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Goldacre paper:

Rapid cell expansion and cellulose synthesis regulated by plasmodesmata and sugar: insights from the single-celled cotton fibre

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Abstract. Higher plants comprise mixtures of some 40 different cell types, and this often complicates the interpretation of data obtained at the tissue level. Studies for a given cell type may provide novel insights into the mechanisms underlying defined cellular and developmental processes. In this regard, the cotton fibre represents an excellent single-cell model to study the control of rapid cell elongation and cellulose synthesis. These single cells, initiated from the ovule epidermis at anthesis, typically elongate to ~3–5 cm in the tetraploid species before they switch to intensive secondary cell wall cellulose synthesis. By maturity, more than 94% of fibre weight is cellulose. To unravel the mechanisms of fibre elongation and cellulose synthesis, two hypotheses have been examined: (a) that sucrose degradation and utilisation mediated by sucrose synthase (Sus) may play roles in fibre development and (b) that symplastic isolation of the fibre cells may be required for their rapid elongation. Reverse genetic and biochemical analyses have revealed the critical role that Sus plays in fibre initiation and early elongation. Late in development, plasma-membrane and cell wall association of Sus protein seems to be involved in rapid cellulose synthesis. Cell biology and gene expression studies showed a temporary closure of fibre plasmodesmata (PD), probably due to the deposition of callose, at the rapid phase of elongation. The duration of the PD closure correlates positively with the final fibre length attained. These data support the view that PD closure may be required for fibres to achieve extended elongation. The branching of PD towards the secondary cell wall stage is postulated to function as a molecule sieve for tight control of macromolecule trafficking into fibres to sustain intensive cellulose synthesis.

Introduction

The growth of higher plants is achieved through a series of coordinated events of cell division, expansion and cell wall synthesis by ~40 different cell types (Goldberg 1988; Martin *et al*. 2001). The diversity in architecture at the tissue or whole-plant level reflects, to a large degree, high level of heterogeneity in the extent of cell expansion and cell wall synthesis among different cell types (Martin *et al*. 2001). For example, leaf mesophyll cells usually expand only several-fold after cytokinensis and undergo primary cell wall synthesis. In contrast, other cell types, such as pollen tubes and xylem cells, can quickly elongate more than a thousand times and synthesise a massive amount of secondary cell wall polymers (Fukuda 1991; Franklin-Tong 1999; Chen *et al*. 2003; Andersson-Gunnerås *et al*. 2006). The accomplishment of the extraordinary elongation of pollen tubes is critical for the delivery of the male gametes to the ovules for double fertilisation, and the synthesis of various wall materials in the xylem provides mechanical strength for the respective tissue and a network for transport water and inorganic solutes. Understanding the mechanisms controlling rapid cell expansion and cell wall synthesis is of fundamental importance for better understanding basic biology as well as for designing strategies to improve crop productivity and stress tolerance (John 1997; Haigler *et al*. 2001; Martin *et al*. 2001; Ruan 2005).

One challenge involved in the study of the molecular and cellular basis of rapid cell expansion and cell wall synthesis is the difficulties in experimentally accessing, isolating and manipulating the cell types of interest *in vivo.* To this end, although the pollen tube and xylem elements have been studied extensively *in vitro* for understanding cell elongation and cell wall biosynthesis, respectively, it is uncertain whether the phenomenon observed *in vitro* reflects that *in vivo* (Taylor and Hepler 1997). The impediment in studying these cell types *in vivo* is largely due to the complex anatomic structure of the tissue where those cells are located (Galbraith and Birnbaum 2006). For example, pollen tubes penetrate within the transmitting tract of the style (Taylor and Hepler 1997) and stem xylem elements are deeply embedded between the ground parenchyma cells of cortex and pith and surrounded by phloem cells (e.g. Wu *et al*. 2000). These inherent anatomical features make it a difficult task to isolate those specialised cell

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types from the surrounding tissues unless through the use of techniques such as laser capture microdissection (see Galbraith and Birnbaum 2006), which is expensive and not available to most laboratories. Thus, the developing cotton fibre represents an excellent system to study the mechanisms of rapid cell expansion and cell wall synthesis for the following reasons. First, unlike the pollen tube, which contains triple nuclei of two sperm cells plus one vegetative nucleus, complicating the genetic makeup of the system (Franklin-Tong 1999), each cotton fibre has one nucleus and does not undergo cell division during development *in planta* (Basra and Malik 1984; Ruan *et al*. 2000; Taliercio *et al*. 2005). Therefore, it represents a true single-cell system to study without the complexity of cell division and multicellular development (Ruan *et al*. 2001). Second, cotton fibres grow outwards from the seed epidermis and are, thus, readily accessible *in vivo* by opening up the pericarp of the fruit. Third, the rapid and synchronised growth of the fibre cells ensures a sufficient amount of samples for experimentation. Here, each cotton seed produces 10 000 to 18 000 fibre cells from its epidermis (Van't Hof and Saha 1997; Taliercio *et al*. 2005) with the average density of one fibre in every 3–4 epidermal cells (Fig. 1*B*, *C*). By 10 days after anthesis (DAA), ~5–6 g of fresh fibre cells can be harvested from each fruit, and this yields sufficient amount of RNA, proteins and metabolites for various analyses (Y-L Ruan, unpublished data). This is in contrast with other cell types isolated through the laser microsampling method, which often results in very small amounts of materials (Galbraith and Birnbaum 2006). Further, developing cotton fibre has distinct and continuous developmental programs, from initiation at anthesis to rapid elongation by ~18 DAA, followed by intensive secondary cell wall cellulose synthesis from ~18 to 40 DAA before maturation and desiccation (Basra and Malik 1984; also see Fig. 1). This provides an invaluable advantage that a particular molecular or cellular event can be precisely mapped or correlated with the corresponding developmental program at the single cell level. Finally, in addition to being an excellent system for studying cell growth, cotton fibre is also the most important source of cellulose for the textile industry, with >94% of mature fibre dry weight being cellulose, a β-1,4 polymer of glucose (Basra and Malik 1984; Delmer 1999). Hence, understanding the mechanisms underlying rapid cell elongation and cellulose production, two key processes that determine cotton fibre quality and yield, could lead to the identification of target genes for genetic engineering of fibre development for the improvement of cotton productivity (John 1997; Kim and Triplett 2001; Ruan 2005).

