FLOWERING AND FRUITING IN COTTON

NUMBER EIGHT
THE COTTON FOUNDATION
REFERENCE BOOK SERIES

Edited by
Derrick M. Oosterhuis
and
J. Tom Cothren

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THE COTTON FOUNDATION

Reference Book Series

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Bill M. Norman
Executive Director
The Cotton Foundation
7193 Goodlett Farms Parkway
Cordova, Tennessee 38016

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Monsanto is proud to be a member of the Cotton Foundation and sponsor of ***FLOWERING AND FRUITING IN COTTON***, the eighth book in the Cotton Foundation's cotton reference book series. The sponsorship is a reflection of the company's belief that continued support of the development of technologies and materials that promote the production of more cost-effective, higher yielding cotton, will make a stronger cotton industry.

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Executive Editor and Publishing Coordinator
WILLIAM C. ROBERTSON

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TABLE OF CONTENTS

COTTON PHYSIOLOGY BOOK SERIES

FOREWORD

The *Cotton Physiology Book* series started with the first publication ***COTTON PHYSIOLOGY*** in 1985, edited by J.R. Mauney and J.M. Stewart, and the second book ***PHYSIOLOGY OF COTTON*** book edited by J.M. Stewart, D.M. Oosterhuis, J.J. Heitholt, and J.R. Mauney published in 2010. This series is being continued using a smaller book format with each future book covering a specific pertinent topic. The smaller book format will facilitate timely publication each year and reduce the cost per book. The books will be published in book form as well as on CD's under the auspices of the National Cotton Council as a continuation of the original Cotton Physiology book published in 1985. Each book will incorporate a special symposium on a topic chosen by members of the National Cotton Council, Agronomy and Physiology Conference and held at the Beltwide Cotton Conferences. Prominent speakers will be invited to partake in the symposium, and together with additional invited authorities, will make up the subsequent book. The first of the new small book physiology of cotton series was on ***STRESS PHYSIOLOGY IN COTTON*** and was published in 2011. The next symposium was held at the Beltwide Cotton Conferences in Atlanta in January 2011 and the subsequent book entitled ***FLOWERING AND FRUITING IN COTTON*** will be published in 2012.

PREFACE

For cotton production to be sustainable and profitable, it is essential to understand the growth of the cotton plant and how the plant responds to environmental stress. The cotton plant has complex growth pattern due to its perennial nature, indeterminate growth habit, and sympodial fruiting pattern. Furthermore, the crop is especially responsive to changes in the environment and management, particularly during reproductive development. Plants in production systems are continually exposed to various stresses, including extreme temperatures, inadequate water, nutrient deficiencies, and pathogens. The effect of these stresses on plant growth and yield depends upon the severity and timing of the stress and the ability of the plant to respond and adapt to the stress. Previous cotton physiology books have covered the fundamental physiological functions, metabolism and responses to stress over the whole growth period of the cotton plant. The current book focuses on reproductive growth and responses to environmental stresses. This phase of development period is particularly sensitive to adverse conditions, and is important for the development for optimum yields and fiber quality. Hence, a sound understanding of physiological processes and how they respond to stress during this period of yield development is essential to formulate strategies, agronomic and genetic, to counteract or ameliorate stress. Plant responses to environment and their ability to adapt determine the success of that plant genotype for successful production to achieve maximum yields. Therefore, understanding the effects of various stresses on the physiology of plants during the critical reproductive development is essential for an understanding of resistance and survival mechanisms for breeding for stress resistance and for formulation of improved management practices. Individuals involved in growing cotton should be familiar with the requirements of the cotton plant in relation to growth and yield development, in order to use management aids to know the most profitable time to irrigate, apply plant growth regulators, herbicides, foliar fertilizers, insecticides, defoliants, etc. The chapters in this book were assembled to provide those dealing with the production of cotton with the basic knowledge of the physiology of the plant during the critical reproductive stage for optimum and profitable management of the cotton crop.

