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MOLECULAR BIOLOGY AND PHYSIOLOGY

The Effect of Calcium on Early Fiber Elongation in Cotton Ovule Culture

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ABSTRACT

Cotton fiber developing on cultured ovules is an important experimental tool, even though *in vitro* grown fibers are shorter than *in situ* grown fibers. Fiber measurements and microscopy show that early fiber elongation increased when calcium concentration in the culture medium was reduced from the traditional 3 mM to 0.1 mM. Fiber length in 0.1 mM calcium responded negatively to trifuoperazine, which antagonizes calcium-dependent calmodulin signaling. Fiber length in 0.1 mM calcium responded positively to calcimycin, which antagonizes intracellular calcium compartmentalization. In contrast, neither compound affected the length of fibers in 1 mM calcium medium. Transferring ovules between low- and high-calcium concentration media showed that fiber elongation was most sensitive to exogenous calcium concentration in the first 1 to 2 d after anthesis (2-3 d in culture). The transcript level of a fiber expansin gene increased when ovules were cultured in low-calcium medium. These data show that use of low-calcium medium in the culture of ovules less than 3 d after anthesis promoted fiber elongation through effects related to intracellular calcium signaling. Future *in vitro* experiments probing the controls of fiber development can be conducted with less restriction on elongation imposed by the culture medium, which should help to uncover more of the mechanisms controlling fiber development.

Cotton fibers are seed trichomes that develop in four overlapping stages to form spinnable fiber (Graves and Stewart, 1988). Fibers initiate in the ovule epidermis, rapidly elongate via a diffuse growth mechanism, produce thick secondary cell walls, and finally mature and dry (Tiwari and Wilkins, 1995). Fibers are single cells that develop fairly synchronously in a cotton fruit (or boll), allowing the investigation of rapidly differentiating cells without the complications of cell division. Additionally, fibers will develop on ovules in tissue culture (Beasley and Ting, 1974). *In vitro* ovule culture has been instrumental in investigating many aspects of early fiber development including defining the stage of fiber initiation and determining the roles phytohormones play in fiber development (Beasley and Ting, 1973, 1974; Shi et al., 2006; Sun et al., 2005; Triplett, 1998). However, fibers grown in ovule culture are shorter than fibers grown on plants indicating that some aspects of fiber development are altered in ovule culture (Kim and Triplett, 2001).

Previous experiments show that fiber initials accumulate calcium around 1 day after anthesis, implying that calcium might play a role in fiber development (Taliercio and Boykin, 2007). Plants are sensitive to high levels of calcium in the cytoplasm and transport calcium against a gradient to the lumen of organelles to reduce calcium levels in the cytoplasm to submicromolar amounts (White and Broadley, 2003). Analysis of gene expression, ultrastructural observations, and histochemical staining all indicated that endoplasmic reticulum (ER) is abundant in young fibers (Berlin, 1986; Ramsey and Berlin, 1976; Taliercio and Boykin, 2007). The increase in ER in calcium-accumulating fiber initials could provide increased sites for the storage of calcium in these cells (Taliercio and Boykin, 2007). Many phenomena stimulate efflux of calcium from the compartments where it is stored to temporarily raise the level of calcium in the cytoplasm. In the cytoplasm, calcium interacts with calcium sensors such as calmodulin, calmodulin-like proteins, calcineurin B-like proteins, and calcium-dependent protein kinases, all of which have been identified in plants. Indeed, transcripts encoding a novel calmodulin and a calcium-dependent protein kinase have been identified in fiber initials (Huang et al., 2008; Taliercio and Boykin, 2007). Genes associated with calcium signal transduction that were expressed in rapidly elongating fibers were reported to be down regulated in a cotton fiber mutant (Gao et al., 2007). The interaction of calcium with the calcium sensor plays important roles in transducing the signal for multiple pathways including response to light, cell division, a variety of developmental pathways, phytohormone response, biotic stress, and abiotic stresses

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(White and Broadley, 2003). In addition to effects on cell signaling, calcium can stabilize membranes and plays a role in secretion (Laohavisit et al., 2009; Legge et al., 1982; Wang et al., 2009). External calcium ions can also cross-link with relatively de-esterified pectins of the cell wall to confer strength and/or resist expansion (Hepler, 2005). Even though cotton fiber is rapidly elongating, its diameter might be controlled by such a mechanism. It is not surprising that a massive morphogenetic transformation of an ovule outer integument cell into a seed trichome shows evidence of calcium involvement given the diverse roles of calcium in various cellular and signaling processes.