This paper appraises and discusses recent progress in understanding cotton fibre elongation and cellulose synthesis, focusing on the role of plasmodesmta (PD) and sucrose metabolism mediated by sucrose synthase (Sus) in fibre development. Where appropriate, comparisons have been made

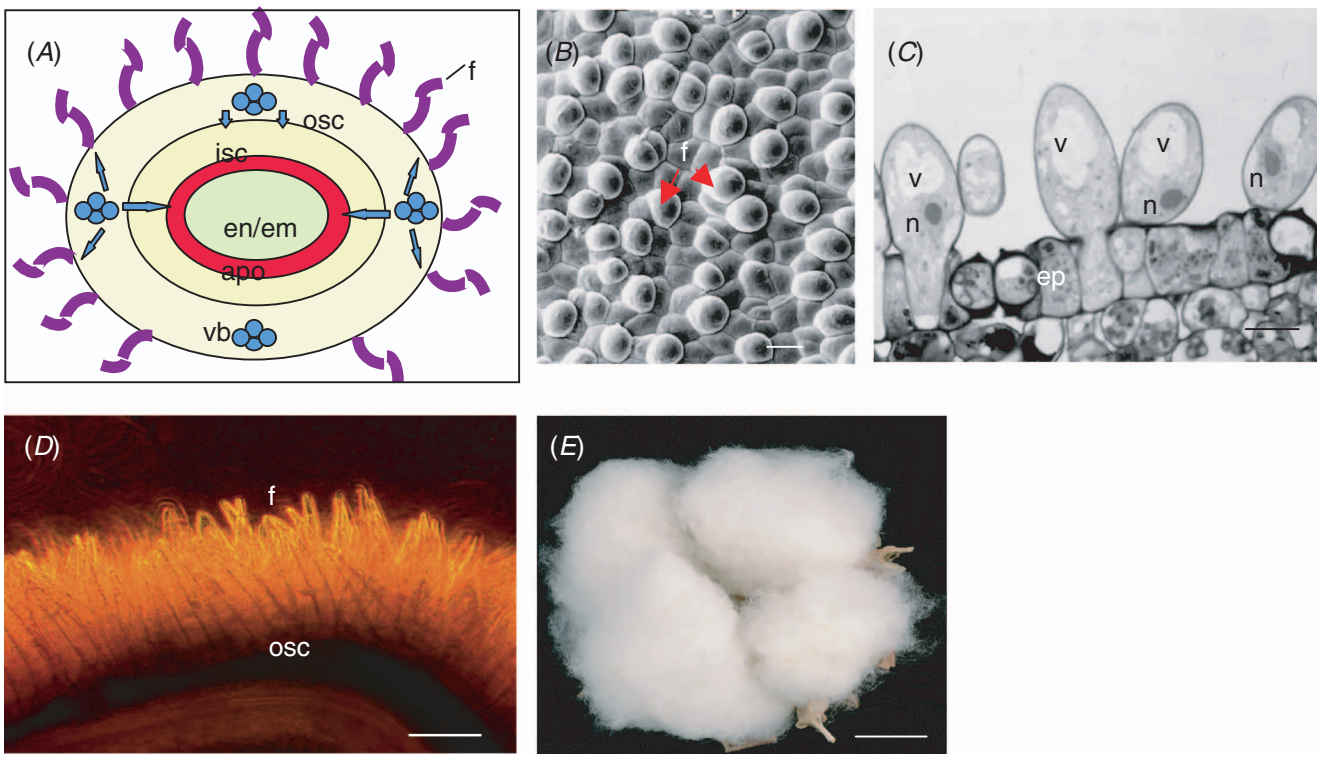

Fig. 1. The development of cotton fibre from initiation to maturation. (*A*) A schematic representation of a developing cotton seed. (*B*) A scanning electron micrograph showing the fibre cells (arrows) initiated from the ovule epidermis on day of anthesis. Note only a quarter to one-third of the epidermal cells become fibres. Scale bar = 15 µm. (*C*) A light microscopic micrograph showing the protrusion of the fibre cells above the ovule epidermis on day of anthesis. Note the enlarged vacuoles and nuclei in the fibre cells. Scale bar = 15 µm. (*D*) A free-hand cross section of a cotton seed at 3 DAA viewed under phase contrast. Note the synchronised elongating fibres from the epidermis of the seed. Scale bar = 120 µm. (*E*) A fully mature cotton fruit at ~60 DAA, showing massive amounts of long fibres. Note, seeds were totally covered by the fibres and therefore were invisible. Scale bar = 1.3 cm. Apo, apolast; em, embryo; en, embryo; f, fibre; isc, inner seed coat; osc, outer seed coat; v, vacuole; vb, vascular bundle.

with other cell systems to stimulate thinking and to conceive new hypotheses for future research.

The control of cotton fibre elongation

The unidirectional and diffusive growth of cotton fibre

Plant cell expansion follows either diffuse growth or tip growth (Yang 1998; Martin *et al*. 2001). Typical unidirectional and fast-growing cells such as pollen tube, root hair and fungal hyphae, follow a tip growth pattern, where expansion is confined to the dome-shaped apex that is filled with secretory vesicles (Franklin-Tong 1999). Rapidly elongating cotton fibre cells, however, seem to grow by diffuse growth based on the following observations (also see Fig. 2). First, the elongating fibre cells lack the 'zonenation' phenomenon of intracellar organelles in the cell tip (Tiwari and Wilkins 1995). The concentration of endoplasmic reticulum (ER), Golgi bodies and mitochondria in the subapical region of the cell tip is a major characteristic of tip growth (Yang 1998). Second, the orientation of cortical microtubules, and, hence, that of the newly deposited cellulose microfibrils, are transversely organised to the growth axis in the cotton fibre, which provides a greater resistance to radial expansion than to a longitudinal expansion (Seagull 1990, 1992; Martin *et al*. 2001). Thus, in response to turgor pressure, the fibre cells elongate perpendicularly to the orientation of cellulose microfibrils, leading to unidirectional outgrowth from the seed epidermis (see Ruan 2005). In tip growth cells such as in the pollen tube, however, microtubules are arranged as bundles in parallel to the growth axis and are absent from the apical dome (Yang 1998), which may allow the apical region to be free of constriction by cellulose microfibrils and, thus, to grow readily forwards.

