Twenty-Five Years after Beasley and Ting: Revealing Fundamental Properties of Fiber-Growth with Cotton Ovule Cultures

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ABSTRACT

Since the initial description of culture conditions for growing fiber in vitro by Beasley and Ting in 1973 many laboratories, including the Cotton Fiber Bioscience Research Unit, have used cotton ovule cultures to answer questions about fiber development. Fiber growth on developmental mutants cultured in vitro was similar to patterns seen in planta, however subtle differences in the production of cell wall polymers were detected by gel permeation chromatography of cultured fibers compared with in planta fibers. Fiber cells from cotton ovule cultures proved to be exceptional starting material for isolating cytoskeletons from plant cells for biochemical characterization. Adding alpha-amanitin to ovule cultures inhibited the normal progression of fiber development depending on when the transcription inhibitor was added. The inhibition was reversible during the early stages of fiber initiation and cell elongation, but irreversible if ovules were left in contact with alpha-amanitin for greater than five days. This observation suggests that there is a window of opportunity for fiber cell initials to develop into elongated fiber cells. If fiber cells do not differentiate within 4-6 days post-anthesis, fibers lose their capacity to differentiate. Current research is directed toward showing that the same mechanisms leading to the loss in capacity to differentiate operate to regulate gene expression during fiber development. Our laboratory is also developing conditions that will be suitable for growing cotton ovule cultures aboard the International Space Station to monitor the effects of microgravity on fiber gene expression and cell wall structure.

Introduction

With the advent of *in vitro* culture methods for plants in the late 1960's, many laboratories around the world aspired to develop a method for culturing immature cotton ovules. While Drs. Beasley and Ting with their co-workers at the University of California-Riverside were ultimately successful in reaching the goal of producing cotton fiber in culture, their work depended upon the pioneering efforts of many other researchers (Joshi, 1960; Murashige and Skoog, 1962; Joshi and Johri, 1972; Eid, 1972). The interest in developing conditions for growing fiber in culture was to have a "working research tool to investigate the physiology and biochemistry of fiber development" (Beasley *et al.*, 1971). Several reviews concerning the early work directed toward this effort have appeared in recent years (Kosmidou-Dimitropoulou, 1986; Stewart, 1991; Beasley, 1992; Mellon, 1998).

Material and Methods

The cotton ovule culture system as originally described (Beasley and Ting, 1973; Beasley and Ting, 1974) has served as an excellent "working research tool" for the researchers at the USDA, ARS Southern Regional Research Centre for nearly a decade (Triplett *et al.*, 1989; Mellon and Triplett, 1989). Ovule cultures have been used to study a variety of biochemical and physiological phenomena associated with fiber development. This paper reviews a few experimental approaches that have been taken by our research team in the past three years and discusses how we are using cotton ovule cultures in current research efforts.

Cell wall polymer characterization

The utility of cotton ovule cultures for cotton research depends upon the assumption that fiber produced in vitro is biochemically equivalent to fiber produced on field-grown plants. Four years after the culture system was first described (Beasley and Ting, 1973), a study was published comparing fibers grown in vitro and in planta (Meinert and Delmer, 1977). No other work comparing the physiology or biochemistry of the two fiber populations was published until three years ago. At that time, the late Dr. Judy Timpa had developed a new analytical technique for the characterization of cotton fiber cell wall components at the Southern Regional Research Centre. The method involved dissolving cotton fiber in a solution of dimethylsulfoxide-lithium chloride and separating the polymers by size exclusion (gel permeation) chromatography (Timpa, 1991). In collaboration with

Dr. Timpa, the profiles of fiber cell wall polymers from many stages of fiber development were determined using fiber from field-grown plants (Timpa and Triplett, 1993). Subsequently, we chose to investigate the polymer molecular weight distribution profiles from fiber grown in vitro and compare the profiles with those from mature and immature plant-grown fiber (Triplett and Timpa, 1995). Our results showed that the molecular characteristics, cumulative molecular weight distribution, and degree of polymer branching of ovule culture fiber after 21 days in culture were more similar to measurements for 30 DPA plant-grown fiber than mature fiber.

Cytoskeleton isolation

The orientation of cellulose microfibrils in the cell wall is an important determinant of fiber tenacity. The arrangement of cellulose polymers in the fiber wall is regulated by the cytoskeleton composed of microtubules and microfilaments (Seagull, 1986). The two major protein components of microtubules are alpha- and beta- tubulin. The main component of microfilaments is a protein known as actin. In higher plants, little is known about the mechanism through which the cytoskeleton influences cellulose microfibril organization. Based on studies in animal cells, it is believed that proteins that associate specifically with the cytoskeleton exert an influence on its organization.