CONTRIBUTORS

Dr. Brian G. Ayre
Department of Biological Sciences
University of North Texas
1155 Union Circle, #305220
Denton, TX 76203
bgayre@unt.edu

Dr. Allen G. Green
CSIRO Division of Plant Industry
PO Box 1600
ACT 2601, Australia
allan.green@csiro.au

Dr. Greg Constable
CSIRO Division of Plant Industry
Cotton Research Unit
P.O. Box 59, Narrabri
NSW 2390, Australia
Greg.Constable@csiro.au

Dr. Dimitra Loka
Department of Crop, Soil, and Environmental Sciences
University of Arkansas
1366 Altheimer Drive
Fayetteville, AR 72704
dloka@uark.edu

Dr. Tom Cothren
Department of Soil and Crop Sciences
Texas A & M University
Heep Center
College Station, TX 77843
jtcothren@tamu.edu

Dr. Jack R. Mauney
161 E. 1st Street, Suite 4
Mesa, AZ 85201
jacmauney@juno.com

Dr. Vladimir A. da Costa
Dow AgroSciences LLC
Sloughhouse, CA 95683
vdacosta@dow.com

Dr. Roisin C. McGarry
Department of Biological Sciences
University of North Texas
1155 Union Circle, #305220
Denton, TX 76203
rmcgarry@unt.edu

Dr. Meredith Errington
University of Sydney
NSW, Australia
meredith.anne.errington@monsanto.com

Dr. William R. Meredith Jr.
USDA-ARS (retired)
P.O. Box 225
Stoneville, MS 38776

Dr. Derrick Oosterhuis
Department of Crop, Soil, and Environmental Sciences
University of Arkansas
1366 Altheimer Drive
Fayetteville, AR 72704
oosterhu@uark.edu

Dr. Michael R. Stiff
Crop Science and Plant Biology
North Caroline State University
4405 Williams Hall
Raleigh, NC 27695
mrstiff@ncsu.edu

Dr. Candace Haigler
Crop Science and Plant Biology
North Caroline State University
4405 Williams Hall
Raleigh, NC 27695
Candace_Haigler@ncsu.edu

Dr. William T. Pettigrew
Crop Production Systems Research Unit
141 Experiment Station Road
PO Box 350
Stoneville, MS 38776
Bill.Pettigrew@ars.usda.gov

Dr. Qing Liu
CSIRO Division of Plant Industry
PO Box 1600
ACT 2601, Australia
Qing.Liu@csiro.au

Dr. Ian Rochester
CSIRO Division of Plant Industry
Cotton Research Unit
22888 Kamillaroi Hwy
Locked Bag 59, Narrabri
NSW 2390, Australia
Ian.Rochester@csiro.au

Dr. Danny J. Llewellyn
CSIRO Division of Plant Industry
PO Box 1600
ACT 2601, Australia
Danny.Llewellyn@csiro.au

Dr. Surinder P. Singh
CSIRO Division of Plant Industry
PO Box 1600
ACT 2601, Australia
Surinder.Singh@csiro.au

Dr. John Snider
Department of Crop and Soil Sciences
University of Georgia
115 Coastal Way
Tifton, GA 31794
jlsnider@uga.edu

Dr. Randy Wells
Crop Science and Plant Biology
North Carolina State University
2210 Williams Hall, Campus Box 7620
Raleigh, NC 27695
randy_wells@ncsu.edu

Photography by Bill Robertson

Chapter 1

ANATOMY AND MORPHOLOGY OF FRUITING FORMS

Jack Mauney,USDA-ARS, retired
Jarman Enterprises, Mesa, AZ 85201

INTRODUCTION

As a woody perennial cotton has the most complex vegetative and reproductive morphology of any annual crop grown. Its growth habit produces both vegetative and reproductive organs simultaneously. The sympodial flowering pattern of the cotton plant causes a very complex production-distribution pattern of carbohydrate throughout the structure of the crop. Maximizing this production and distribution of energy is, of course, the goal of all growers' and research effort.

One of the earliest comprehensive descriptions of cotton plant morphology is that of J.M. Hector in ***INTRODUCTION TO THE BOTANY OF FIELD CROPS*** (1936) and the Russian monograph ***THE STRUCTURE AND DEVELOPMENT OF THE COTTON PLANT*** (Baranov and Maltzev, 1937). Mauney (1968, 1984, 1986) updated these descriptions based on anatomical details observed by Mauney and Ball (1959) which more accurately analyzed the association of the two branch buds found at the base of each leaf. More recently Oosterhuis and Jernstedt (1999) provided an overview of the morphology and anatomy of cotton No new observations which alter our understanding of the structure of the plant have developed over the decades since these earlier descriptions, therefore this review will use them to interpret several interesting features of highly productive modern cultivars.

VEGETATIVE GROWTH

The primary axis is the framework upon which the floral branches develop. This axis develops only roots, stems, and leaves. Because cotton is an indeterminate perennial, it continues to produce additional numbers of these organs until some stress such as drought, freezing, nutrient deficiency, terminates growth (Fig. 1).

