of CF may be present as the undissociated form in some regions of the fiber cytoplasm in which pH might be slightly acidic (e.g., 6.3 to ~ 7.3 ; see Wright and Oparka, 1996). Under this condition, the undissociated form of CF may become membrane permeant, thus moving across the tonoplast of the vacuole (Wright and Oparka, 1996). Given that each fiber cell is ~ 2.5 to ~ 3.0 cm long, with a huge vacuole present at 16 DAA (Basra and Malik, 1984), a localized penetration and accumulation of CF into the vacuole could result in patchy fluorescence in fibers (Figure 1K).

The observation that the fiber plasmodesmata were closed for CF import at 10 DAA and reopened at 16 DAA (Figure 1) is unusual. To confirm this, we loaded CF locally into fibers of attached fruit for 20 min through a window cut in the pericarp and examined its possible movement into underlying seed coats. The CF was initially confined in fibers upon completion of the loading at both 10 and 16 DAA (Figures 2A and 2C, respectively). For seed at 10 DAA, the CF in fibers did not spread into the underlying seed coat after a 2-hr incubation in buffer solution (Figure 2B). On the other hand, at 16 DAA, the movement of CF from the fibers into the outer seed coat was evident after the same duration of incubation (Figure 2D). This observation (Figure 2) is consistent with that shown in Figure 1.

Structure of Cotton Fiber Plasmodesmata

Electron microscopy was performed to examine possible structural changes of fiber plasmodesmata during the elongation period. At 6 DAA, all the plasmodesmata observed were in simple form (Figure 3A). However, by 10 DAA, approximately half of the plasmodesmata became branched toward the fiber side (Figure 3B). The proportion of the branched-form plasmodesmata increased at 18 DAA. However, a small percentage of plasmodesmata were still present in simple form at this stage (data not shown).

Coordinated Expression of Sucrose and K⁺ Transporters and Expansin in Elongating Cotton Fibers

The closure of plasmodesmata for CF import into fibers at 10 DAA (Figures 1 and 2) would necessitate solute uptake across the plasma membrane of fibers at the base region connecting to the seed coat. Further experiments were therefore conducted to examine the possible expression of sucrose and K⁺ transporters in fibers. To achieve this, we cloned partial cDNAs—*GhSUT1* and *GhKT1*—encoding plasma membrane sucrose and K⁺ transporters, respectively, from fiber tissue using reverse transcription-polymerase chain reaction. A Blast search of the GenBank database showed that the amino acid sequences encoded by *GhSUT1* and *GhKT1* shared high homology exclusively to known plasma membrane sucrose and K⁺ transporters in the first 35 and 15 matches, respectively. In the first two matches, *GhSUT1* shared 82 and 80% amino acid identity with sucrose transporters from *Alonsoa meridionalis* and *Ricinus communis* (GenBank accession numbers AF191025 and AJ224961), respectively, whereas *GhKT1* showed 67

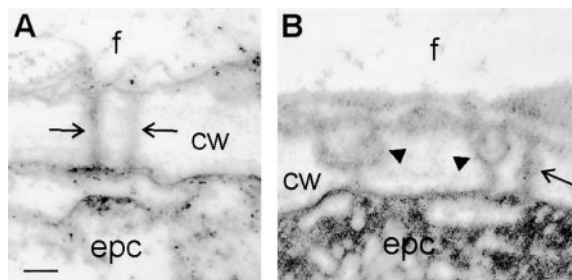


Figure 3. Plasmodesmata of Elongating Cotton Fibers.

(A) Simple plasmodesmata (arrows) at 6 DAA in longitudinal orientation.
(B) Branched (arrowheads) and “swollen” simple (arrow) plasmodesmata at 10 DAA. Branching occurred at the fiber side.
 cw, cell wall; epc, epidermal cell; f, fiber cell. Bar in **(A)** = 100 nm for **(A)** and **(B)**.

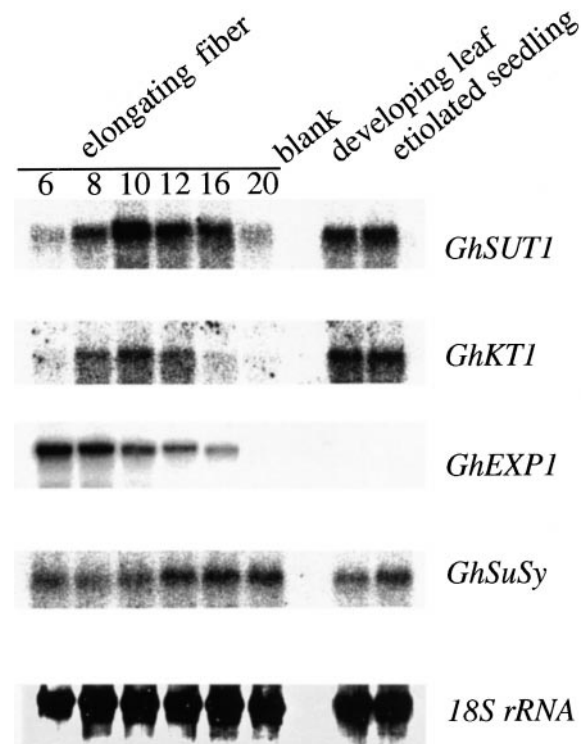


Figure 4. Transcript Expression of *GhSUT1*, *GhKT1*, *GhEXP1*, and *GhSuSy* in Elongating Cotton Fibers and Other Sink Tissues.

RNA gel blot with 25 µg total RNA in each lane was sequentially hybridized with *GhSUT1*, *GhKT1*, *GhEXP1*, and *GhSuSy* cDNA probes. The blot was finally hybridized with a maize rRNA cDNA probe to show equal loading and transfer of RNA in each lane. Number on fiber samples indicates days after anthesis.

and 60% identity to high affinity K⁺ transporters from *Arabidopsis* and *Schwanniomyces occidentalis* (GenBank accession numbers AL031394 and AC004473), respectively.