The goal of these experiments was to determine if optimization of calcium concentration in ovule culture promotes early fiber elongation. We determined the effect of varying calcium levels in the medium on *in vitro* development of young fibers and a temporal window for maximum sensitivity to changes in calcium concentration. We measured fiber length after perturbation of calcium signaling and calcium compartmentalization to determine if these aspects of calcium function played a role in early fiber development. Pharmaceuticals used for this purpose included: trifuoperazine (TFP), which is an inhibitor of calcium-dependent calmodulin signaling (Frohnmeyer et al., 1998; Vandonselaar et al., 1994); and calcimycin, which is an ionophore that disrupts calcium gradients across membranes (Kohno and Shimmen, 1988; Minibayeva et al., 2000). Finally, we investigated the effects of altered calcium levels in the expression of selected genes previously shown to be increased in expression in elongating fibers (Taliercio and Boykin, 2007).

MATERIALS AND METHODS

Plant Material and Ovule Culture. *Gossypium hirsutum* L. (cv. ST4793R) plants were grown in the greenhouse without supplemental light in Raleigh, NC, March 2009 to December 2009 following standard procedures. The timing of fiber development is described by days after anthesis (daa). Flowers were harvested on the day prior to anthesis (-1 daa). The petals, anthers, and sepals were removed and the bolls were washed for 5 min successively in 0.1% triton, three times in water, and finally in 95% ethanol. The bolls were dried in the air flow from a sterile hood and the ovules excised into a modified Beasley and Ting (BT) medium. The components of the baseline BT medium are listed in Table 1. The BT medium (pH 5) was made using 3% (wt/vol) glucose instead of a mixture of glucose and fructose following the modification of Triplett (Beasley and Ting, 1974; Triplett, 1998). To facilitate altered concentrations of calcium in BT medium, the medium was made without calcium, which was then added from a 100 mM $CaCl_2$ stock prior to autoclaving. All phytohormones were added from sterile dimethyl sulfoxide (DMSO) stocks after the media were autoclaved and cooled. Consistent results were obtained with media less than a week old that had not been frozen. TFP (5 µM final concentration) and calcimycin (0.5 µM final concentration) were made as 1000X concentrated DMSO stocks. These stocks were aliquoted and stored frozen at -20°C until a fresh aliquot was thawed for each experiment. Unused portions of thawed aliquots were discarded, which was critical for reproducibility of the data. The working concentrations were determined by first identifying the maximum amount of each pharmaceutical that allowed ovule survival and development without regard to fiber development (data not shown). Ovules treated with pharmaceuticals were pooled and randomized in media without the added pharmaceutical and then 15 ovules were transferred to media with the indicated amount of the pharmaceutical or an equal amount of DMSO as a control. Ovules were cultured at 30°C in the dark without supplemental CO_2. All chemicals were purchased from Sigma unless otherwise noted.

Table 1. BT media composition.

BT media	
BT 10X (made as a 10X stock) concentration	
calcium chloride Anhydrous	0-6 mM
magnesium sulfate 7H2O	2 mM
potassium phosphate, monobasic	2 mM
borax	0.1 mM
BT 100X (made as a 100X stock)	
sodium molybdate	0.001 mM
potassium iodide	0.005 mM
manganese (II) sulfate	0.1 mM
zinc sulfate	0.03 mM
CuSO4 x 5 H2O	0.0001 mM
CoCl2 X 6H20	0.00009 mM
Fe-EDTA (made as 100X stock)	
Na2EDTA	0.03 mM
FeSO4 x 7H2O	0.03 mM
vitamins (made as 1000X stock)	
nicotinic acid	0.004 mM
pyridoxine HCl	0.004 mM
thiamine HCl	0.004 mM
potassium nitrate	50 mM
myo-inositol	1 mM
glucose	167 mM
Phytohormones (made as 4000X stock)	
IAA	5.0 µM
GA3	0.5 µM