The plant produces three types of leaves, cotyledons, prophylls, and true leaves. At the base of each leaf, a branch (axillary) bud primordium is left behind by the advancing apical meristem. This branch bud forms the axis for the axillary branches. The prophyll, the first inconspicuous leaf, resembling a stipule, is the first leaf to be formed by the branch axis. Since there is no internode formed below it, the prophyll resides in the axil where it was formed, though technically it is a part of the branch axis. In the axil of this prophyll an additional branch bud is formed to create what Mauney and Ball (1959) termed the "second axillary". This bud is ordinarily dormant, but on very vigorous plants it may elongate and produce an additional branch axis (Fig. 2).

The representation depicted in Figure 2 is of a node from which vegetative branches (monopodia) develop. Morphologically these branches are indistinguishable from the primary axis.

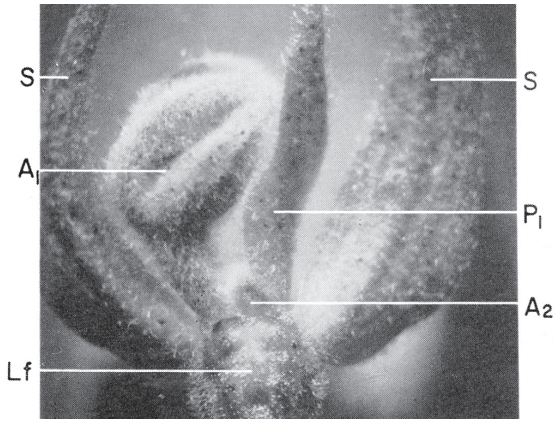

Figure 1. Young vegetative node of cotton. Lf = Leaf scar ; S = Stipules ; A_1 = First axillary; P_1 = Prophyll of first axillary; A_2 = Second axillary.

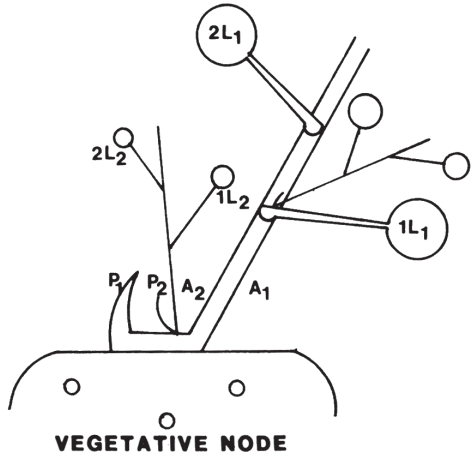

Figure 2. Schematic representation of a vegetative axillary branch . Sawtooths at left = prophylls; open circles = true leaves; P_1 = Prophyll of first axillary (A_1) ; P_2 = Prophyll of second axillary (A_2); 1, $2L_1$ = First and second leaf of first axillary; 1, $2L_2$ = First and second leaf of second axillary.

REPRODUCTIVE GROWTH

At some point early in the growth of the cotton seedling, the primary axis receives a signal from the leaves and begins developing nodes at which the first axillary shifts from vegetative branching to reproductive (sympodial) branching (Fig. 3). Morphologically these axes are identical to vegetative branches until the first true leaf is formed after which the meristem terminates in a flower. The branch continues by means of the axillary at the base of the prophyll of that true leaf breaking dormancy, elongating into a true leaf, and producing an additional flower.

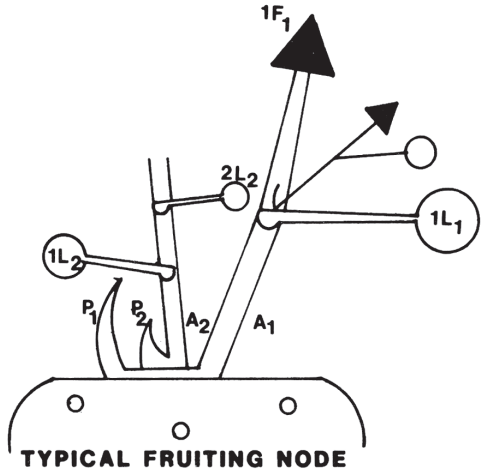

Figure 3. Schematic representation of a reproductive branch, sympodium. Black triangles = Flower buds, squares. $1F_1$ = First flower of first axillary. All other symbols are the same as Figure 2.

In robust plants with strong signals for flowering, the second axillary, which lies at the base of each branch, may also develop into a sympodium (Fig. 4). These fruiting structures often do not have a leaf opposite the flower, and as such do not have an axillary to continue the branch. Thus, they can appear as a single flower on a spur at any node on a fruiting branch. They are frequently functional flowers and contribute to productive yield. In certain heavy fruiting cultivars and open spacing situations the second axillary sympodia have true leaves, and may produce several flowers.

