Title: Cellulose and the control of growth anisotropy
Active dates: 7/1994 — 6/2003

18 peer reviewed publications resulted from the grant and cite the award.

Reviews and Methods:

1. Baskin TI, Miller DD, Vos JW, Wilson JE, Hepler PK (1996) Cryofixing single cells and multicellular specimens enhances structure and immunocytochemistry for light microscopy. **Journal of Microscopy** 182: 149 - 161.

2. Baskin TI (2000) On the constancy of cell division rate in the root meristem. **Plant Molecular Biology** 43: 545 - 554.

3. Baskin TI (2001) On the alignment of cellulose microfibrils by cortical microtubules: A review and a model. **Protoplasma** 215: 150-171.

4. van der Weele CM, Jiang H, Palaniappan KK, Ivanov VB, Palaniappan K, Baskin TI (2003) A new algorithm for computational image analysis of deformable motion at high spatial and temporal resolution applied to root growth: Roughly uniform elongation in the meristem and also, after an abrupt acceleration, in the elongation zone. **Plant Physiology**, 132:1138-1148.

Growth Physiology:

5. Baskin TI, Bivens NJ (1995) Stimulation of radial expansion in arabidopsis roots by inhibitors of actomyosin and vesicle secretion but not by various inhibitors of metabolism. **Planta** 197: 514 - 521.

6. Liang BM, Sharp RE, Baskin TI (1997) Regulation of growth anisotropy in well watered and water-stressed maize roots I. Spatial distribution of longitudinal, radial and tangential expansion rates. **Plant Physiology** 115: 101 - 111.

7. Beemster GTS, Baskin TI (1998) Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. **Plant Physiology** 116: 1515 - 1526.

8. Beemster GTS, Baskin TI (2000) *STUNTED PLANT 1* mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of *Arabidopsis thaliana*. **Plant Physiology** 124: 1718 - 1727.

9. Baskin TI, Remillong EL, Wilson JE (2001) The impact of mannose and other carbon sources on the elongation and diameter of the primary root of *Arabidopsis thaliana*. **Australian Journal of Plant Physiology** 28: 481-488.

Cortical Microtubules and Cellulose Microfibrils:

10. Liang BM, Dennings AM, Sharp RE, Baskin TI (1996) Consistent handedness of microtubule helical arrays in maize and arabidopsis primary roots. **Protoplasma** 190:8-15

11. Assmann SM, Baskin TI (1998) The function of guard cells does not require an intact array of cortical microtubules. **Journal of Experimental Botany** 49: 163-170.

12. Baskin TI, Meekes HTHM, Liang BM, Sharp RE (1999) Regulation of growth anisotropy in well watered and water-stressed maize roots. II. Role of cortical microtubules and cellulose microfibrils. **Plant Physiology** 119: 681 - 692.

13. Schindelman G, Morikami A, Jung J, Baskin TI, Carpita NC, Derbyshire P, McCann MC, Benfey PN (2001) COBRA encodes a putative GPI-anchored protein, which is polarly

localized and necessary for oriented cell expansion in *Arabidopsis*. **Genes and Development** 15: 1115-1127.

14. Lane DR, Wiedemeier A, Peng L, Höfte H, Hocart CH, Birch RJ, Baskin TI, Burn JE, Arioli T, Betzner AS, Williamson RE (2001) Temperature-sensitive alleles of *radially swollen2* link the KORRIGAN endo-1,4-ß-glucanase to cellulose synthesis and cytokinesis. **Plant Physiology** 126: 278-288.
15. Wiedemeier AMD, Judy-March JE, Hocart CH, Wasteneys GO, Williamson RE, Baskin TI (2002) Mutant alleles of arabidopsis *RADIALLY SWOLLEN 4* and *RSW7* reduce growth anisotropy without altering the transverse orientation of cortical microtubules or cellulose microfibrils. **Development** 129: 4821—4830.
16. Andème-Onzighi C, Sivaguru M, Judy-March J, Baskin TI, Driouich A (2002) The *reb1-1* mutation of Arabidopsis alters the morphology of trichoblasts, the expression of arabinogalactan-proteins and the organization of cortical microtubules. **Planta** 215: 949—958.
17. Sivaguru M, Pike S, Gassmann W, Baskin TI (2003) Aluminum rapidly depolymerizes cortical microtubules and depolarizes the plasma membrane: Evidence that these responses are mediated by a glutamate receptor. **Plant and Cell Physiology**, 44: 667–675.
18. Tian GW, Smith D, Glück S, Baskin TI (2004) The higher plant cortical microtubule array analyzed in vitro in the presence of the cell wall. **Cell Motility and the Cytoskeleton,** 57: 26-36.