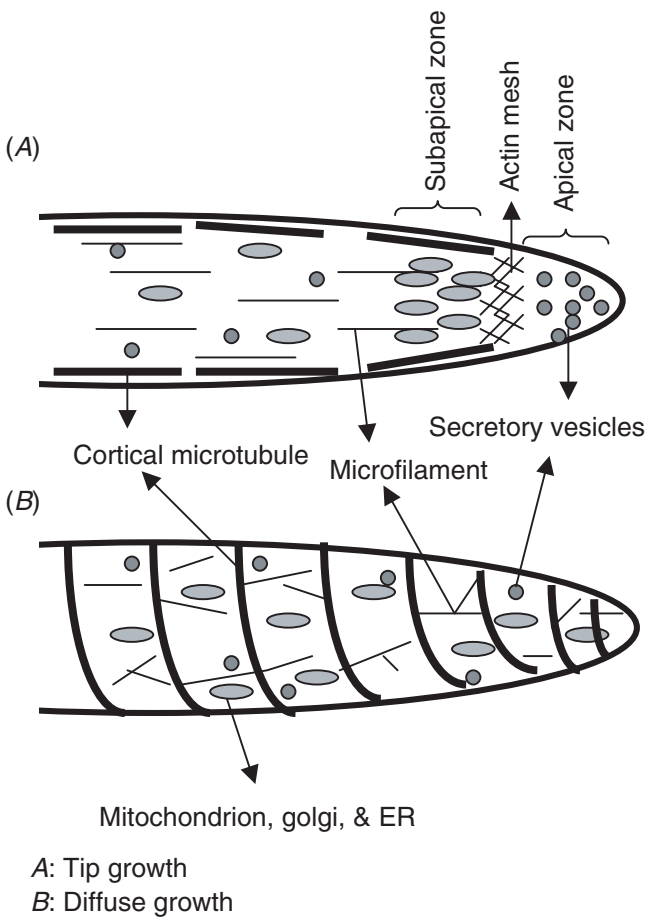

Fig. 2. A schematic representation of tip growth (e.g. in pollen tube and root hair, *A*) and diffuse growth of cotton fibre (*B*). Modified from Franklin-Tong (1999) and Martin *et al*. (2001). The major difference between the two growth patterns are (1) during tip growth, there is a zonation of mitochondrion, Golgi and ER in the subapical of the cells and vesicles containing cell wall precursors move towards the very tip of the cells. Such polarised concentration of organelles and secretory vesicles are, however, absent in diffuse growth; (2) during tip growth, the microtubules are arranged as bundles along the longitudinal axis of the cell, whereas in diffuse growth (elongation) of cotton fibre, the microtubules are oriented transversely to the axis of growth. Note that the orientation of microtubules, hence, the cellulose microfibrils, undergo a shift from transverse to ~45° helical arrays at the onset of secondary cell wall synthesis of cotton fibre (see Seagull 1990).

Another cytoskeleton component, microfilaments composed of actin filaments, support the intracellular trafficking of organelles and secretory vesicles (Chen *et al*. 2003). Actin filaments are arranged as long bundles along the length of the cells during tip growth but randomly during diffuse growth (Fig. 2). In addition, actin filaments may form a meshwork in the subapical region of tip growth cells (Franklin-Tong 1999), a phenomenon that does not exist in cotton fibre (Seagull 1990, 1992). The meshwork of actin may facilitate vesicle transport and docking within the apex and could also function as a filter to prevent the large organelles from entering the apical region, thus, creating a so called 'clear zone' in the tip (Martin *et al*. 2001). The essential role of actin in the tip growth of pollen tube has been shown by the overexpression of Rac/Rop GTPases that disrupts actin cytoskeleton and converts polar growth into isotropic growth (Chen *et al*. 2003). In cotton fibres, silencing the expression of a fibre-specific actin gene, *GhACTIN1*, reduces the amount of F-actin and inhibits fibre elongation, probably through decreasing the number of organelles (such as ER and Golgi bodies) travelling along the filamnents, and hence, the number of vesicles transported to the cell periphery for membrane and cell wall biosynthesis (Li *et al*. 2005).

It is not known why the polar growth of some cell types displays a tip growth feature but cotton fibre follows a pattern of diffuse growth. One possibility is that it may relate to the environment where these cells were evolved. Here, the growth of pollen tube and root hair needs to overcome the resistance imposed by the transmitting tissue of the style and soil particles, respectively. The tip growth of these cells may be advantageous in penetrating through the physical barrier encountered. In contrast, the outgrowth of cotton fibre occurs relatively 'freely' in the aqueous space between the seed coat and the pericarp without the need to overcome the physical resistance that the pollen tube and root hair face. Similar to a cotton fibre, the aerial protrusion of trichomes from the leaf epidermis also follows a pattern of diffuse growth (Mathur *et al*. 1999; Szymanski *et al*. 1999).

The power of symplastic isolation in fibre elongation

Cotton fibres typically elongate over 2000–3000-fold within 3 weeks after initiation. The final length attained can reach 3–5 cm in the tetraploid cotton of *Gossypium hirsutum* and *G. barbadense*, respectively. This renders the cotton fibre as the

fastest growing and longest single cell known in plants (Ruan 2005). The adjacent seed coat cells, however, expand by only 5–10-fold during this period. This indicates a high degree of cell autonomy of the fibres. One mechanism for such a discrepancy in cell growth and function is the symplastic isolation between the respective tissues or cell types through the closure of PD, the plasma membrane and ER-lined cytoplasmic channels connecting plant cells (see Roberts and Oparka 2003; Zambryski 2004). To examine the symplastic continuity between fibres and underlying seed coat epidermis, a membrane-impermeant fluorescent dye, carboxyfluorescein (CF), was ester-loaded into phloem of a fruit-bearing shoot. Confocal microscopic imaging was then used to monitor whether or not the CF unloaded from the phloem of the seed coat moves into the fibres (Ruan *et al*. 2000). The presence of the fluorescent signals of CF in the fibre indicates that the fibre PD is open or permeable to CF import; otherwise, the PD is closed. As the molecular mass of CF is similar to sucrose, the kinetics of CF movement is likely to reflect that of endogenous solute transport (Ruan and Patrick 1995).