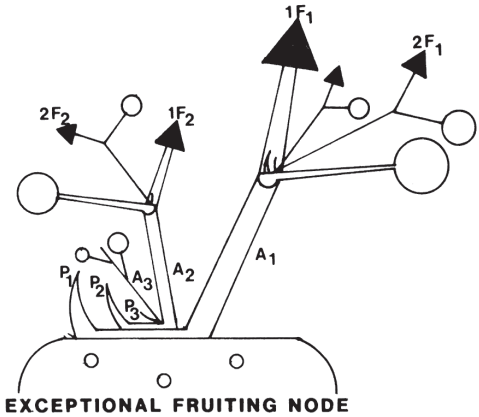

Figure 4. Schematic representation of a second axillary sympodium (A_2) and the resulting development of the third axillary (A_3). Other symbols are the same as Figures 2 and 3.

Since vegetative branches have the same potential as the primary axis, i.e., if they are vigorous, they will also bear sympodia. The signal to commence flowering seems to be received by all axes at the same time so that there are approximately the same number of nodes to the first flower on branches as on the main stem (Fig 5).

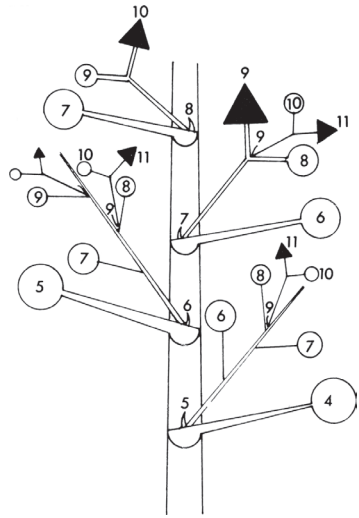

Figure 5. Schematic representation of square on sympoda on vegetative limbs and those of successive sympodia on the main stem. Numerals represent succession of leaf units (true leaves, prophylls, and bracts of squares). Other symbols are the same as Fig. 2 – 4. The plant represented had first flower at node 6. Though all leaf units are not precisely equal amounts of time, the events with equivalent leaf units should happen within a few days.

Flower Development

Reproductive branches are identical to vegetative branches throughout the development of the first true leaf. At that point the development diverges in that a whorl of three bracts occurs on the meristematic dome rather than the second true leaf of that axis (Fig. 4). These three bracts can be seen as a modification of the leaf and two stipules of vegetative development. The three bracts surround the meristem of the axis and terminate its vegetative elongation. Subsequently the floral meristem continues with whorls which become the calyx (fused sepals), corolla (sympetalus petals), androecium (with anthers), and gynoecium (pistil of 3-5 carpels with several ovules in each axil placentation) (Fig. 6).

The process of floral development from initiation to blossom takes about 40-45 days. At about the midway point of this process the leaf in which the branch develops begins to unfold and presents itself to sunlight. It is the top-most leaf of the vegetative axis and the flower bud (square) in its axil is at “pinhead” stage. About 21 days remain in the development of that flower before it opens as a white blossom and anthesis and pollination occurs (Fig. 7).

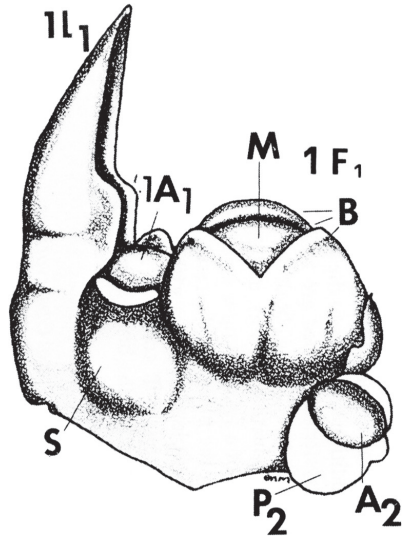

Figure 6. Artistic depiction of the orientation of the meristems which result in the formation of flower buds**.** The node depicted is about five nodes below the apex in the axil of a juvenile true leaf which has not yet expanded. At this point in the development of the branch, the three bracts (B) of the flower ($1F_1$) surround the dome of meristematic tissue which will form the petals and reproductive structures. The formation of the second axillary (A_2) and the second node of the sympodium ($2F_1$) can also be seen. Other symbols are the same as Fig.2-5. Drawing from Mauney (1984) by Edward Mulrean.