Microscopy. Two daa ovules (after 3 d in culture) were prepared for microscopy by first floating them for 30 sec on 0.05% Toluidine Blue in culture medium to mark the side that was submerged during culture, followed by floating on three changes of medium to remove excess dye. Then the ovules were transferred to Histochoice® (Amresco, Solon, Ohio) and stored at 4°C. Prior to observation, the ovules were soaked in water (3 x 20 min) to remove excess fixative and stained 5 min with 0.02% (w/v) Ruthenium red, which stains the pectin in the cell walls of young fibers (Turley and Vaughn, 1999). The ovules' surface where fibers had not initiated remained white (Fig. 1). After rinsing in water, ovules immersed in water were photographed with the original aerial side up using a dissecting microscope and a digital camera (Model Q5 QImaging, Surrey, BC, Canada).

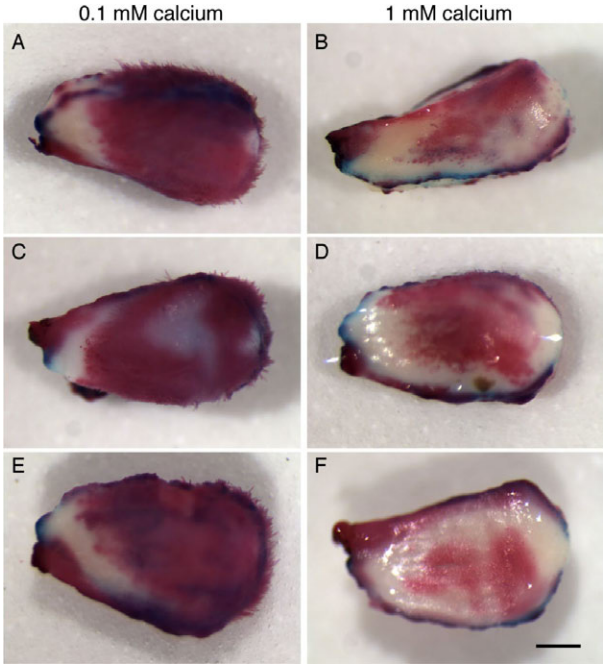

Figure 1. Micrographs of -1-daa ovules cultured for 3 d in BT media with either 0.1 mM or 1 mM calcium (left and right columns, respectively), along with treatment with DMSO (A and B), calcimycin (C and D), or TFP (E and F). DMSO was used as a control for the other treatments. The pectin in the cell wall of initiating and elongating fibers was stained with Ruthenium red. The top side of the floating ovule is shown in each case, and the single ovule shown was representative of the larger set. The magnification bar in (F) = 0.25 mm and applies to all micrographs.

Measurement of Fiber Length and Statistical Methods. Unless otherwise noted all fiber was analyzed at 7 daa (in culture 8 d). An efficient method was developed to determine the average relaxed length of the fiber. Ovules with attached fiber were heated to 95°C in 1% NP40 for 30 min and transferred to staining buffer (0.0005% toluidine blue, 1% NP40) for 3 min with vigorous agitation. The stained fibers were gently stretched onto a piece of moist, white filter paper. After the filter paper dried, the fiber length was measured from the bottom of the ovule to the tip of the observable fibers. Each treatment represents measurements of fiber from 5 ovules. Experiments to identify the window of response to calcium and the effects of TFP and calcimycin were replicated at least three times, with fiber of at least 5 ovules measured in each experiment (n = 15). At least one and usually all three replications represent ovules cultured in media prepared at different times. Standard errors of the means were determined with EXCEL and statistical significance was assessed with the mixed model function in SAS.

RNA Isolation and Analysis. RNA was isolated from cotton ovules, 1-daa fibers, 3-daa fibers, and 10-daa fibers using the Qiagen plant RNA isolation kit and included an on-column DNAse digestion to remove contaminating genomic DNA (Qiagen, Valencia, CA). Real-time polymerase chain reaction (PCR) was performed using a BioRad kit following the manufacturer's instructions using primers that measure levels of the expansin transcript and the rRNA to normalize expression. Primer sequence and other details are published elsewhere (Singh et al., 2009; Taliercio, 2010). Expression was calculated using the method of Pfaffl (2001) on at least two and usually three samples of RNA prepared from samples grown at different times or in different media preparation.