A systematic imaging analysis of the CF movement revealed that the fibre PD is initially open but become closed for ~5 days during the rapid phase of elongation before they reopen at ~16 DAA, at the end of elongation (Ruan *et al*. 2001). Further comparison among genotypes differing in fibre length shows that the duration of PD closure correlates positively with the final fibre length attained, and the closure always occurs at the onset of rapid phase of elongation (Ruan *et al*. 2004, 2005). These observations strongly suggest that the closure of PD in the fibre base is important for the rapid and extensive elongation of cotton fibre.

The closure of PD may provide a mechanism for the fibre cells to generate and maintain a higher turgor to drive the extensive elongation. Cell turgor is generated through the influx of water driven by the accumulation of osmotically active solutes. In elongating cotton fibre, malate, potassium and soluble sugars account for most of the osmotic potential (see Ruan 2005). Although malate is produced within the fibre through re-fixation of CO_2 by phosphoenolpyruvate carboxylase (Smart *et al*. 1998), potassium and sucrose are imported from the underlying seed coat cells, either symplastically through the PD or apoplastically across cell wall space and plasma membrane. The closure of fibre PD would necessitate solute uptake into fibres across their plasma membranes. Indeed, transporter genes, *GhSUT1* and *GhKT1*, which encode a H^+/sucrose symporter and a potassium transporter, respectively, are maximally expressed when the PD is closed at the fibre base (Ruan *et al*. 2001). The high expression of these transporter genes facilitates the uptake of sucrose and potassium, hence, the generation of the observed low osmotic potential (Ruan *et al*. 2001). The closure of the fibre PD likely enables the estimated high turgor to be maintained to drive the elongation as long as osmotically active solutes are continuously imported to attract water (Cosgrove 1997; Pfluger and Zambryski 2001) and the cell wall is expandable through the expression of expansin genes (Ruan *et al*. 2000; Harmer *et al*. 2002). Consistent with this hypothesis is the reopening of fibre PD, which correlates with the decrease of estimated fibre turgor and the termination of the elongation process (Ruan *et al*. 2001).

It is possible that the hydraulic isolation of the elongating fibres from the remainder of the seed coat may be a requirement for rapid restoration and maintenance of cell turgor following cell wall relaxation through osmotic adjustment mediated by membrane transport. In addition, the hydraulic isolation would render fibre water relations independent of the vegetative plant, and that this may prevent or reduce the backflow of water from elongating fibres to leaves down a water potential difference (X-W Fang, Y-L Ruan, unpublished data).

Developmentally-reversible gating of PD has been reported in the shoot apex of birch trees (Rinne *et al*. 2001) and *Arabidopsis* (Gisel *et al*. 2002). The transient closure of PD correlates with dormancy in the former, and with flowering time in the later. In both cases, however, it has not been possible to locate the cellular site where the restriction and restoration of the symplastic connection occurs because of the multicellular differentiation processes in the shoot apical meristem (Gisel *et al*. 2002). The findings in cotton fibre (see above) provide evidence for a direct role of PD gating in plant growth at the single cell level (Pfluger and Zambryski 2001).

The molecular basis of PD gating in plants is unclear since the components that control the structure and function of PD remain virtually unknown (Roberts and Oparka 2003), and mutational screening for alteration in PD function could have lethal effects (Zambryski 2004). In some cases, the switch between symplastic and apoplastic pathways relates to the decrease or absence of PD connection (Patrick 1997) or the presence of an apoplastic barrier (e.g. Walsh *et al*. 2005). In cotton fibres, several studies indicate that deposition and degradation of callose, a β-1,3 polymer of glucose, may be involved in the closure and opening of PD, respectively. Here, by using a monoclonal antibody against callose and a callose-specific stain, aniline blue, the glucose polymer is specifically localised in the neck region of PD at the fibre base when PD is closed but not at the time when PD is open (Ruan *et al*. 2004). The expression of a fibre-specific β-1,3 glucanase gene, *GhGluc1*, is strong at the time of callose degradation but undetectable when callose is deposited at the fibre base, suggesting that expression of *GhGluc1* may be responsible for the degradation of callose and consequently the re-opening of the fibre PD (Ruan *et al*. 2004). It would be of significance to examine if and how PD gating and fibre elongation are affected when *GhGluc1*expression is genetically altered.

The critical role of Sus in fibre elongation

As illustrated above, the closure of fibre PD coincides with the strong expression of transporter genes for the uptake of sucrose and K^+ across the plasma membrane of the fibre cells (Ruan *et al*. 2001). To this end, the subsequent utilisation of sucrose within the fibres may play multiple roles in fibre development, from contributing to the generation of turgor potential to the supply of substrate for cellulose biosynthesis (Ruan 2005; Ruan and Chourey 2006). In most of the sink cells, sucrose is either hydrolysed by invertase into glucose and fructose or degraded by Sus into uridine 5′-diphosphate (UDP) glucose and fructose for subsequent metabolism and biosynthesis (Geigenberger and Stitt 1993; Koch 1996). A series of cellular, biochemical and molecular studies has provided strong evidence that Sus plays a

critical role in cotton fibre development. During the initiation and early elongation period, sucrose moves into fibres symplastically based on imaging of CF movement and plasmolysis analyses (Ruan *et al*. 2000, 2001). This would render Sus as the major enzyme to degrade incoming sucrose in the cytoplasm because the activity of the second sucrose-degrading enzyme in cytosol, neutral/alkaline invertase, is nearly 10-fold lower than Sus in fibre cell (Y-L Ruan, unpublished data). Indeed, Sus protein and mRNA are abundantly and specifically localised in initiating fibres but not in the adjacent non-differentiating epidermal cells nor in the ovule epidermis of a fibre-less mutant (Nolte *et al*. 1995; Ruan and Chourey 1998). Consistent with a role of Sus in fibre development, microarray studies have also shown that expression of Sus, among other genes including expansin and some transcription factors, is strongly reduced in the ovule epidermis of several mutants defective in fibre initiation (Wu *et al*. 2006). Significantly, the suppression of Sus expression in the ovule epidermis leads to shrunken or collapsed fibre initials and the repression of fibre elongation in transgenic cotton plants (Ruan *et al*. 2003), demonstrating the essential role of Sus in fibre growth.