In order to characterize cotton fiber cytoskeleton-associated proteins and study their influence on cellulose deposition, a procedure for isolating these proteins had to be devised. Once again, ovule cultures proved to be an excellent starting material. A procedure for isolating fiber cytoplasts (protoplasts without nuclei) first described by Gould *et al.* (1986) was modified for our experiments. Cytoplasts were extracted with the detergent Triton X-100 to remove the plasmalemma and other non-cytoskeleton proteins and purified on a sucrose step gradient (Andersland *et al.*, 1998). The extracted cytoplast preparation was enriched in alpha- and beta-tubulin and actin, but contained no other major protein component.

Immunological techniques were used to determine if two proteins previously shown to be associated with plant cytoskeletons were present in cotton fiber cytoskeleton preparations. Specifically, neither elongation factor 1-alpha (Durso and Cyr, 1994) nor spectrin (Yang *et al.*, 1992) was present in isolated cytoskeletons. These results suggest that the major components of cotton fiber cytoskeleton are the two tubulins and actin and that associated proteins are present in much smaller proportions than in other plant cells for which this type of analysis has been performed. More recently, a 115 kDa protein has been identified from isolated cytoskeleton preparations that appears to associate specifically with microfilaments and may represent a true actin-associated protein in cotton fiber (Andersland and Triplett, unpublished).

Transcription inhibitor studies

The highly elongated nature of cotton fiber cells, where cell length is often 1000-3000 times larger than the cell's diameter, presents an interesting problem as to how the single nucleus communicates with the remainder of the cell in a co-ordinated fashion. Two models can be proposed to account for this enigma: (1) mRNA is made throughout fiber development and there is a mechanism for delivery of mRNA molecules from the nucleus or the translated gene product to the cellular location where the gene product will be used, or (2) mRNA is produced early in fiber development and distributed evenly throughout the fiber as it elongates. In order to differentiate between these two working models, a study was conducted to examine the effect of adding alpha-amanitin to cotton ovule cultures (Triplett, 1998). Alpha-amanitin is a cyclic peptide of fungal origin that specifically inhibits the expression of genes transcribed by RNA polymerase II, i.e. polyadenylated mRNA molecules. Addition of alpha-amanitin on the day of anthesis to 2 days post anthesis resulted in fewer epidermal cells developing into fiber. When alpha-amanitin was added to ovule cultures at early times after culture initiation, fiber length was reduced compared to cultures in which the inhibitor was added at later time periods. This result suggests that continual mRNA synthesis takes place throughout fiber development, a result directly substantiated by two other research groups (Orford and Timmis, 1997; Smart *et al.*, 1998)

Inhibition of fiber growth was reversible when ovules were transferred to inhibitor-free medium within two days of culture initiation. If left in contact with alpha-amanitin for more than 5 days, ovules could not overcome the effects of alpha-amanitin and failed to produce fiber when transferred to inhibitor-free medium. These results suggest that there is a window of opportunity during which time fiber development must begin. Currently, we are investigating several possible molecular mechanisms that could regulate this important developmental event.

Preparing ovule cultures for space flight

A number of studies from both U.S. and Russian space programs concluded that higher plants grown in microgravity have altered plant cell wall structure and composition (Dutcher *et al.*, 1994; Kordyum, 1997). Since cotton fiber cell walls are not required for the physical support of any plant organ, analysis of the effects of microgravity on cotton fiber development seems like an ideally suited system to define how cell wall biogenesis is affected in the space environment. More importantly, analysis of fiber growth in microgravity will enable our research team to evaluate

how cellulose microfibril biosynthesis or deposition may be modified.

Before we can propose to send ovule cultures aboard the International Space Station, some modifications of the growth conditions are necessary. Normally in culture, ovules are floated on the surface of liquid media. Any ovules that sink to the bottom of the culture dish do not develop fiber nor do the ovules grow. Many of the early investigators found that incorporation of agar into culture media either inhibited fiber differentiation or led to prolific callus growth. Recently, our research team has investigated the use of a bacterial fermentation product, gellan gum, to solidify ovule culture media (Triplett and Johnson, manuscript in review). Addition of the gelling agent had no effect on the length of fibers produced in culture, however the cellulose content was reduced by one half. At present, it is unknown how cellulose biosynthesis is inhibited on solid media. Nevertheless, such cultures should prove useful in determining how micronaire and yield differences are generated.

Conclusions

The in vitro culture technique established by Beasley and Ting over 25 years ago continues to be an important research tool for dissecting the complex cellular processes that occur during fiber development. In vitro culture of cotton ovules facilitates some types of physiological and biochemical experiments that would be difficult with field-grown or greenhouse-grown material, most notably experiments requiring radioisotopes and toxic inhibitors. Nevertheless, it is incumbent on the investigators using cotton ovule culture as a model for fiber development to verify findings with in planta grown material to the extent possible. By continuing to establish links between the performance of cotton ovules in culture with ovules developing on whole plants, this important research tool will continue to be used well into the next century.

In Memorium

This paper is dedicated to the memory of C.A. “Bud” Beasley.