RESULTS

Length of fibers produced on ovules cultured for 11 d in BT media containing 0.09 mM to 6 mM calcium was measured to determine if early elongation was sensitive to calcium concentrations (Fig. 2A). BT media typically contained 3 mM calcium, however this concentration of calcium did not result in the longest fibers after 11 d in culture (10-daa ovules). Instead, fiber elongated fastest with 0.09 to 0.19 mM exogenous calcium. Medium without calcium supported some fiber development, but by 5 d in culture, ovules showed signs of death when a necrotic spot developed on the ovule and quickly

spread (data not shown). Evidence that early fiber elongation was sensitive to calcium concentration was extended by measuring fiber length on ovules cultured in 0.1 mM or 1 mM calcium between 6 to 10 daa (Fig. 2B). At 6 daa, the fibers in the high-calcium condition were less than half as long as fibers in the low-calcium condition. The percentage difference had narrowed by 10 daa, which suggested that the concentration of calcium had the strongest effect prior to 6 daa.

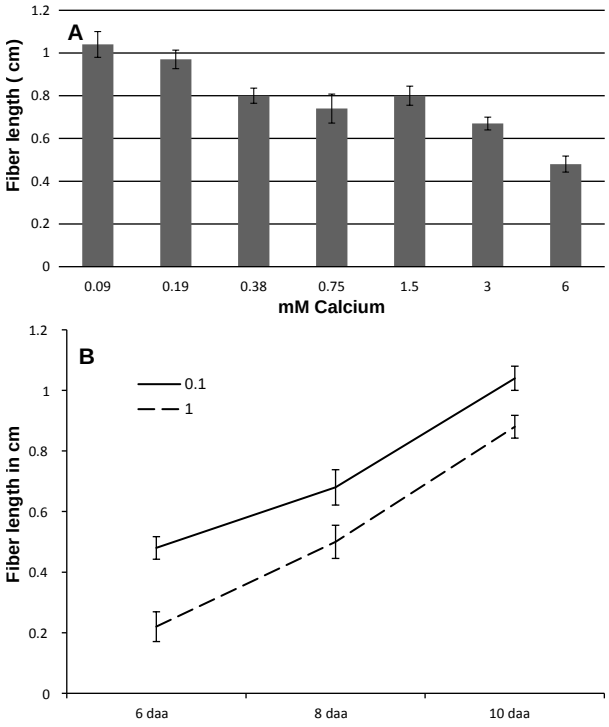

Figure 2. Effects of altered calcium levels in ovule culture on fiber length. (A) Fiber length was measured after -1-daa ovules were cultured for 11 d in BT media containing 0.9 mM to 6 mM calcium. (B) Increase in fiber length for -1-daa ovules cultured up to 10 daa in BT media with high- (1 mM) or low- (0.1 mM) calcium levels.

The role of calcium in early fiber elongation was investigated by culturing ovules in media containing 0.1 mM or 1 mM calcium. Ovules that were harvested at -1 daa and cultured for 3 d in 1 mM calcium (plus DMSO, the carrier for other drug experiments) had less fiber covering the white ovule surface than in 0.1 mM calcium and they showed no signs of elongated fiber extending beyond the edges of the ovule (Fig. 1A, B). These observations were consistent with the inhibition of fiber development by the higher calcium concentrations.

Further evidence for a role of calcium in regulating early fiber development was obtained through treatment of ovules cultured in both low and high calcium with calcimycin or TFP. These pharmaceuticals perturb calcium compartmentalization in the cell or calcium signaling through calmodulin, respectively. The appearance of typical ovules stained with Ruthenium red after exposure to these drugs for 3 d is shown in Figure 1. Ruthenium red stains fibers but not the ovule surface, so red color indicated that some fiber development occurred in all treatments at both low and high calcium (Fig. 1C-F). As described previously for the control, the extent of early fiber development was suppressed by high calcium in the presence of both TFP (compare Fig. 1E, F) (an inhibitor of calmodulin signaling) and calcimycin, an ionophore that disrupts calcium compartmentalization across membranes (compare Fig. 1C, D). To analyze the treatment effects quantitatively and over a longer time, fiber length was measured after 9-d exposure to the drugs. Fiber grown in low calcium in the presence of TFP was shorter than the control (Fig. 3A). Fiber grown under low calcium in the presence of calcimycin was statistically longer than the control (Fig. 3B). Neither drug affected fiber length in the high-calcium medium. The ability of the fibers to elongate in 1mM calcium media in the presence of TFP to the same length observed in the DMSO-treated control (Fig. 4A) indicated that this level of TFP is not generally cytotoxic. The effects of the calcium channel blocker, Verapamil, were also investigated, but no definitive evidence for effects on fiber elongation was observed (data not shown). Attempts to use other calcium channel blockers such as nifedipine and lanthanum failed because they caused a precipitate to form in the media.