RNA gel blot analysis with *GhSUT1* showed a readily detectable 2.0-kb mRNA in fibers (Figure 4). The transcript intensity was weak at 6 DAA but increased significantly to a maximal level at 10 DAA and remained relatively higher up to 16 DAA. The blot was stripped and rehybridized with *GhKT1*. A temporal expression pattern similar to that of *GhSUT1* was observed, except that the transcript detected by *GhKT1* decreased much faster after its peak at 10 DAA (Figure 4). The *GhKT1* transcript was also found to be specifically induced by K⁺ starvation treatment (data not shown), a feature indicative of the high-affinity K⁺ uptake transporter (Kim et al., 1998). Both the *GhSUT1* and *GhKT1* transcripts were abundant in etiolated seedlings and developing leaves (Figure 4). Rehybridization of the blot with *GhEXP1*, an expansin cDNA isolated from cotton fiber (Orford and Timmis, 1998), revealed that the expansin

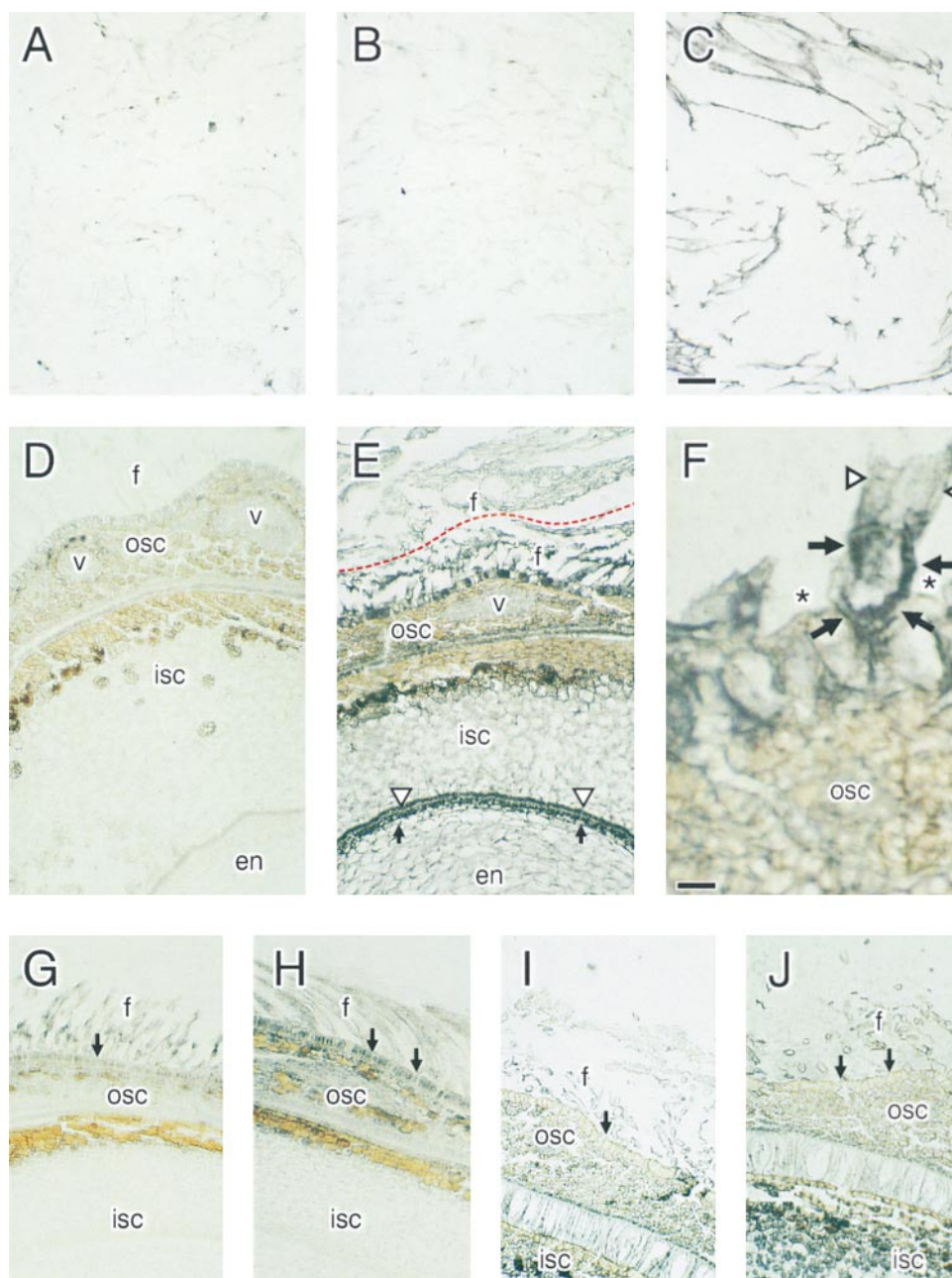


Figure 5. Immunogold Localization of SUT Proteins in Developing Cotton Seed at 10, 5, and 18 DAA.

For samples at 10 and 18 DAA, fibers were excised from the seed surface at their base region and treated separately. The black signal represents SUT proteins.

(A) Fibers at 10 DAA treated with preimmune serum.

(B) Fibers at 10 DAA treated with antiserum against SUT. No signals were detected compared with **(A)**.

(C) Fibers at 10 DAA treated with antibody against cotton SuSy, showing strong signals of SuSy protein.

(D) Cross-section of seed at 10 DAA treated with preimmune serum.

(E) A consecutive section of **(D)** but treated with antiserum against SUT. Note strong SUT signals at the fiber base region interconnecting the outer seed coat (below the red line). The signals became much weaker beyond the base region (above the red line). Also note specific strong signals at the innermost of inner seed coat (triangles) and at the outermost of the endosperm (arrows).