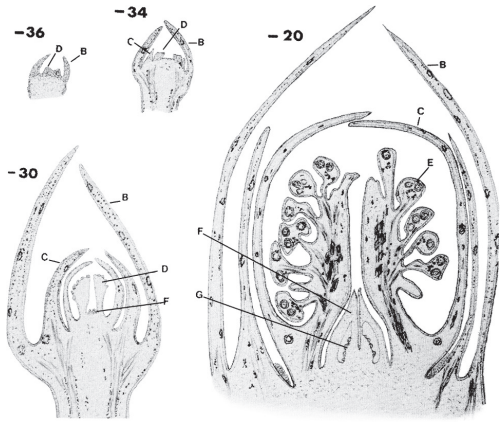

Figure 7. Development of a flower bud. Numbers refer to the estimated days prior to anthesis (Quintanilha *et al.* 1962). B = Bracts; C = Corolla; D = androecium; E = anthers; F = Ovary (locules); G = Ovules. Drawing adapted from Baranov and Maltzev, 1937.

Environment is influential throughout the development of the floral organs. The signal to initiate production of sympodia can be enhanced or delayed by excessively high or low temperatures (Mauney, 1966). Meiosis resulting in pollen and ovule initiation takes place about 20-22 days before anthesis (DBA) (Stewart, 1986). This process is temperature sensitive, so that at high temperatures sterile pollen may result (Meyer, 1969; Fisher, 1975). It is also affected by certain herbicides (including Glyphosate) if applied during this time frame (Yasour, *et al.*, 2006).

On the day the flower matures and opens (anthesis), the anthers dehisce, pollen is deposited on the stigma, it germinates, and pollen tube growth begins. Pollen tube growth is sensitive to high temperatures (Snider, *et al.* 2011) which may result in poor fertilization and reduced seed number. Fertilization of the egg and endosperm occurs about 12 to 30 hours after anthesis (Stewart, 1986). Embryo growth begins about 4 to 5 days after anthesis (Quintanilha, *et al.* 1962).

The embryo sac expands rapidly after anthesis driven by the expanding endosperm. The ovule and developing boll reaches 90% of mature volume about 20 days post anthesis (DPA).

Growth of the embryo lags behind that of the ovule and endosperm, but follows a sigmoid growth pattern (Fig 8). In early growth the embryo is nourished by the endosperm, which becomes cellular and is absorbed by the cotyledons as they mature. At about 24 DPA the boll reaches mature volume, and the cotyledons fill the ovule. (Leffler, 1976) Mature boll weight occurs at about 35 DPA, though environmental stresses may delay boll opening for several weeks.

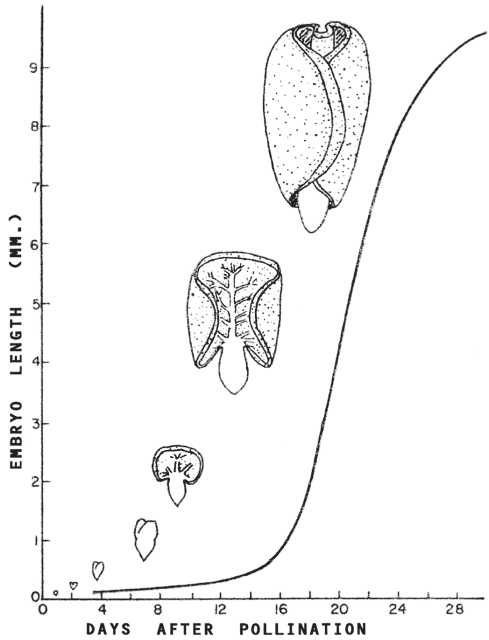

Figure 8. Growth of the cotton embryo. Sigmoidal curve is a plot of length of the embryo against age from pollination. Illustration shows degree of differentiation at various stages of development.

Vascular Connections of the Boll

The developing cotton fruit receives water and nutrients from subtending leaves and the rest of the plant in an elaborate manner related to the vascular network of the stem and connections of individual bolls. Brown (1968) used ^{14}C-labelled sucrose to show that leaf assimilate moved in well-defined strands of phloem related to the 3/8 phyllotaxy of the plant. He found the pattern of translocation down the plant occurred mainly to sympodia which arose on the same side of the main axis.