A flow chart of the experiments used to define the window of maximum calcium response is shown in Fig. 4A. Ovules cultured in low-calcium media (0.1mM, treatment 1) for 0 to 5 d were transferred to either low-calcium or high-calcium media (1mM, treatment 2) for the remaining time in culture until 7 daa (total time in culture was 8 d) when fiber was measured (Fig. 4A, B). The ovules not exposed to high-calcium media (0 d) but grown exclusively in low-calcium media had significantly longer fibers than ovules grown exclusively in high-calcium media, as predicted (Fig. 4B). Fibers from ovules exposed to low-calcium media for up to 2 d were significantly shorter when transferred to high-calcium media than those transferred to low-calcium media. After 3 d there was no difference or a much smaller difference between fiber lengths when the ovules cultured in low-calcium media were transferred to either new medium.

Reciprocal experiments were performed by culturing ovules in high-calcium media (treatment 1) for 0 to 5 d and transferring to high- or low-calcium media (treatment 2) (Fig. 4A, C). Again, 0-d treatments confirm the original inhibitory effect of fiber length by 1mM calcium. After 2 d there was little (though still significant) difference between fiber lengths when the ovules cultured in high-calcium media were transferred to either new medium (Fig. 4C). The data presented in Fig. 3 show that the window of fiber length response to calcium closed around 2 to 3 d in culture when the ovules were 1 to 2 daa.

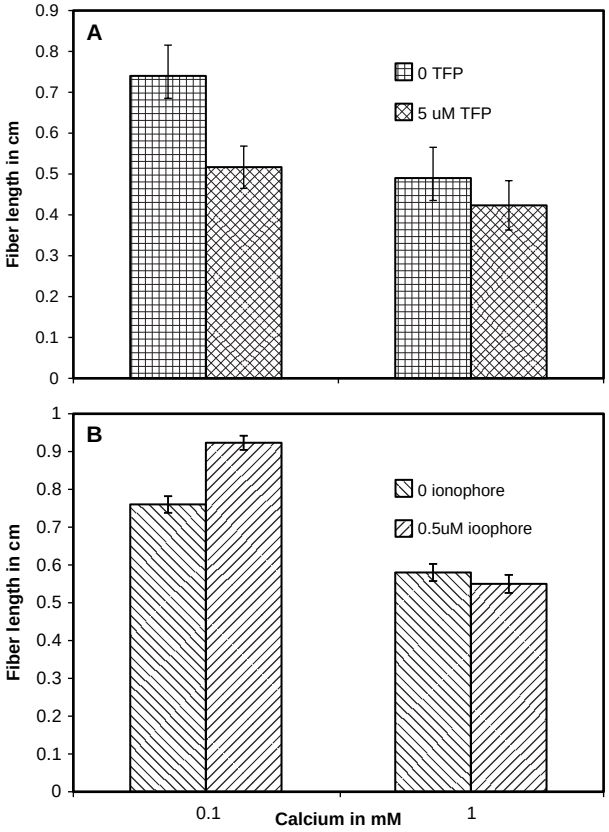

Figure 3. Effects of TFP and calcimycin on fiber length for ovules cultured with high and low calcium for 9 d. (A) TFP caused a decrease in fiber length with low-calcium medium ($p < 0.0001$). (B) Calcimycin, a calcium ionophore, caused an increase in fiber length in the low-calcium medium ($p < 0.0001$). The bars indicate standard error (n = 15 from three replications).