(F) Magnified view of a base region of fibers shown in **(E)**. Note strong SUT signals confined at the base region of fibers (arrows) with much de-

mRNA (~1.1 kb) was abundant at 6 DAA but decreased gradually and became undetectable by 20 DAA (Figure 4). This expansin mRNA is fiber specific because it was detected neither in seedlings and leaves (Figure 4) nor in other parts of the seed (data not shown). Finally, the blot was re-hybridized with SS3, a cotton sucrose synthase (SuSy) cDNA (Ruan et al., 1997). The SuSy transcript (~2.6 kb) was readily detectable in fibers throughout the stages examined, with a slight increase in its level of expression at the later stage of elongation (≥ 12 DAA). The hybridization signal for rRNA is shown as a loading and transfer control for each sample (Figure 4).

The cotton fiber connects with the outer seed coat epidermis only at the base end (Figure 1A), where influx of nutrients occurs (Ryser, 1992). To determine whether the sucrose transporter (SUT) proteins were present at the base region or throughout the fiber cells, we conducted immunogold localization experiments using an antiserum against a synthetic SUT peptide (see Ruan et al., 2000). The sample shown in Figure 5B showed no SUT proteins in 10-DAA fibers cut from ~1 mm above the seed epidermis compared with the preimmune control (Figure 5A). For comparison, consecutive fiber sections were treated with a polyclonal antibody against cotton SuSy protein (Ruan et al., 1997). As expected (see Ruan et al., 1997), strong immunogold labeling of SuSy protein was detected in fibers (Figure 5C). Significantly, SUT antiserum treatment of cross-sections of 10-DAA seed revealed strong SUT protein signals at the base region of fibers as well as at the innermost cell layer of the inner seed coat and outermost cells of the endosperm (Figure 5E), as compared with preimmune control (Figure 5D). No immunogold label was found in vascular bundles and other parts of the seed coat (Figure 5E). Under high magnification, strong immunogold signal for SUT protein was evident at the fiber base, with much decreased signals toward the upper part of the fibers (Figure 5F). SUT signals were lacking in the adjacent undifferentiated epidermal cells (Figure 5F). It is interesting that the width of the fiber increased very little, only ~100 μm at 10 DAA (Figure 5F), despite the huge increase in its length, ~1 cm, at this stage (Figure 1). Incubation of cross-sections at 5 and 18 DAA with the SUT antiserum showed a very weak reaction and no signals at the fiber base (Figures 5H and 5J, respec-

Table 1. Effect of PCMBs and EB on ^{14}C -Sucrose Uptake by Developing Cotton Fiber

DAA	^{14}C -Sucrose Uptake ($\mu\text{mol} [\text{g FW}]^{-1} \text{hr}^{-1}$) ^a		
	Control	PCMBs	EB
5–6	1.45 \pm 0.12 (100)	1.50 \pm 0.10 (103)	1.37 \pm 0.13 (95)
10–12	1.95 \pm 0.40 (100)	0.88 \pm 0.06 (45)	1.02 \pm 0.07 (52)
16–18	1.58 \pm 0.17 (100)	1.25 \pm 0.05 (79)	1.43 \pm 0.10 (91)

^aConcentrations of sucrose, PCMBs, and EB were 1.0, 0.2, and 0.1 mM, respectively. Values in parentheses are percentages of the control. Each value is the mean \pm SE of six replicates. FW, fresh weight.

tively), as compared with the respective preimmune controls (Figures 5G and 5I).

Sucrose transporter function was further examined in fibers using ^{14}C -labeled sucrose uptake. Table 1 shows that ^{14}C -sucrose uptake into fibers at 10 to 12 DAA was significantly slowed by PCMBs and EB, a nonpermeating sulfhydryl-group modifier and an inhibitor of plasma membrane H^+ -ATPase, respectively (M'Batchi et al., 1986; Beffagna and Romani, 1988). The inhibitory effect was much reduced at 16 to 18 DAA and was undetectable at 5 to 6 DAA (Table 1).

Fiber Turgor Rises after the Closure of Plasmodesmata and Maximal Expression of Sucrose and K^+ Transporters

To determine whether the gating of plasmodesmata (Figures 1 and 2) and expression of the transporters and expansin (Figures 4 and 5 and Table 1) correlate with possible changes in fiber turgor, the osmotic potential of elongating fibers and seed coat were measured. Figure 6A shows that the osmotic potential in fibers was initially similar to that of the seed coat from 5 to 10 DAA and elevated after 10 DAA, returning to the seed coat level at 20 DAA. As estimated from the measured osmotic potentials (Figure 6A) and published water potentials in developing cotton seed (van Iersel

Figure 5. (continued).

creased signals in regions above the base area (triangles). Also note that little signal was detected in undifferentiated seed coat epidermal cells (stars), flanked by the fiber cell.

(G) Cross-section at 5 DAA treated with preimmune serum.

(H) Cross-section at 5 DAA treated with antiserum against SUT, showing weaker signals at the fiber base region (arrows).

(I) Cross-section at 18 DAA treated with preimmune serum.

(J) Cross-section at 18 DAA treated with antiserum against SUT. No SUT signals were detected in the fiber base (arrows).

en, endosperm; f, fiber; isc, inner seed coat; osc, outer seed coat; v, vascular bundle.

Bar in (C) = 200 μm for (A) to (E) and (G) to (J); bar in (F) = 50 μm .

and Oosterhuis, 1996), the turgor potential was found to be similar between the fibers and seed coats at the early phase of fiber elongation before 10 DAA (Figure 6B). A sharp increase in fiber turgor was, however, evident afterward, with the peak value of 0.69 MPa at 16 DAA, which is 0.47 MPa higher than in the underlying seed coat (Figure 4B) and within the range of the turgors reported in most growing plant cells (Cosgrove, 1997). The fiber turgor subsequently decreased to levels similar to that in the seed coat at 20 DAA and later (Figure 6B).