My research aims to understand morphogenesis, focusing on growth anisotropy, a process that is crucial to make organs with specific and heritable shapes. For the award, the specific aims were to test hypotheses concerning how growth anisotropy is controlled by cell wall structure, particularly by the synthesis and alignment of cellulose microfibrils, the predominant mechanical element in the cell wall. This research has involved characterizing the basic physiology of anisotropic expansion, including measuring it at high resolution; and second, characterizing the relationship between growth anisotropy, and cellulose microfibrils. Important in this relationship and also to the control of anisotropic expansion are structures just inside the plasma membrane called cortical microtubules, and the research has also investigated their contribution to controlling anisotropy and microfibril alignment. In addition to primary experimental papers, I have also developed improved methods relating to these objectives as well as written relevant reviews . Major accomplishments in each area will now be described. Numerical citations refer to the list above.

REVIEWS AND METHODS

A novel hypothesis for microfibril organization by microtubules. The major objective of my DOE funded work is to understand the role of cellulose microfibrils in controlling the anisotropy of expansion. An outstanding unresolved issue is cellulose orientation itself, and particularly the role of cortical microtubules in effecting this organization. While often taken for granted that cellulose is oriented by cortical microtubules, there is no convincing mechanism and there are examples known where microtubules appear not to be involved. I looked into the literature on this and wrote a review of the subject running to 22 pages in Protoplasma and with more than 250 citations (3). As a result, I came up with a new hypothesis for how cellulose microfibrils are oriented, namely by virtue of binding the nascent microfibril as opposed to steering the synthesizing complex. The hypothesis accounts for orientation of microfibrils by microtubules by supposing that microtubules orient trans-membrane proteins that bind nascent microfibrils, and for the orientation of microfibrils without microtubules by supposing that the extant cell wall is also capable of orienting proteins that bind nascent microfibrils.

Cryofixation of arabidopsis roots for light microscopy. One methodological advance made by my lab is cryofixation for *light* microscopy (1). In cryofixation, the sample is cooled in milliseconds to -180 ˚C, thus "fixing" the sample. This fixation is then made permanent through freeze substitution for several days at -80 ˚C, when the water in the sample is exchanged with a solvent. The sample is then warmed, embedded in methacrylate and sectioned as usual. We found this method improved sample preservation and immunocytochemistry. Plunge freezing multicellular objects, such as a root, has been dismissed, owing to the inevitable presence of damage from ice crystals at depths greater than 10 μm or so from the surface of the tissue, damage which is overwhelming at the high magnifications of electron microscopy. However, we have shown that this damage is not detectable at the lower magnifications of light microscopy, and that the cryofixed tissue is superior to its chemically fixed counterpart by several criteria. This work has spurred other labs to use cryofixation for their light microscopy.

Structural analysis of the cell wall with Field-Emission Scanning Electron Microscopy. While on sabbatical with Geoffrey Wasteneys (ANU), I helped develop methods for imaging cell wall ultrastructure with the Field-Emission Scanning Electron Microscope (FESEM). This instrument combines the topographic contrast of a conventional SEM with the high resolution of a transmission electron microscope. Sample preparation is easier than that required to make replicas, and FESEM can produce striking images of cell wall ultrastructure. Although FESEM has been used for years to study tracheid cell walls in wood, it is only recently becoming applied to the primary cell wall. For the primary cell wall, one must remove the cytoplasm and plasma membrane. I helped develop a method based on using a cryo-ultramicrotome to open the arabidopsis root and I developed an alternative method based on embedding roots in polyethylene glycol. I also found that material with large cells, such as maize roots or cucumber hypocotyls, can be imaged simply and reliably. We have extended this work to include a comparison of ultrastructure revealed by FESEM and the atomic force microscope, and to enable immuno-labeling for the FESEM with gold-conjugated probes. These methods have been essential for two publications (15, 18).

High resolution quantification of relative elongation rate. To understand how growth anisotropy is controlled, it must be measured. In pursuing objectives of an NSF-sponsored project on the relation between cell division and elongation in the root, we developed software to recover the spatial profile of velocity (and of relative elongation rate) of a growing root based on digital image processing, in collaboration with Dr K. Palaniappan of the University of Missouri's Computer Science and Computer Engineering Department (4). The input is a stack of nine images taken at 10 sec intervals and the output is the velocity at each pixel. The algorithm uses methods from time-sequence analysis of digital images. The method does not require marking the root, and represents an improvement of spatial and temporal resolution each of more than an order of magnitude. This rapid and reliable method for measuring the velocity profile enhances the ability to quantify the profile of expansion anisotropy.