References

Andersland, J.M., D.C Dixon, R.W. Seagull and B.A. Triplett. (1998): Isolation and characterisation of cytoskeletons from cotton fiber cytoplasts. In: Vitro: Cell. Develop. Biol. Plant. 34:173-180.

Beasley, C.A. (1992): In vitro cotton ovule culture: a review. Proceedings of Cotton Fiber Cellulose: Structure, Function, and Utilization Conference. National Cotton Council, Memphis TN. Pp.65-89.

Beasley, C.A., I.P. Ting and L.A. Feigen. (1971): Test tube cotton. Calif. Agric. 25:6-8.

Beasley, C.A. and I.P. Ting. (1973): Effects of plant growth substances on *in vitro* fiber development from fertilized cotton ovules. Am. J. Bot. 60:130-139.

Beasley, C.A. and I.P. Ting. (1974): Effects of plant growth substances on in vitro fiber development from unfertilized cotton ovules. Am. J. Bot. 61:188-194.

Durso, N.A. and R.J. Cyr. (1994): A calmodulin-sensitive interaction between microtubules and a higher plant homolog of elongation factor-1. Plant Cell. 6:893-905.

Dutcher, F.R., E.L.Hess and T.W. Halstead. (1994): Progress in plant research in space. Adv. Space Res. 14:159-171.

Eid, A.A.H. (1972): In vitro culture of fertilized cotton ovules. Ph.D. dissertation, Rijksunversiteit, Ghent, Belgium.169 pp.

Gould, J.H., R.L. Palmer and W.M. Dugger. (1986): Isolation an culture of anucleate protoplasts from cotton fiber: assessment of viability and analysis of regenerated wall polymers. Plant Cell Tissue Organ Cult. 6:61-72.

Joshi, P.C. (1960): In vitro growth of cotton ovules. Symp. Plant Embryology. Council of Scientific and Industrial Res., New Delhi. Pp. 199-204.

Joshi, P.C. and B.M. Johri. (1972): In vitro growth of ovules of Gossypium hirsutum. Phytomorphol. 22:195-209.

Kosmidou-Dimitropoulou, K. (1986): Hormonal influences on fiber development. In: Cotton Physiology. Mauney, J.R.; Stewart, J.McD. (Eds.), The Cotton Foundation Reference Book Series, No. 1. Memphis, TN. P pp. 361-373.

Kordyum, E.L. (1997): Biology of plant cells in microgravity and under clinostating. Int. Rev. Cytol. 171:1-78.

Meinert, M.C. and D.P. Delmer. (1977): Changes in biochemical composition of the cell wall of the cotton fiber during development. Plant Physiol. 59:1088-1097.

Mellon, J.E. (1998): In vitro culture of cotton ovules. In Biotechnology in Agriculture and Forestry, vol. 42: Cotton. Bajaj, Y.P.S. (Ed.) Springer-Verlag, Berlin. Pp. 107-120.

Mellon, J.E. and B.A. Triplett. (1989): De novo synthesis of peroxidase in cotton ovule culture medium. Physiol. Plant. 77:302-307.

Murashige, T. and F. Skoog. (1962): A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Orford, S.J. and J.N. Timmis. (1997): Abundant mRNAs specific to the developing cotton fiber. Theor. Appl. Genet. 94:909-918.

Seagull, R.W. (1986): Changes in microtubule organisation and microfibril orientation during in vitro cotton fiber development: an immuno-fluorescent study. Can. J. Bot. 64:1373-1381.

Smart, L.B., F. Vojdani, M. Maeshima and T.A. Wilkins. (1998): Genes involved in osmoregulation during turgor-driven cell expansion of developing cotton fibers are differentially regulated. Plant Physiol. 116:1539-1549.

Stewart, J. McD. (1991): Biotechnology of cotton. CAB International, Wallingford. pp. 1-50.

Timpa, J.D. (1991): Application of universal calibration in gel permeation chromatography for molecular weight determinations of plant cell wall polymers: cotton fiber. J. Agric. Food Chem. 39:270-275.

Timpa, J.D. and B.A. Triplett. (1993): Analysis of cell wall polymers during cotton fiber development. Planta. 189:101-108.

Triplett, B.A., W.H. Busch and W.R. Goynes. (1989): Ovule and suspension culture of a cotton fiber development mutant. In Vitro: Cell. Develop. Biol.-Plant. 25:197-200.

Triplett, B.A. and J.D. Timpa. (1995): Characterisation of cell-wall polymers from cotton ovule culture fiber cells by gel permeation chromatography. In Vitro: Cell. Develop. Biol.-Plant. 31:171-175.

Triplett, B.A. (1998): Stage-specific inhibition of cotton fiber development by adding alpha-amanitin to ovule cultures. In Vitro: Cell. Develop. Biol.-Plant. 34:27-33.

Yang, C., I. Xing and Z. Zhai. (1992): Intermediate filaments in higher plant cells and their assembly in a cell-free system. Protoplasma. 171:44-54.