DISCUSSION

The Developmentally Reversible Gating of Fiber Plasmodesmata

The gating of plasmodesmata has been increasingly recognized to play a central role in controlling cell-to-cell commu-

nication and in establishing cell identity and function (Lucas et al., 1993; van Bel and Oparka, 1995; Mclean et al., 1997; Schulz, 1999). The anatomical and developmental complexity of multicellular plant tissues, however, often imposes technical difficulties for a clear assessment of plasmodesmatal function in a given cell or cell type (Gisel et al., 1999). The single-celled cotton fibers interconnect with the underlying seed coat only at their base regions (Figure 1A), where a high number of plasmodesmata are present (Ryser, 1992). Through that region, nutrients are taken up from the phloem in the seed coat (van Iersel and Oosterhuis, 1996; Ruan et al., 1997) at sufficient rates to support fiber growth (Ryser, 1992). Thus, the cotton fiber is an excellent system in which to study the control of molecular trafficking through plasmodesmata at a single-cell level in a defined direction. In this study, we have shown that the initially permeable fiber plasmodesmata became impermeable to CF transport at ~ 10 DAA but only temporarily, because the symplastic connection was reestablished at ~ 16 DAA (Figures 1 and 2). Interestingly, the restriction of CF import into fibers at 10 DAA was accompanied by a gradual switch from simple to branched forms of plasmodesmata (Figure 3). A similar structural change of plasmodesmata has been observed in tobacco leaves during the sink-source transition, when the size exclusion limit of the plasmodesmata was seen to decrease (Oparka et al., 1999). It is, however, difficult to assign functional implications for such a structural change. The fact that CF was able to move into fibers again at 16 DAA (Figures 1J to 1L), when a large proportion of fiber plasmodesmata become branched, suggests that the change of plasmodesmata structure from simple to branched form may not be related to decreased plasmodesmata permeability.

Developmentally programmed symplastic isolation has been reported in a wide range of tissues and cells. This includes stomatal guard cells (Palevitz and Hepler, 1985), sieve element-companion cell complex in developed leaves (Turgeon, 1996), stem (van Bel and van Rijen, 1994), and potato tuber (van Bel and Oparka, 1995), differentiated root epidermis and hairs (Oparka et al., 1994; Duckett et al., 1994), pericarp of developing tomato fruit (Ruan and Patrick, 1995), germinating embryos (Duckett et al., 1994; Mclean et al., 1997), and central-egg cell apparatus during the fertilization process (Han et al., 2000). The symplastic isolation is believed to be required for the respective cells and tissues to perform distinct functions and morphogenesis (Duckett et al., 1994; Gisel et al., 1999) and is largely due to the occlusion or changes of gating properties of plasmodesmata, not the degradation of their structure (Palevitz and Hepler, 1985; Duckett et al., 1994). Importantly, developmental changes in symplastic continuity are not restricted to isolation of cells. Indeed, new symplastic connections can be established between cells and tissues (Mclean et al., 1997). For example, emerging lateral roots of *Arabidopsis* are symplastically isolated initially but become symplastically connected to the main root after differentiation of phloem (Oparka et al., 1995). The molecular permeability of plasmodesmata in stamen hairs of *Setcreasea purpurea* is

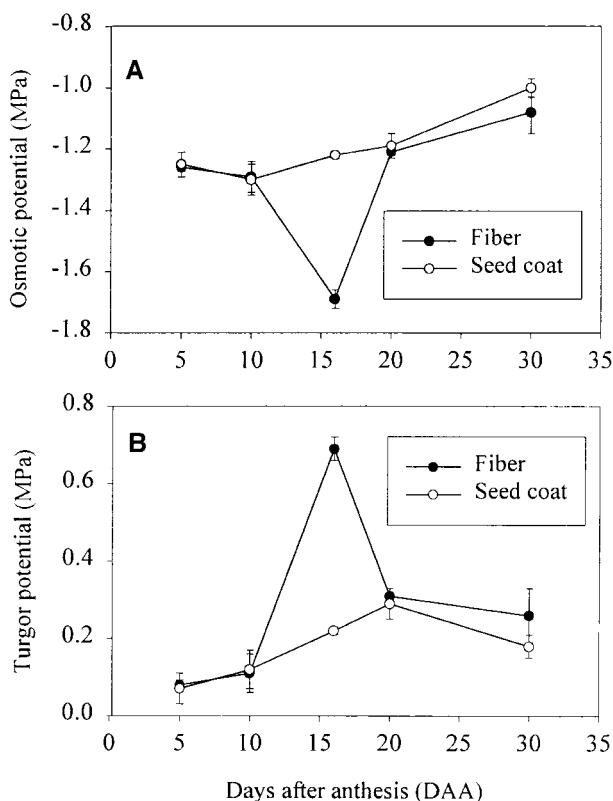


Figure 6. Developmental Changes of Osmotic and Turgor Potentials in Elongating Cotton Fibers and Seed Coats.

(A) Osmotic potentials.

(B) Turgor potentials.

Bars represent \pm SEM (four replicates).

upregulated from early flowering to senescent stage (Yang et al., 1995). Evidence is lacking, however, that plasmodesmatal permeability of a particular cell or cell type displays a developmentally programmed down- and upregulation. To the best of our knowledge, our results on the movement of CF into cotton fibers (Figures 1 and 2) represent an unprecedented example of plasmodesmatal gating that is developmentally reversible at the single-cell level. This observation highlights the complexity and dynamics of plasmodesmata in controlling cell function in plants (see below). It is noteworthy that a similar model of temporal regulation of symplastic domains has been recently observed in the shoot apex of *Arabidopsis* during the transition from vegetative to flowering status (Gisel et al., 1999). However, due to the active cell division and complex differentiation processes in the shoot apical meristem, it is not possible to locate the cellular site at which the restriction of symplastic tracer movement occurs (Gisel et al., 1999).