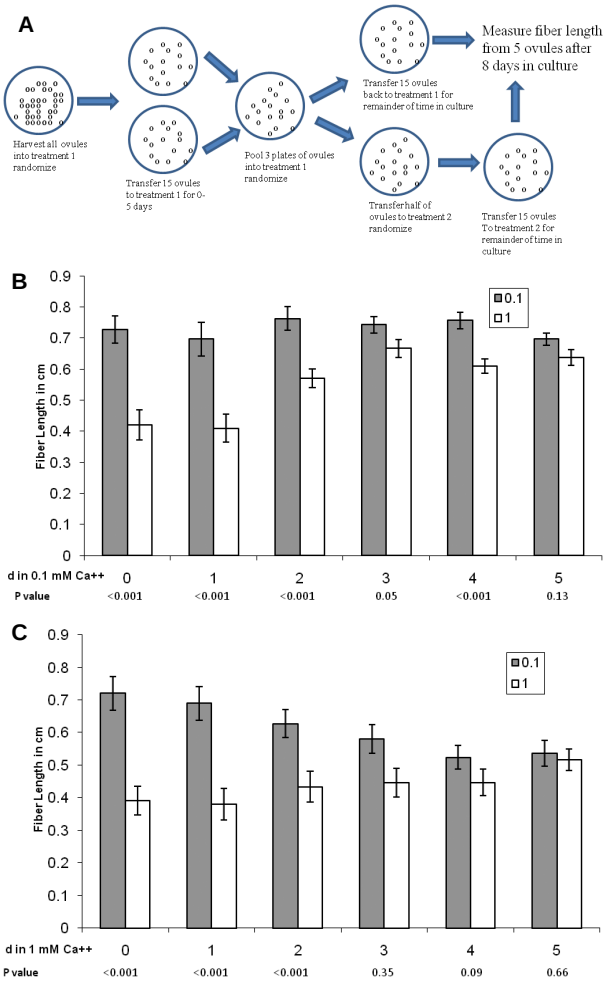

Figure 4. Temporal windows of sensitivity of fiber elongation to calcium levels. (A) Flow chart for experiments to determine the temporal sensitivity of fiber elongation to calcium concentration. (B) For cultures initiated in low-calcium BT medium (treatment 1), ovules were transferred after 0 to 5 d to either high-calcium or new low-calcium medium (treatment 2), followed by fiber length measurement after 9 d in culture. For example, the second set of bars represents ovules that were cultured for 1 d in low calcium medium followed by transfer to media containing 1mM calcium or back to media containing 0.1 mM calcium. (C) For cultures initiated in high-calcium BT medium (treatment 1), ovules were transferred after 0 to 5 d to either new high-calcium or low-calcium medium followed by fiber length measurement as above (treatment 2). Error bars indicate standard error of all replications (n = 15). The measure of the confidence, *p* value, of the difference between treatments is shown below the chart.

The effects of zero, high-, and low-calcium concentrations on the levels of transcripts encoding a calcium pump and an expansin, two transcripts previously shown to increase in elongating fiber, were investigated. Transcripts encoding both the calcium pump and the expansin were equally abundant in 1-daa fibers and in whole ovules harvested from plants (Fig. 5A). The levels of both transcripts, but particularly expansin increased substantially as the fiber rapidly elongated between 3 daa to 10 daa. Figure 5B shows that levels of expansin, but not calcium pump, transcripts increased when -1-daa

ovules were cultured for 24 h in media with lower levels of calcium. Transcripts encoding an ER-lumen protein or a Rho-GDP dissociation inhibitor were not affected by calcium levels, although the levels of these transcripts increased in 10-daa fiber compared to 1-daa fiber (data not shown). The response of the expansin transcript levels to calcium concentration indicated that the control of this gene lies in a signaling pathway that responds to calcium levels.