GROWTH PHYSIOLOGY

Specificity of swelling. In the use of genetics to untangle the strands of a complex phenotype like root shape, there is the problem that morphogenesis could be connected, through layers of indirect interactions, to any cellular process. To see how easily arabidopsis roots can be made to swell, an undergraduate student, Nathan Bivens, surveyed the effects on root elongation and lateral expansion of a variety of metabolic inhibitors (5). He found no swelling in response to 16 different compounds with known metabolic sites of action, such as inhibitors of amino acid synthesis, folate metabolism, respiration, DNA synthesis, protein synthesis, sterol synthesis and others. These results indicate the processes that control growth anisotropy are reasonably well isolated from cellular metabolism, and make the outcome less likely that distorted morphology in

the mutants arises from a mutation in a purely metabolic enzyme. Furthermore, he did find a modest but significant stimulation of swelling caused by compounds that interfere with vesicle secretion or with actin filaments. These results suggest that vesicle secretion and actin filaments may play roles in morphogenesis.

Metabolism and morphology In fungi, morphology can be disrupted by imbalanced carbohydrate metabolism. To determine whether this is true in higher plants, undergraduate students in my lab exposed seedlings to a variety of carbon compounds and assessed the effect on root shape. First, we looked at the effects of replacing the sucrose customary in growth medium with a different carbon source. Although many sugars inhibited elongation, only one changed the shape of the root, 2-deoxyglucose, which is known to partly inhibit cellulose synthesis. Second, elongation was inhibited by extremely low levels of mannose, this project was extended to study the effects of to reveal a hexokinase-based sugar sensing mechanism regulating root elongation (9).

Analysis of cell division and cell expansion in the arabidopsis root We developed a new method for measuring the spatial distributions of expansion rate and cell division rate in arabidopsis roots. In the first publication (7), we validated the method, and showed that the acceleration of root growth in this species is caused by the number of dividing cells increasing, which in turn increased the number of cells in the zone of rapid elongation. Rates of cell division did not change, nor did the elongation behavior of single cells. In the second publication (8), we showed that auxin and cytokinin exert opposing effects on cell number in the meristem but surprisingly, only cytokinin inhibits relative elongation rate. The data in these papers are among the only quantification of local expansion rates within a plant meristem and present a baseline for understanding growth physiology in the root. Also, a review of cell division rate in the root meristem was commissioned from me by Plant Molecular Biology (2).

Spatial distribution of growth anisotropy We quantified longitudinal, radial, and tangential expansion rates in well-watered and water-stressed maize roots, and we did so separately for the cortex and stele (6). The magnitude of growth anisotropy was obtained as the ratio of longitudinal to radial or tangential expansion. We found that the spatial profiles of radial and tangential expansion rates differed between stele and cortex. Water-stress caused maize roots to thin by sharply limiting lateral expansion rate in the apical 5 mm of the root; basal to this, lateral expansion was unaffected. Because in contrast, the profile of elongation rate was unchanged apically but reduced basally, we hypothesized that rates of longitudinal and lateral expansion are regulated independently. Independent variation longitudinal and lateral expansion rate is significant because it is unexpected and, in present day models of the cell wall yielding, unexplained. This publication is significant in general because it marks to my knowledge the first measurements of growth anisotropy for defined tissues of any plant organ, and specifically for the award because this analysis is a prerequisite to achieving a major aim, namely, to discover what controls the degree of anisotropic expansion.

CORTICAL MICROTUBULES AND CELLULOSE MICROFIBRILS

Microtubules and guard cell function Sarah Assmann (Pennsylvania State University) and I have shown that cortical microtubules are not needed by mature stomata to open or close in response to several stimuli; additionally, the organization of the cortical array is the same in open and closed stomata (11). This work was undertaken, in part, to find a system in which cortical microtubules have a function other than orienting the deposition of cellulose microfibrils. Such a system would offer a useful comparison to growing cells for interpreting experiments in which microtubules were depleted or disorganized. Unfortunately, we detected no functional requirement in guard cells for microtubules.

Cortical microtubules form helical arrays with a unique handedness. We discovered that, at the base of the growth zone, maize and arabidopsis roots form exclusively right-handed helical arrays of cortical microtubules (10). Subsequently, we showed that cortical arrays are composed microtubules of random polarity (18). Thus, microtubule organization contains a fundamental asymmetry, and this may indicate a hitherto unknown mechanism specifying polarity of cells or organs.