The mechanism(s) responsible for the reversible gating of fiber plasmodesmata observed here is unknown. To date, studies of plasmodesmatal regulation have concentrated on physiological rather than developmental modulation of their size exclusion limit (Oparka et al., 1999). Callose deposition at the neck region of plasmodesmata has been implicated in “closing” plasmodesmata (Schulz, 1999). We examined this possibility using a monoclonal antibody against callose (Meikle et al., 1991). Immunogold labeling on callose was undetectable in fibers at 4 to 6 DAA but became evident at the fiber base at 10 DAA and increased further throughout the fiber cells at 18 DAA (data not shown). Given that fiber plasmodesmata “reopened” at 16 DAA (Figures 1 and 2), this observation suggests callose deposition does not appear to be involved in “closing” plasmodesmata in fibers but rather seems to be associated with the onset of secondary cell wall cellulose synthesis at 16 to ~18 DAA (Maltby et al., 1979). One recent study shows that physiological elevations in cytoplasmic free Ca^{2+} level result in rapid transient closure of plasmodesmata (Holdaway-Clarke et al., 2000), possibly in an energy-dependent manner (van Bel and Oparka, 1995; Oparka and Turgeon, 1999). On the other hand, low ATP levels induced by, for example, anaerobic or osmotic stress and metabolic inhibition could substantially increase plasmodesmatal permeability (Schulz, 1999). The molecular components controlling plasmodesmata gating may involve unique Ca^{2+} -dependent protein phosphorylation events or an actin-myosin system (Overall, 1999). These results provide useful clues for future studies on the mechanisms controlling the reversible gating of cotton fiber plasmodesmata.

Coordination between Plasmodesmata Gating and Gene Expression in Relation to Fiber Elongation

Cotton fiber elongation is a specialized single-cell expansion, which must be accomplished through a combination of “pushing” against the cell wall exerted by turgor and

“loosening” of the wall mediated mainly by expansins (Cosgrove, 1997). The hypothesis that fiber elongation is driven by higher turgor (e.g., Smart et al., 1998) assumes that fibers are symplastically isolated from their neighboring cells, thus making the generation of higher turgor possible (Ruan et al., 2000). The fact the symplastic tracer, CF, moved readily into fibers from 0 (Ruan et al., 2000) to ~9 DAA (Figures 1 and 2) demonstrates a symplastic pathway for solute import into fibers at the early phase of elongation. Further supporting evidence that sucrose moves into fiber initials symplastically comes from the observation that initiating fibers are enriched in cytoplasmic SuSy mRNA and protein but not cell wall invertase (Ruan and Chourey, 1998) and plasma membrane sucrose transporter (Ruan et al., 2000). The functional symplastic connection between fibers and adjacent cells would lead to rapid water equilibrium among these cells. Consistently, plasmolysis studies of cotton ovules revealed that fiber initials and neighboring cells plasmolyse at approximately the same concentration of osmoticum, suggesting similar turgor potentials in these cells (Ruan et al., 2000). Indeed, the measured osmotic and estimated turgor potentials were similar between fibers and seed coats at the early phase of elongation (Figure 6). These results are unexpected (Ruan and Chourey, 1998; Smart et al., 1998) and suggest that early in elongation, fibers must have higher cell wall extensibility than the adjacent cells (see Cosgrove, 1997). This hypothesis is supported by the high-level expression of the expansin gene in fibers at the early stage (Figure 4). The fact that *GhEXP1* is solely expressed in fibers but not in other sink tissues (Figure 4) further highlights its specific role in fiber cell wall loosening.

After slow elongation for ~9 days at an average rate of 0.7 mm day^{-1} , the fibers enter a rapid phase of elongation at ~2.3 mm day^{-1} for ~6 days before they slow down and eventually stop at ~16 to 18 DAA (Dhindsa et al., 1975; Basra and Malik, 1984). Several significant changes in fiber plasmodesmata gating and gene expression were found at the beginning of the rapid elongation phase. First, the permeability of fiber plasmodesmata was dramatically down-regulated such that CF import was completely blocked at ~10 DAA (Figures 1 and 2). This results in a mandatory shift for solute import into fibers from an initially symplastic to an apoplastic pathway. Consistently, the plasma membrane sucrose and K^{+} transporter genes *GhSUT1* and *GhKT1* were expressed at maximal levels in fibers at this stage (Figure 4). The strong transcription of *GhSUT1* and *GhKT1* appears to correlate with increased transporter protein levels and activities. This is evidenced by the finding that sucrose transporter proteins were abundantly localized at the fiber base region at 10 DAA but not in earlier or later stages (Figure 5) and that ^{14}C -sucrose uptake into fibers was significantly slowed by PCMBs at 10 to 12 DAA (Table 1) and the rate of K^{+} accumulation in fibers peaks between 10 to 15 DAA (Dhindsa, 1975). It is noted, however, that PCMBs inhibited ^{14}C -sucrose uptake by only 50% (Table 1). This suggests that a diffusion component of sucrose influx could

coexist with the transporter-mediated uptake at the base region of the fibers (see Maynard and Lucas, 1982). The expression of plasma membrane H^+ -ATPase in fibers (Smart et al., 1998) and the reduction of ^{14}C -sucrose uptake by EB (Table 1) suggest that the activities of sucrose and K^+ transporters could be coupled with the transmembrane H^+ gradient (Ruan and Patrick, 1995; Kim et al., 1998).