These observations are important for two reasons. First, unlike those found in other sink tissues, including maize kernel (Chourey *et al*. 1998), potato tubers (Zrenner *et al*. 1995) and tomato fruit (Chengappa *et al*. 1999), where a large amount of Sus activity seems to be dispensable, the initial spherical expansion and subsequent elongation of cotton fibre is sensitive to a small reduction of Sus expression (Ruan *et al*. 2003). Second, Sus has been previously shown to be involved mainly in the storage of protein and starch in sink tissues (Weber *et al*. 1997). The elongating cotton fibre, however, does not show detectable levels of these storage activities (Basra and Malik 1984). Apparently, the fast-growing cotton fibre represents a very strong sink, the activity of which depends strongly on the level of Sus expression. The biochemical basis for the critical role of Sus in fibre growth most likely relates to its ability in providing hexoses and UDPglucose to the fibre cells. A large amount of hexose could be required for the fibre cells to generate osmotic potential, hence, turgor for their protrusion above the ovule surface as well as to produce energy (ATP) and substrates for protein and membrane biosynthesis. Support for this view comes from the observation that the levels of glucose and fructose are significantly reduced in the fibre-suppressed transgenic ovules (Ruan *et al*. 2003). Further, Sus may control fibre development by providing UDPglucose, the immediate substrate for cell wall cellulose biosynthesis (Delmer 1999). Both of these possibilities could account for the collapsed phenotype of fibre cells when Sus is suppressed (Ruan *et al*. 2003). Figure 3 represents an integrated model of fibre development regulated by Sus, PD gating and branching (see below).

The switch from elongation to cellulose synthesis: possible role of PD branching

Cotton fibre elongation slows down dramatically at ~16 DAA and terminates by ~20 DAA, accompanying concurrently with the onset of intensive secondary cell wall cellulose synthesis (Basra and Malik 1984). The molecular basis of this developmental switch remains largely unknown, although it may involve hormonal signaling, rearrangement of the cytoskeleton and oxidative burst mediated by small GTPases (see Ruan 2005 for a recent review).

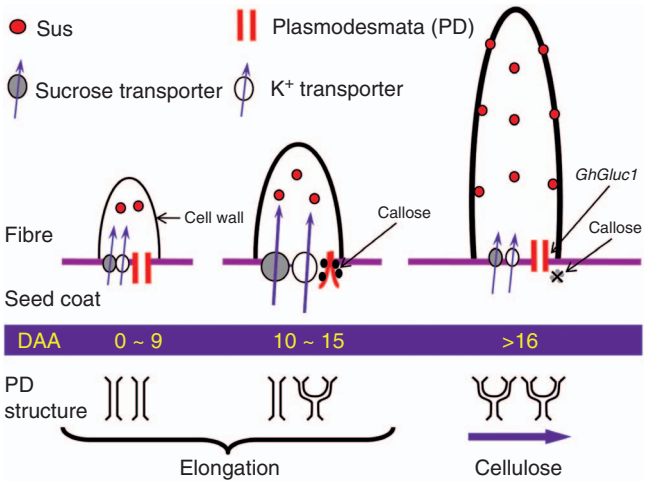

Fig. 3. An integrated model on cotton fibre elongation and secondary cell cellulose synthesis mediated by PD and Sus (based on Ruan *et al*. 2001, 2003, 2004, 2005). Early in elongation from 0–9 DAA, PD is open for solute import. At this stage the Sus-mediated sucrose utilisation and the expansin-regulated cell wall loosening are critical for elongation. By the middle of elongation, the PD is closed, coincided with maximal expression of transporters for uptake of sucrose and K^+, which together could generate and maintain a high turgor in the fibres to sustain an extended elongation. Finally, at ~16 DAA onwards, the PD is re-opened for solute exchange, which may release the turgor from the fibres. This, together with the increased cell wall rigidity (indicated by the diminished expression of expansin) and re-organisation of cytoskeletons, terminates the elongation process. At this stage, part of the Sus proteins starts to associate with the plasma membrane and even move to the cell wall region to support the intensive secondary cell wall cellulose synthesis. The branching of the PD towards the secondary cell wall thickening is postulated to serve as a 'molecule sieve' for tight control of macromolecule trafficking between fibres and adjacent normal epidermal cells such that input of activators for cellulose synthesis may be permitted, whereas negative regulators, such as repressors, are excluded (see text for more details).

In light of the distinct features of elongation and secondary cell wall thickening of the fibre cells, it is reasonable to envisage that different mechanisms may be operative for the control of molecular trafficking between the fibres and underlying seed coat cells at the two developmental stages. Studies in other systems have shown that PD may function as a 'molecular sieve' in regulating intercellular movement of macromolecules including transcription factors during development and cell patterning (Cilia and Jackson 2004; Kurata *et al*. 2005). For example, in *Arabidopsis*, myeloblastosis-like transcription factors, encoded by *TRY* and *CPC*, could selectively move from trichome cells and root hair to adjacent epidermal cells via PD and act as lateral inhibitors for trichome and root hair formation, respectively (Schellmann *et al*. 2002). In this regard, it is noted that the structural changes of PD in cotton fibres before the onset of secondary cell wall cellulose synthesis. Here, the fibre PD are all in the simple form early in elongation, but about half become branched towards the fibre side at 10 DAA (Ruan *et al*. 2001).