Cortical microtubules, cellulose microfibrils and growth anisotropy Paul Green hypothesized that the magnitude of growth anisotropy is controlled by the degree of alignment among cellulose microfibrils; however, this has never been tested. We quantified the angular distribution of microtubules and microfibrils for longitudinal-radial cell walls of the root cortex of both well-watered and water-stressed maize roots, and we compared those distributions to the measured rates of radial expansion and growth anisotropy for the same tissue (12). For microfibrils, angular distributions were quantified directly from electron micrographs of microfibrils at the innermost layer of the cell wall, and indirectly with measurements of cell wall retardation with polarized-light microscopy. Throughout nearly the entire growth zone, the average orientation of microtubules and microfibrils was transverse, consistent with rates of elongation being everywhere greater than rates of radial expansion (i.e., anisotropy magnitude greater than 1); however, the degree of alignment was totally unrelated to either radial expansion rate or to the magnitude of growth anisotropy. Therefore, Green's hypothesis is clearly refuted.

The analysis of the maize root described above represents by far the most detailed study ever made of the relationship between the orientation of microtubules and microfibrils and growth anisotropy. Few studies have quantified cellulose orientation, or microtubule orientation, or growth anisotropy with the spatial resolution we have used, and no study has quantified all three parameters for the same material. The above work reaches the major goal of the funded proposal: namely, to find out whether the degree of growth anisotropy is controlled by the degree of alignment among cellulose microfibrils. For the maize root, the answer appears to be no.

A genetic analysis of growth anisotropy in arabidopsis As a complementary approach to using water-stress or drugs to uncover mechanisms underlying the control of anisotropic expansion, I have pursued a genetic approach, based on mutants in which the highly anisotropic expansion of the root is lost. This phenotype is called radially swollen (Rsw). I began this project with Richard Williamson (A.N.U.) and we have identified mutants at eight genetically distinct loci. A specific aim of was to analyze whether the swelling phenotype involved microfibril orientation. Microfibril alignment was assessed with measurements of birefringent retardation of cell walls in sections, and with electron microscopy of replicas of the innermost wall layer. In one mutant, *rsw2*, retardation was unaffected in the epidermis, but became undetectable in interior tissues (14). This shows that the *RSW2* gene product participates in cellulose synthesis, or in the regulation of cellulose microfibril longevity in the cell wall, and has helped confirm the function of the gene, which is now known to be an endoglucanase called KORRIGAN.

Another mutant in which the loss of control of anisotropy occurs in root hair cells specifically has been analyzed to show that instead of cellulose synthesis, a major defect occurs in the synthesis of a particular type of cell wall component called arabinogalactan proteins (16). This work identified this class of cell wall component as playing a role in the control of anisotropic expansion.

Phenotypic analysis of *rsw4* and *rsw4* In two mutants, *rsw4* and *rsw7,* we found that radial swelling was accompanied by disorganization in neither microtubules nor microfibrils (15). This is the first demonstration of a loss of control of anisotropic expansion without involvement of either microtubules or microfibrils. These mutants present a puzzle: why do they swell? The mutants also present an opportunity: finding out what makes them swell should enhance our understanding of growth anisotropy. Swelling in *rsw4* and *rsw7* is accompanied by

the production of ectopic cells, at seemingly random orientations, and in principle, swelling could be driven by these extra cells instead of by a specific defect in growth anisotropy. To check this, we exposed seedlings to a cell division inhibitor, aphidicolin, and learned that nearly all extra cell production could be eliminated without reducing the radial swelling. We also found that following transfer to the 30˚C, swelling begins before extra cells appear. These results indicate that radial swelling in the mutants is not caused by ectopic cell production and buttress our conclusion that the mutants are defective specifically in the control of expansion anisotropy.

To explain swelling by a means other than cellulose alignment, we hypothesized that the cell walls in the mutants differ from the wild type in polysaccharide composition. This hypothesis has been consistently falsified by staining roots with six different well characterized monoclonal antibodies to specific cell wall components. Additionally, we analyzed cell wall composition biochemically. In collaboration with Richard Williamson, cell walls were separated into acid-soluble and insoluble fractions and the monosaccharides were analyzed by GC/MS; in collaboration with Karl Hasenstein (Univ. of Louisiana), we analyzed the sugar content of an alcohol-insoluble residue after methanolysis and trimethylsylilation of monosaccharides, which allowed us to assay the growing root tips specifically. Results from both methods consistently falsified the hypothesis that swelling in *rsw4* and *rsw7* results from a compositional difference among cell wall carbohydrates.