The increased expression of sucrose and K^+ transporters (see above) and phosphoenolpyruvate carboxylase (Smart et al., 1998) would lead to the increased accumulation of the major osmoticum, that is, soluble sugars (Ruan et al., 1997), K^+ , and malate (Dhindsa, 1975), which together account for the elevation of osmotic potential (Figure 6A). As expected, the estimated turgor increased rapidly in fibers from 10 to 16 DAA (Figure 6B). This higher turgor can be maintained in fibers due to the closure of plasmodesmata, analogous to stomatal guard cells (Palevitz and Hepler, 1985). Given the increased rigidity of the fiber cell wall, indicated by the decreased expression of expansin (Figure 4), it is almost certain that the higher turgor in fibers at this stage plays a critical role in driving the rapid-fiber elongation (see Figure 7). In this regard, field trials have shown that an increase in fiber K^+ concentrations, presumably leading to higher turgor

(Dhindsa et al., 1975), has indeed yielded longer fibers with higher quality (Cassman et al., 1990).

Possible Basis for the Termination of Fiber Elongation

Cotton fiber cells do not synthesize secondary cell wall cellulose until their elongation process stops at ~16 to 18 DAA (Basra and Malik, 1984). Little is known about the molecular and cellular basis for the termination of fiber elongation. Here, we found that the transcript of the fiber-specific wall-loosening gene, *GhEXP1*, was dramatically reduced to undetectable levels at 20 DAA (Figure 4). This contrasts with its abundance in fibers at the early stage (Figure 4), indicating that the initially highly extendable primary cell wall of elongating fiber has become quite rigid at the turning point to cellulose synthesis. Consistent with this change is the suberization of fiber secondary cell wall (Ryser, 1992). The deposition of hydrophobic suberin in the basal part of fibers excludes the apoplastic pathway for solute import (Ryser, 1992). This could be the structural basis for the reopening of fiber plasmodesmata for CF import (Figures 1 and 2). The dramatically reduced expression of sucrose and K^+ trans-

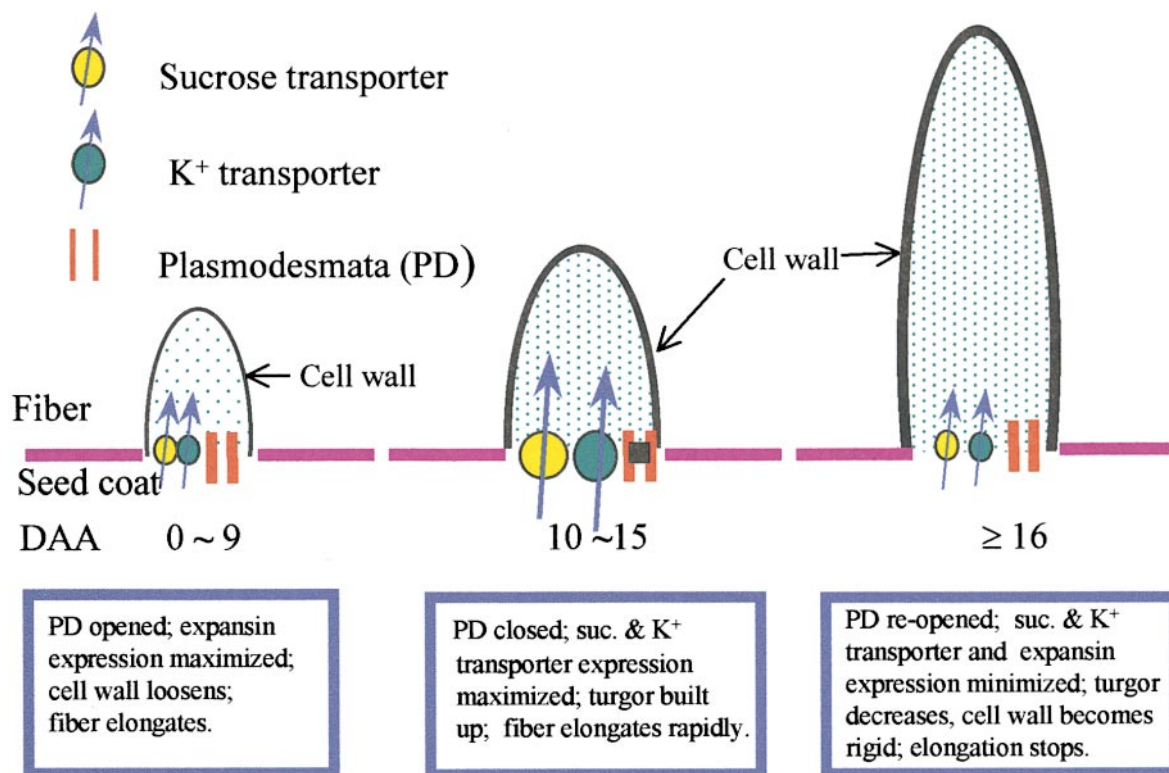


Figure 7. An Integrated Model of the Control of Cotton Fiber Elongation by Reversible Gating of Plasmodesmata and Coordinated Expression of Plasma Membrane Sucrose and K^+ Transporters and Expansin.

The thicker the curve representing fiber cell wall, the less the expansin expressed and the more rigid the wall is.

porter genes at 16 DAA and later (Figure 4) indicates a high turnover rate of these transcripts (Kühn et al., 1997). This reduction in transporter expression is in agreement with the shift back to the symplastic pathway of solute import into fibers. Although symplastic sucrose import into fibers is sustained by the activity of sucrose synthase in the cytosol of fibers (Figure 4; Ruan et al., 1997), the import of K^+ into fibers was greatly reduced after 15 DAA (Dhindsa et al., 1975). This may contribute to the decrease of osmotic and turgor potentials in fibers (Figure 6) and slow down the elongation (Cassman et al., 1990). Furthermore, the reopening of fiber plasmodesmata at ~ 16 DAA would release higher turgor in fibers, if any, to a level similar to that present in the seed coat (Figure 6B). Together, the results suggest that the fiber elongation could be terminated by the combination of increased cell wall rigidity and loss of higher turgor (Figure 6).