Most of the fibre PD becomes branched by 16 DAA onwards at the secondary cell wall cellulose synthesis stage (Ruan *et al*. 2001; also see Fig. 3). The branching of PD does not correlate with the changes of its permeability to small molecules, because the initially opened PD is closed at 10 DAA and reopens at 16 DAA for CF transport (see previous discussion). Rather, the modification of the PD structure may provide a basis for tighter control of macromolecular trafficking into and out of fibre cells, similar to those proposed for the PD connecting companion cells of phloem (Oparka and Turgeon 1999). It is postulated that the stringent control of molecular trafficking by the branched PD may be required for the secondary cell wall cellulose synthesis in fibres through, for example, blocking and facilitating the import of putative repressors and activators for cellulose synthesis, respectively. This hypothesis is supported by the observation that the timing and frequency of PD branching positively correlates with the commencement of secondary cell wall cellulose synthesis among three cotton genotypes examined (R White, RT Furbank, Y-L Ruan, unpublished data).

The transition from simple to branched PD during development has been reported from green algae (Franceschi *et al*. 1994) to leaves and roots of plants (Itaya *et al*. 1998; Zhu *et al*. 1998). In tobacco, for example, there is a clear structural change of PD from simple form in sink leaves to branched shape in source leaves (Ding *et al*. 1992; Oparka *et al*. 1999). Importantly, whereas the simple PD allows non-specific trafficking of green fluorescent protein (GFP) or GFP-fused protein at the molecular mass up to 50 KDa, the branched PD does not permit the passage of GFP, a molecule of 27 KDa (Oparka *et al*. 1999). In addition, viral movement proteins from tobacco and cucumber mosaic viruses only target and dilate the branched PD but not the simple one in tobacco leaves (Ding *et al*. 1992; Itaya *et al*. 1998). These results show not only a much reduced size exclusion limit but also possibly different molecular components (e.g. putative receptors and chaperons) of the branched PD as compared to the simple PD (Ding *et al*. 1992; Roberts and Oparka 2003). Further support for the suggestion that branched PD have different functions from the simple PD comes from the observation that expression of a yeast invertase in transgenic tobacco inhibits the formation of branched PD but not the simple PD (Ding *et al*. 1993). Such modifications in PD structure and function may be necessary for tight screening of the input and output of proteins and RNA, so that specific functions can be performed in the cells or tissues at more advanced stage of development (Lucas *et al*. 1993; Oparka and Turgeon 1999). The progressive branching of PD in cotton fibre development (Fig. 3) mirrors that in tobacco leaves during sink-to-source transition (Ding *et al*. 1992; Oparka *et al*. 1999). It would be of interest to examine if the size exclusion limit of the branched PD in cotton fibre is different from that of simple form and if so, whether and how it relates to the onset of secondary cell wall cellulose synthesis.

The channelling of carbon by Sus for rapid secondary cell wall cellulose synthesis

Cellulose, the most abundant renewable biomass produced on earth, accounts for 28–30% of dry matter in typical forage grasses, 42–45% of wood and more than 94% of mature cotton fibre (see Haigler *et al*. 2001). It is a major polymer in plant cell walls that play critical roles in the establishment of plant structure and morphogenesis (Doblin *et al*. 2002). Clearly, understanding how carbon is partitioned into cellulose is a critical question in both plant biology and biotechnology. Several studies discussed below point to the important role of Sus in this process.

A remarkable characteristic of cotton fibre development is that at the onset of secondary cell wall formation stage, the rate of cellulose synthesis abruptly rises over 100-fold compared with the elongation and primary cell wall synthesis stage (Delmer 1999). To support this rapid cellulose synthesis, a virtually irreversible carbon sink, some 80% of total carbon entering the fibres is partitioned to cellulose (Mutsaers 1976). It is intriguing how so much imported carbon, mainly in the form of sucrose (Ruan *et al*. 1997), is channelled to cellulose synthesis on the plasma membrane. To this end, Amor *et al*. (1995) made a surprising discovery that a substantial amount of Sus, a protein traditionally thought to be exclusively located in the cytosol (reviewed by Winter and Huber 2000), is tightly associated with the plasma membranes of cotton fibre. It is estimated that sucrose entering the fibre cells is partitioned in the ratio of 3 : 1 between membrane (PM-) and soluble (S-) Sus at the secondary cell wall stage (Delmer 1999). Further immunolocalisation analyses at the electron microscopic level showed that the occurrence of PM-Sus is developmentally regulated. Here, the immuno-gold labelled PM-Sus is only sporadically present in fibres at 10 DAA (Fig. 4*B*) but becomes more evident at 20 DAA (Fig. 4*C*) when the fibres are at the elongation and secondary cell wall cellulose stages, respectively (Basra and Malik 1984). Enzyme assay for putative PM-Sus from ethylene glycol tetracetic acid-eluted pellet fraction (see Haigler *et al*. 2001) also shows undetectable activity in 10-day-old fibres but some 390 nmol mg^{-1} protein min^{-1} from 20-day-old fibres. It is of interest to note that in 20-day-old fibres the gold-labelled Sus particles are also abundantly present in the cell wall region of the fibre cells towards the plasma membrane side (Fig. 4*C*), so called exoplasmic zone (see Salnikov *et al*. 2003). This Sus protein is hereafter termed CW-Sus. It is unlikely that the CW-Sus is an artifact associated with sample preparation because it has also been observed in cryogenic-fixed and freeze-substituted cotton fibres harvested at the secondary cell wall stage (Salnikov *et al*. 2003). Cryogenic methods accurately preserve cellular structure and block molecular movement during sample processing (Nicolas and Bassot 1993). The developmental correlation of PM-/CW-Sus with the secondary cell wall cellulose synthesis indicates its role in channelling carbon from sucrose to cellulose (Delmer 1999; Haigler *et al*. 2001). Consistently, abolishing PM-/CW-Sus through transgenic suppression (data not shown) leads to little no or fluorescent signals of cellulose in cotton fibre at 20 DAA (Fig. 4*E*), which is in contrast to the abundant signals of cellulose in the wild type cotton fibre at this stage (Fig. 4*D*). These observations indicate that the PM-/CW-Sus plays an important role in the secondary cell wall cellulose synthesis of cotton fibre.