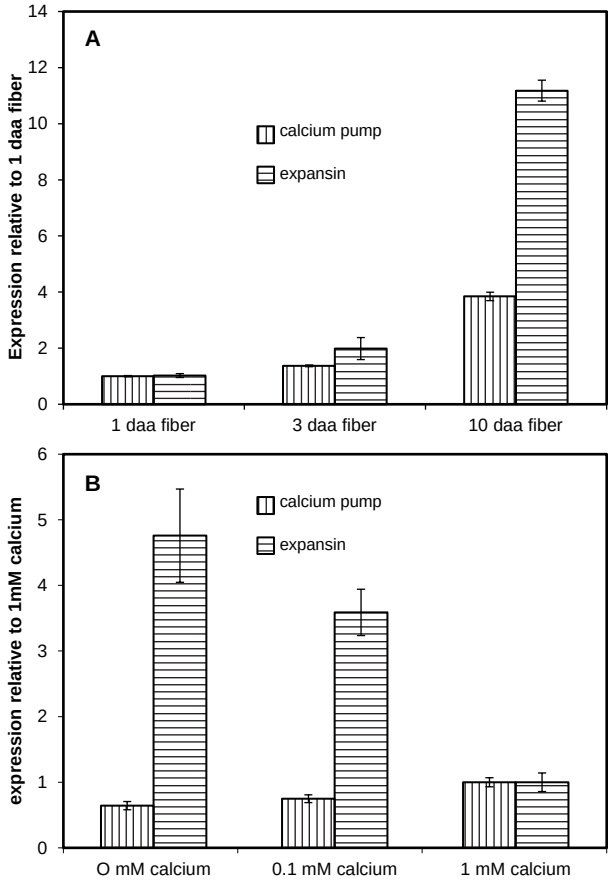

Figure 5. Measurements of levels of transcripts encoding a calcium pump and expansin using real-time rt-PCR. (A) Transcript levels were measured 1-, 3-, and 10-daa fiber harvested from greenhouse grown plants using real-time rt-PCR. (B) Transcript levels were measured in ovules cultured in media containing the indicated levels of $CaCl_2$. Bars represent the standard error of three replications.

DISCUSSION

These data indicated that optimal levels of calcium were needed to maximize early fiber growth in tissue culture. Higher levels of calcium, for example, the 3mM calcium used in the origin BT media, actually inhibited early fiber growth. The effect of calcium concentration on fiber elongation was greatest during the first 2 to 3 d in culture, suggesting that fiber elongation in ovules was especially sensitive to calcium levels before 3 daa, which was corroborated by microscopic observations. We note that the end of calcium-sensitive fiber elongation *in vitro* coincides with the end of the fiber-initiation stage of development identified by Triplett (1998). The expression level of expansin also increased in low-calcium medium, and expansin is well known to aid the cell wall loosening that supports cell expansion (Sampedro and Cosgrove, 2005).

Optimization of calcium concentration in ovule culture might allow the production of longer *in vitro* grown fiber more similar to fiber found on plants. Furthermore, the ability to transfer ovules after 3 d in low-calcium medium to new media without affecting fiber elongation will allow assessment of the effects of calcium concentrations on the transition to the elongation, cell wall biosynthesis, and maturation stages of fiber development.

The inhibitory effects of the calmodulin inhibitor TFP on early fiber elongation indicated that calmodulin played a positive role in fiber elongation under low calcium levels. However, this positive effect was negated at higher calcium levels, possibly because the native cytoplasmic calcium levels required for signaling processes to operate were overwhelmed. The fact that the calcium ionophore promoted fiber elongation in low-calcium medium was consistent with calcium being compartmentalized in young fibers as previously suggested by the increase in ER and calcium levels early in fiber development (Taliercio and Boykin, 2007). Possibly the stored calcium released by the ionophore was synergistic with the native regulatory processes operating when exogenous calcium was low. However, we note that calcimycin might not change levels of cytoplasmic calcium when used at lower levels than reported in this work (Bush, 1995; Felle et al., 1992). Therefore internal calcium levels in fiber initials treated with calcimycin should be measured to definitively determine if elevation of cytosolic calcium by the ionophore was responsible for enhanced fiber elongation measured in low-calcium medium. Similar measurements would help to determine if the cytoplasmic calcium levels were supraoptimal so that elongation was suppressed when fibers developed in the presence of high exogenous calcium. It would also be interesting to know if cytoplasmic calcium levels vary during early fiber development *in situ*.

Although 0.1 mM calcium was optimal for early fiber development, the ovules began to die at about 12 d (data not shown). Therefore, low-calcium medium will not support the development of mature fiber. In contrast, 3 mM calcium in the traditional BT medium (Beasley and Ting, 1974) allows fibers to progress through all the stages observed *in situ,* but at the expense of early fiber elongation. These results show that calcium concentrations should be optimized for each stage of fiber development in ovule culture, which might promote more similarity between *in vitro* and *in situ* fibers and more meaningful outcomes of *in vitro* experiments (Kim and Triplett, 2001). The data also demonstrate the need to do additional experiments to understand particular cellular mechanisms that may be modulated by calcium and calmodulin signaling to support fiber development.

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DISCLAIMERS

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

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