In summary, the results obtained suggest that (1) the initial fiber elongation is largely achieved by cell wall loosening, indicated by the fiber-specific expression of *GhEXP1*; (2) the transient closure of plasmodesmata and the maximal expression of the sucrose and K^+ transporters at ~ 10 DAA raise the fiber turgor to drive the rapid elongation; and (3) the elongation is terminated by the increased wall rigidity and loss of higher turgor due to the downregulation of the transporter genes and reopening of plasmodesmata. Figure 7 shows a model for such a regulation. Together, these results provide a remarkable demonstration that the gating of plasmodesmata in a given plant cell is developmentally reversible and is highly coordinated with the expression of membrane transporters and cell wall expansin, which together control plant cell elongation.

METHODS

Plant Material

Cotton (*Gossypium hirsutum* var Coker 315) plants were grown in soil mixture under controlled conditions as previously described (Ruan et al., 1997). Cotton fruit age was determined by tagging the flowering truss when the flower was fully opened. Shoots, each bearing two to three fruits and developed leaves, were excised from the plant for loading of 5(6)-carboxyfluorescein (CF). For RNA extraction and osmolality measurement, samples were frozen in liquid N_2 and stored at -70°C until analysis. Fresh samples were used for ^{14}C -sucrose uptake studies.

Loading of CF, and Confocal Laser Scanning and Fluorescent Microscopy

The membrane-permeant, nonfluorescent dye 5(6)-carboxyfluorescein diacetate (CFDA; Sigma) was prepared as a 2.0% (w/v) stock solution in acetone and stored at -20°C . After excision, the fruit-bearing detached shoots were immediately recut under water with or without $100\ \mu\text{g mL}^{-1}$ of CFDA and illuminated at a photon flux density of $500\ \mu\text{mol m}^{-2}\text{ sec}^{-1}$ at 22 to 25°C for 24 hr, unless otherwise

stated. Upon entering cells, the CFDA is cleaved by cytoplasmic esterase to produce the membrane-impermeant symplastic fluorescent probe CF (Goodall and Johnson, 1982). Because the subsequent confocal imaging of CF in seed took place ~ 20 cm away from the loading end of shoots, the disturbance to the CF movement into the seed was minimized.

After the loading of CF for a specified duration, its movement into fibers from the seed coat phloem was imaged using a Leica confocal laser scanning microscope. Intact seeds were dislodged from fruit and immediately placed onto a glass slide for viewing CF signals in fibers. Alternately, thick hand sections (~ 1 mm) were cut and mounted on the glass slide for viewing CF in seed coat and interconnecting fibers. The CF was excited with 488-nm light generated from a 2- to 50-mW argon-ion laser. Each image is the average combination of seven optical sections. In some cases, the individual images were montaged using Photoshop software (Adobe, Mountain View, CA) to reconstruct a single image of the entire area of interest.

For local loading of CF directly into fibers, a piece of pericarp ~ 1.0 cm in diameter was removed from an attached fruit. This exposed the fiber surface. A well was established on the fruit surface around the corresponding area using vacuum grease. CFDA ($50\ \mu\text{g mL}^{-1}$) was prepared in a solution containing 0.5 mM CaCl_2 , 20 mM KCl, 0.2% BSA, and 0.2% PVP buffered at pH 6.0 with Mes/Tris. Final solution osmolalities were adjusted to $250\ \text{mOsm kg}^{-1}$ with sorbitol. Approximately $200\ \mu\text{L}$ of CFDA was loaded into the well. After 20 min, the dye was removed from the fiber apoplast by 3-min washes with cold buffer solution. The seeds were then dislodged and free-hand sectioned for checking that CF was confined to fibers. The fiber-attached seed sections were subsequently incubated in the buffer at 25°C for 2 hr. Possible movement of CF from fiber into seed coat cells was then monitored under epifluorescence (Ruan and Patrick, 1995).

Electron Microscopy

Small pieces of outer seed coat from seed at 6, 10, and 18 days after anthesis (DAA) were fixed in 3% glutaraldehyde in 25 mM phosphate buffer, pH 7.2. Fibers were trimmed from the 10- and 18-DAA seed epidermis. The fixative was vacuum-infiltrated into tissue pieces. After fixation for 2 hr at room temperature, tissues were rinsed 4×15 min in the buffer followed by 2-hr post-fixation in 2% OsO_4 in the buffer and then rinsed again in the buffer solution. The fixed tissues were dehydrated in an ethanol series and then infiltrated in Spurr's resin, which was polymerized at 70°C for 16 hr. Ultrathin sections were collected on formvar-coated grids, stained with aqueous uranyl acetate and lead citrate, and observed at 80 kV in a 100 CX transmission electron microscope (JEOL, Tokyo, Japan).

Immunolocalization

Cotton seeds at specified developmental stages were fixed in formalin-acetic acid, dehydrated in ethanol, and embedded in paraffin. Fibers were excised from seed coat epidermis at 10 and 18 DAA and treated in parallel. Immunogold silver staining was then conducted as previously described (Ruan and Chourey, 1998) using the HISTOGOLD kit (ZYMED HISTOGOLD SYSTEM for immunohistological staining; ZYMED Laboratories, Inc., San Francisco, CA). Briefly, 10- μm paraffin-embedded cross sections were cut, affixed to slides, deparaffined, rehydrated, and washed with PBS. Thereafter, slides were incubated with serum-blocking solution for 10 min followed by

incubation with 1:500 diluted polyclonal antiserum against a conserved synthetic peptide of H⁺/sucrose symporter (see Ruan et al., 2000 for details) or preimmune serum for 1 hr. In some case, sections were incubated with 1:1500 diluted cotton sucrose synthase (SuSy) polyclonal antibodies (Ruan et al., 1997) for the same duration. After washing with PBS, slides were incubated for 30 min in a solution of secondary antibody (goat anti-rabbit IgG linked to colloidal gold). Slides were then washed thoroughly with PBS (four times for 3 min each), incubated for 4 min with freshly prepared silver enhancement reagents, and washed with excess distilled water. Slides were dehydrated in an ethanol series and permanently mounted in Permunt for microscopic examination. Pairs of immunostained and preimmunostained sections were treated on the same slide for better comparison.