Evidence that supports the role of Sus in cell wall cellulose synthesis also comes from studies in other systems. For example, in the tension wood of poplar trees, two Sus genes *PttSUS1* and *PttSUS2* are among the most highly expressed genes and coordinately up-regulated with two cellulose synthase

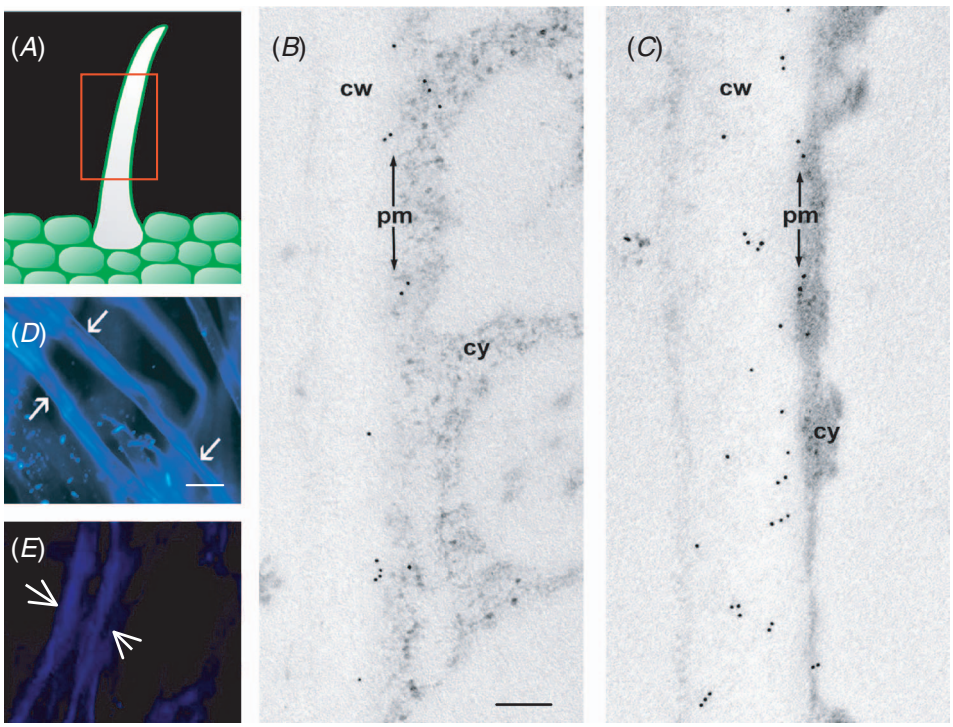

Fig. 4. Developmentally-regulated plasma membrane (PM)- and cell wall (CW)- association of Sus protein in relation to secondary wall cellulose synthesis in cotton fibre. (*A*) A diagram of cotton fibre. The boxed area indicates region where the samples were taken for Sus and cellulose labelling studies. (*B*) Longitudinal thin sections of primary wall stage cotton fibres at 10 DAA, labelled with anti-serum to cotton Sus. Only some sporadic gold-labelled black Sus proteins were detected in the PM and CW region. Samples were fixed in 2% paraformaldehyde plus 0.1% glutaraldehyde in phosphate buffer, embedded in LR White. Transmission electron microscopic sections were incubated in anti-sucrose synthase antibody followed by secondary antibody with 10 nm gold label. (*C*) Longitudinal thin sections of secondary wall stage cotton fibres at 20 DAA, labelled with anti-serum to cotton Sus and colloidal gold. The black Sus protein particles were evident in the region of PM and adjacent CW space, but were much fewer in the wall region distant to the cytoplasm. (*D*) Fluorescent signals of cellulose from wild-type cotton fibres at 20 DAA. The fluorescent labelling of cellulose by Calcofluor White (CFW) was carried out as described by Blanton (1993). Briefly, fresh cotton fibre was excised from seeds, washed in water three times (1 min each time) and then incubated for 5 min in an aqueous CFW solution at 0.01% (w/v) in the dark. After three rinses (1 min each) in water to remove unlabelled CFW, the samples were viewed immediately under ultraviolet light for the fluorescent signals of cellulose. (*E*) Little fluorescent signals of cellulose were detected in fibres at 20 DAA harvested from Sus-suppressed transgenic cotton (Ruan *et al*. 2003). Scale bar = 200 nm in (*B*, *C*) and 25 µm in (*D*, *E*). Cy, cytoplasm; cw, cell wall; pm, plasma membrane. The scale in (*C*) and (*E*) is the same as that in (*B*) and (*D*), respectively.

genes *PttCESA1* and *PttCESA1* during the secondary cell wall cellulose synthesis stage of developing xylem (Hertzberg *et al*. 2001; Andersson-Gunneråas *et al*. 2006). Sus is also highly expressed in wall thickening transfer cells and endosperm cells in the developing seed of maize (Chen and Chourey 1989) and cotton (Ruan *et al*. 1997). Mutation of one Sus gene, *Sh1*, in maize de-generates some endosperm cells, which have extremely thin and fragile cell walls (Chen and Chourey 1989). A similar cell wall phenotype is also seen in transgenic cotton plants, where Sus is repressed in the endosperm (Ruan *et al*. 2003). These observations indicate a role of Sus in cell wall synthesis. Although the precise intracellular location of Sus is unknown in these cells, it is proposed that the cell wall synthesis is dependent on PM-Sus (Haigler *et al*. 2001). Consistent with this view is that the level of PM-Sus in maize endosperm peaked during cellularisation and significantly dropped at the stage of starch synthesis (Carlson and Chourey 1996). In tracheary elements differentiated from mesophyll cells of *Zinnia elegans* L., Sus is abundantly localised near the plasma membrane underlying wall thickening and near patterned aggregates of cellulose synthase (Salnikov *et al*. 2001), suggesting to a direct linkage between PM-Sus and cell wall cellulose synthesis.