The developing boll receives its water mainly from the phloem and not the xylem (Van Iersel, *et al.* 1994). They presented evidence that xylem vessel to bolls to not mature until after the three-week period of rapid increase in boll size. No significant apoplastic water moved into developing bolls during the first three weeks after anthesis indicating that that xylem was not the main source of water for the expansion of the young fruits. These authors (Van Iersel, *et al.* 1995) also used specific phloem tracers to show that phloem was functional in the capsule wall and central column of the boll from the early stages of development. Phloem was the major source of water for the developing bolls. The isolation of the apoplast from the rest of the plant explained why cotton fruits had higher water potential than subtending leaves (Trolinger, *et al.*, 1993) and were less sensitive to drought (Van Iersel and Oosterhuis, 1996).

Fiber development has been described in an excellent article by Delanghe (1986). In summary, fiber elongation begins in certain epidermal cells of the ovule on 1 DPA. Elongation continues for about 20 DPA during which stresses have an effect on fiber length. At 16 DPA secondary thickening begins and environmental factors affect fiber strength and maturity.

Four Bract Squares

Certain environmental conditions cause the plant to produce four-bract squares instead of the common three-bract flower bud. If we interpret the typical 3-bract square as a modified leaf and two stipules, then it is easy to interpret the fourth bract as a modification of the second leaf on the branch. What happens is that in conditions where the plant is in transition from vegetative to flowering the meristematic dome on which the three bracts arises is not consumed by those bracts and continues its activity as if were making the next node on a vegetative branch. This modified leaf becomes the fourth bract (Fig 9). In extreme cases that modified leaf (bract) may have modified stipules and the flower bud may have five or six bracts.

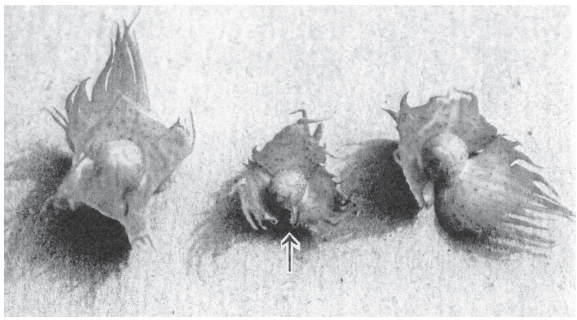

Figure 9. Photograph of three and four bract squares. The square on right is typical three-bract square. The square on left has four well form bracts. The fourth bract (arrow) on the square in the center is not well formed and the air gap produced as it expands can allow thrips to enter and feed on the unprotected androecium (20 DPA Fig.7).

Morphologically these extra bracts do not cause abnormalities in fruit development, but they do present an opportunity for insect damage. Because the fourth bract is usually not well formed, it does not seal tightly against the other bracts, and structural gaps appear as the bud matures. These gaps afford an opportunity for thrips (which are always present) to enter the square while the petals are not yet protecting the developing ovary and androecium. Feeding of thrips on these structures will cause the square to abort (Mauney and Henneberry, 1984).

The well formed thicket of young leaves, internodes, and developing squares that comprise the apical growing point above the youngest expanding leaf of the healthy plant is too tightly matted to be invaded by casual feeders such as thrips. At the point when the leaf of a given node begins to unfold and expand, the square in its axil is approximately three weeks before anthesis (20 DBA, Fig 7). At this stage the petals have not yet expanded sufficiently to envelope the ovary and androecium. The interlocking serrations of the bracts ordinarily prevent thrips from these tissues. However, the fourth bract does not interlock with the others, allowing thrips to introduce bacteria which trigger a soft rot, causing the square to abort.

Reversion to Vegetative Growth

Early fruiting cultivars are sometimes subjected to excessive temperature and humidity after they have set the first fruiting branch. These extreme conditions can lead to production of vegetative branches after the development of sympodial branches has commenced. It is no longer rare to have vegetative branches develop at nodes above the lowest sympodia. In the monsoon climates of India and Pakistan this reversion has always been a concern and limited the season on some cultivars.

Morphologically this phenomenon demonstrates that each axillary primordium receives its own signals for development, and that high temperature is a negative signal for floral development (Mauney, 1966). Fortunately for the productive season these vegetative branches remain relatively rare in the canopy. Development of sterile pollen, discussed above, is also an affect of high temperature-humidity, and is more likely to affect productivity than location of vegetative branches.

Cutout

Vegetative elongation and production of additional nodes by the plant apex is more sensitive to internal and external stresses than is the development of floral buds into flowers. Thus, once flowering commences the progression of the flowers up the stem is usually faster than the addition of new flowering nodes. That is, the rate of flower movement up the stem begins at a rate of about three days per node (0.33 nodes/day) and declines to about four days per node (0.25 nodes/day) as the season progresses, whereas the rate of new node production begins at three days per leaf and declines to ten days per leaf as the season progresses (Fig. 10).