For immunolocalization of callose, deparaffined sections were incubated with 1:100 diluted monoclonal antibody to (1→3)- β -glucan (Biosupplies Australia, Parkville, Victoria, Australia) for 1 hr. After washing in PBS, the sections were incubated for 45 min in 1:300 diluted goat anti-mouse IgG linked to colloidal gold (ICN Biomedicals, Aurora, Ohio). The sections were then silver enhanced, washed, and mounted as described above.

Reverse Transcription-Polymerase Chain Reaction and Cloning

Total RNA was isolated from cotton fibers at 10 DAA and other sink tissues (Ruan et al., 1997). First-strand cDNA was obtained by reverse transcription of 2 μ g RNA with a gene-specific reverse primer (see below) or an oligo(dT)₂₀ primer for sucrose transporter and K⁺ transporter, respectively. For cloning a partial plasma membrane sucrose/H⁺ symporter, a pair of degenerated primers, forward 5'-CA(AG)TT(CT)GG(GT)TGGGC(CT)(CT)T(AGT)CA-3' and reverse 5'-GC(AC)AC(AG)TC(AG)AG(AG)ATCCA(GA)AA-3', was synthesized. The sequences of the primers encode regions at the predicted first and fourth plasma membrane-spanning helices, respectively, and share 100% amino acid identity with the same regions of all of the published sucrose transporters from dicotyledonous plants. A putative sucrose transporter DNA fragment (*GhSUT1*) at the expected size of 333 bp was amplified with the following conditions: 45 cycles of denaturation at 94°C for 0.5 min, annealing at 54°C for 0.5 min, and elongation at 72°C for 2 min. Using the same conditions except for annealing at 60°C, a polymerase chain reaction product corresponding to a 417 bp of K⁺ transporter (*GhKT1*) was amplified with the forward 5'-GACGAAGATGCAGATTCCGAGGAG-3' and reverse 5'-AACCATGTAAGTCATGCCACCTG-3' primers. This set of primers was based on a cDNA expressed sequence tag (EST) sequence of a putative K⁺ transporter from cotton fiber (GenBank accession number AF731506). The EST sequence is located immediately upstream of the gene's stop codon.

The two PCR products, *GhSUT1* and *GhKT1*, were purified with the Wizard PCR Preps DNA purification system (Promega), cloned into pGEM-T Easy vector (Promega), and sequenced using a big dye terminator (377XL DNA sequencing system; Perkin-Elmer, Foster City, CA).

RNA Gel Blot Analysis

After denaturing at 65°C for 5 min in MOPS buffer, pH 7.0, with 50% (v/v) formamide and 18% (v/v) formaldehyde, 25 μ g of total RNA from each sample was loaded on a 1.4% agarose gel containing 5% formaldehyde (v/v) for electrophoresis in the MOPS buffer. The frac-

tionated RNA samples were then transferred to nylon membrane, hybridized with ³²P-labeled probe at 60°C, and washed in 6 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate) and then 0.2 \times SSC at the same temperature as previously described (Ruan et al., 1997). The entire lengths of *GhSUT1* and *GhKT1* were released for probe-making by digestion with EcoRI. A 1.1-kb insert of a cotton fiber expansin cDNA, *GhEXP1*, was derived from a double digestion with ClaI and XbaI. The membranes were initially hybridized with *GhSUT1* and subsequently stripped in a boiling solution (1% SDS, 0.1 \times SSC, and 40 mM Tris buffer, pH 8.0) for re-hybridization with probes of *GhKT1*, *GhEXP1*, and *GhSuSy* (SS3; see Ruan et al., 1997).

¹⁴C-Sucrose Uptake

All solutions used for sucrose uptake were the same as that used for locally loading CF into fibers except that 1 mM sucrose replaced 50 μ g mL⁻¹ of CFDA.

Cotton fibers with a layer of outermost seed coat attached at their bases were separated from the rest of seed on ice. Cellular debris was removed by 2 \times 1-min washes in ice-cold carrier solution. Fiber samples were preequilibrated in the carrier solution for 25 min with or without transporter inhibitors (0.2 mM p-chloromercuribenzenesulphonic acid [PCMBs] or 0.1 mM erythrosin B [EB]) at 25°C. After removing excessive PCMBs and EB by 2 \times 5-sec washes in carrier solution, pretreated tissues were transferred to the same carrier solution plus ¹⁴C-sucrose (3.7 \times 10⁻² MBq mL⁻¹) for 10 min at 25°C. Fluxes of sucrose were estimated as previously described (Ruan and Patrick, 1995).

Osmolality Measurement and Turgor Estimation

Fiber and seed coat osmolalities were determined as previously described (Ruan et al., 1995). Measured osmolalities were converted to MPa using the equivalence 2.48 MPa per Osm kg⁻¹ (Schmalstig and Cosgrove, 1988). Turgor was estimated from the difference between the obtained osmotic potentials and water potentials in developing cotton seed (van Iersel and Oosterhuis, 1996).

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The Control of Single-Celled Cotton Fiber Elongation by Developmentally Reversible Gating of Plasmodesmata and Coordinated Expression of Sucrose and K⁺ Transporters and Expansin

Yong-Ling Ruan, Danny J. Llewellyn and Robert T. Furbank

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