How PM- or CW-Sus regulates cellulose synthesis is a subject being intensively studied by several laboratories. Data from cotton fibre (Amor *et al*. 1995; Haigler *et al*. 2001; Salnikov *et al*. 2003; Fig. 4) and developing xylem (Andersson-Gunneråas *et al*. 2006) collectively support the hypothesis that the PM-Sus may form a complex, either directly or indirectly, with the cellulose synthase on the plasma membrane to channel carbon directly from sucrose to cellulose as originally proposed by Delmer (1999). Coupling between the PM-Sus and glucan synthase has the advantage of (a) promoting synthesis of cellulose from sucrose with no additional energy input, (b) avoiding competition for use of UDPglucose by other pathways, and (c) allowing immediate recycling of UDP, a compound that inhibits the reaction catalysed by cellulose synthase (see Haigler *et al*. 2001 for a recent review). In this model, fructose released from PM-Sus could be phosphorylated by fructokinase or kexokinase (Andersson-Gunneråas *et al*. 2006) and then recycled to sucrose by the joint action of sucrose

phosphate synthase and sucrose phosphate phosphatase, whereas the energy and hexoses required for the maintenance of cell growth is provided by the soluble Sus in the cytosol. Figure 5 represents a schematic model on carbon partitioning to cellulose synthesis mediated by Sus.

The mechanism(s) of the membrane association of Sus is largely unknown, but might involve de-phosphorylation of the serine residue(s) at the *N*-terminal of the protein and interaction with actin (Winter and Huber 2000). In maize, phosphorylation of Ser 15 of the Sus1 protein promotes a soluble phase localisation of Sus, and de-phosphorylation tends to stimulate membrane association (Winter and Huber 2000). The phosphorylation of Ser 15 is developmentally regulated in maize leaves and affects *N*-terminal conformation in a way that may stimulate the catalytic activity of Sus and influence membrane association (Hardin *et al*. 2004). Similar to that in maize, the membrane-Sus protein in mature soybean nodules is also hypophosphorylation specifically on Ser 11 relative to the soluble form (Komina *et al*. 2002). However, in cotton fibres, the level of ^{32}P-labelling of Sus is similar between membrane and soluble fractions and the role of de-phosphorylation in plasma membrane association is yet to be shown (Haigler *et al*. 2001). Nothing is known of how Sus moves to the cell wall region (Fig. 4; Haigler *et al*. 2001; Salnikov *et al*.2003). This is particularly puzzling because the Sus protein does not have an identifiable secretory signal sequence. Further work in this area may reveal novel mechanisms of Sus in intracellular partitioning and cell wall cellulose synthesis.

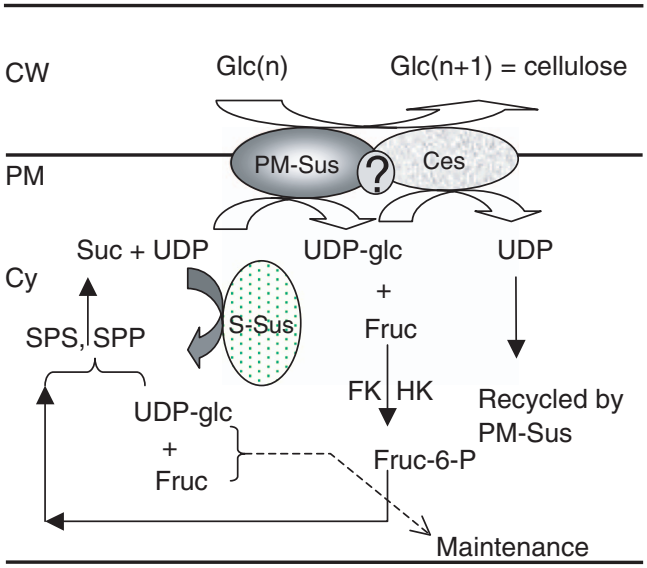

Fig. 5. A model on channelling of carbon from sucrose into secondary cell wall cellulose synthesis mediated by plasma membrane associated Sus in cotton fibre. At this stage of fibre development, a substantial portion of the Sus protein is associated with the plasma membrane (PM-Sus), which may form a complex, directly or indirectly, with cellulose synthase (Ces) to channel carbon from sucrose into cellulose. The fructose may be recycled to sucrose through the sequential action of fructokinase (FK) or hexokinase (HK), sucrose-phosphate synthase (SPS) and sucrose-6′-phosphate phosphatase (SPP). The soluble Sus (S-Sus) degrades sucrose for maintenance and survival metabolism through glycolysis.

Conclusions and future perspectives

Recent studies discussed in this paper establish a mechanistic model on the control of cotton fibre elongation and cellulose synthesis, mediated by PD gating, branching and expression of Sus and transporters for the uptake of sucrose and K^+ (Fig. 3). The model not only provides novel insights into the understanding of rapid cell expansion and cellulose synthesis, but also raises new issues for further studies. Some of the immediate questions are: how important is the PD closure for fibre elongation? For example, will the fibre elongation be affected when the fibre PD is dilated by some virus movement proteins? Are there any differences in the size exclusion limit and selectivity of molecular trafficking between simple and branched PD? How does Sus protein associate with plasma membrane and move to the cell wall? What will happen to fibre development once Sus is overexpressed? In addition to the issues relating to PD and sucrose utilisation, many other equally important biological questions can also be addressed in this system. For example, the large size of the fibre cell indicates that sufficient water needs to be imported and maintained to sustain the elongation process. What is the cellular and molecular basis of water influx into and possibly efflux from the fibre cells? How does the water movement relate to fibre cell elongation, desiccation and maturation? What is the signaling pathway that triggers the transition from elongation to secondary cell wall cellulose synthesis? Answers to these questions will come from studies using multidisciplinary approaches with strategies ranging from hypothesis-driven research to global gene expression studies at RNA, protein and metabolite levels. There is no doubt that developing cotton fibre represents an excellent system in which mechanisms underlying rapid cell expansion and cellulose synthesis can be addressed at the single cell level in defined developmental stages.

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