The disparity between these rates means that at some point in the season the plant will run out of sympodia and must pause in the flowering process until the carbohydrate stress produced by boll maturation is relaxed and vegetative elongation of the apex can resume. This process is known as “cutout” and is always seen in a well fruited cotton planting. In locations with a very long productive season, the crop may have time to produce additional productive nodes from this regrowth and thus resume the fruiting cycle after cutout.

The slope of the intersecting trend lines in Figure 10 determines the timing of cutout for any particular cultivar and season. Ordinarily 10 to 15 fruiting nodes are produced by each plant in any 150-day productive season. The trend lines are influenced by the timing of floral initiation, the tendency of the cultivar to partition nutrients to fruit versus vegetative structures, particularly roots, and seasonal stresses (Landivar *et al.*, 2010; Mauney *et al.*, 1978; Patterson *et al.*, 1978).

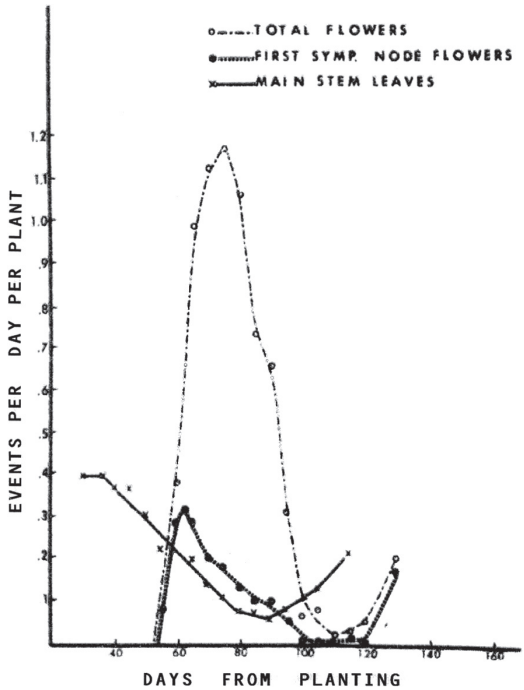

Figure 10. Relationship of production of new flower positions represented by new leaf formation and flowering rate from those positions. "Events per day" is the inverse of "Days between events".

Several crop monitoring programs have been devised to predict the timing of cutout (Kerby, *et al.* 2010, Landivar, *et al.* 2010, Oosterhuis and Bourland, 2008). These programs use the number of main-stem nodes above the uppermost first-position flower (NAWF) as a measure of the approach of cutout. Though in intensively managed plantings, such as those with sub-surface drip irrigation and fertilization, it has been observed that harvestable bolls can be set from flowers very close to the terminal (Mauney, personal observation), it has been usually found that when NAWF is five or less cutout has occurred. That is, few harvestable bolls will be set until the planting has resumed vegetative growth with the production of additional sympodia.

CONCLUSIONS

As a perennial shrub the cotton plant is unique among annual crops in the USA. Because each of the fruiting structures depicted in Figure 5 has an individual time line of development with points of sensitivity to internal and external stresses which differ from all the neighboring fruiting forms, the analysis of effects of stress may be complex indeed. Thus, stress at any time in the entire 150 to 200 days between planting and harvest will affect some developing fruit.

Beginning with the transition from production of vegetative branches to sympodial flowering structures the plant demonstrates sensitivity to environment at every stage. The number of bracts in the flower, the viability and growth of pollen, the distribution of nutrients to the bolls, and the continued production of fruiting sites through vegetative growth are all dependent on favorable temperature and moisture conditions.

By the same token the crop has a remarkable ability to "compensate" for stress by maturing fruit before or after the stress. The marvel is that the crop is so resilient and production and is rather predictable.

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Chapter 2

GENOTYPIC DIFFERENCES IN COTTON FRUITING

Randy Wells[1] and William R. Meredith, Jr.[2]
[1]Department of Crop Science, North Carolina State University, Raleigh, NC
[2]USDA-ARS, Stoneville, MS

INTRODUCTION

Upland cotton (*Gossypium hirsutum* L.) is a perennial and started in its long association with man as more of a tree than the growth habit that we presently manage (Hutchinson *et al.*, 1947). The perennial growth habit was a useful survival adaptation due to generally deep rooting and good drought acclimatization (De Souza and Vieira da Silva, 1987). Through breeding selection cotton has been made into "annual types" which have an intensive reproductive development with reduced vegetative production. However, defoliation is still required and the "annual" moniker is still not reflective of its true growth habit.

Fiber yields in the United States of Upland cotton have increased somewhat linearly since 1937 with an average 8.7 kg per hectare (7.8 pounds per acre) increase per year (Fig. 1). Prior to 1937, a long period of little gain in yield was observed starting in 1866, the start of yield recording. Since 1937 two periods of decline or no gain have been witnessed (Table 1.). The first is 1965 to 1980 and was tied to both a narrow genetic breeding base resulting in a lack of new improved cultivars, and the loss of efficacy of available insecticides (Meredith, 2002). The second decline in the 1990s may have been tied to the backcrossing of new molecular genetic modifications into existing cultivars to give herbicide and insect resistance. Backcrossing, while transferring the trait of interest, allows little opportunity for improved yield. There were two periods of yield gain since 1980. The first, from 1981 to 1990, was reportedly due to the introduction of pyrethroid insecticides and new higher yielding cultivars. Presently, there is a gain starting around 2000 showing a yearly gain of 15.2 kg ha^{-1} (13.5 lb/acre).

Genetic gain through cotton improvement has best been determined by testing obsolete and modern cultivars in the same environments. There is a good synopsis of the studies performed by Schwartz (2005) which has been adapted in Table 2. There have been fifteen studies covering the range of years of release from 1905 through 2002. The average gain in yield from these studies was 7.0 kg ha^{-1}. The question is whether these gains were realized through physiological or morphological modifications of new cultivars, or external factors such as pesticide control options.

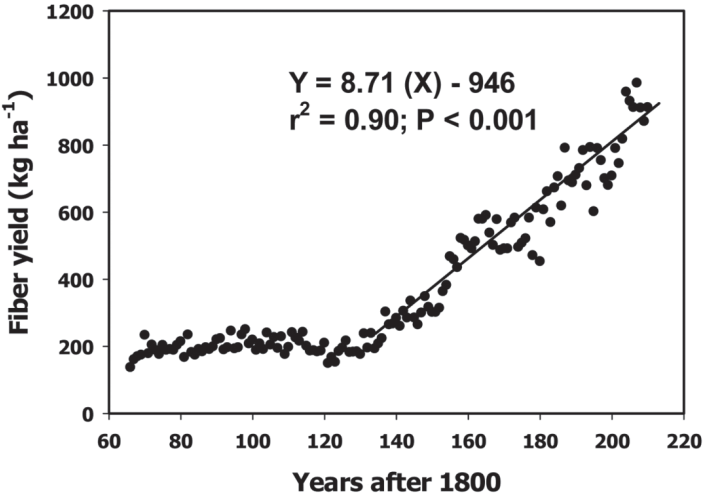

Figure 1. Mean yearly cotton fiber yields in the United States from 1866 to 2010.

Table 1. Regression analysis of yield with year over certain periods of years since 1866.

Period	Regression Equation	r^2	P-value
1866 - 1936	Y = 0.19(X) + 179	0.03	0.175
1937 - 1964	Y = 11.7(X) - 1,348	0.83	<0.001
1965 - 1980	Y = -1.5(X) + 790	0.02	0.59
1981 - 1990	Y = 12.2(X) - 1,583	0.35	0.07
1991 - 2000	Y = -4.6(X) - 1,615	0.05	0.53
2001 - 2010	Y = 15.2(X) - 2,234	0.35	0.07

Table 2. Reported genetic gain determined through comparison of commercially grown obsolete and modern Upland cotton cultivars in the same tests.

Time span of cultivar release†	Genetic Gain (kg ha^{-1} yr^{-1})	Number of cultivars	Reference
1945-1978	10.5	9	Culp and Green (1992)
1922-1962	10.2	13	Bridge *et al.* (1971
1910-1979	9.5	17	Bridge and Meredith (1983)
1937-1965	9.0	8	Meredith *et al.* (1997)
1939-1979	9.0	9	Bassett and Hyer (1985)
1905-2002	8.7	9	Schwartz and Smith (2009)
1937-1974	7.2	6	Hoskinson and Stewart (1977); Culp and Green (1992)
1905-1978	6.8	12	Wells and Meredith (1984b); Meredith *et al.* (1997)
1918-1982	5.6	12	Bayles *et al.* (2005)
1938-1993	5.3	38	Meredith (2002)
1983-1999	3.9	23	Meredith (2002)
1918-1982	3.7	12	Bayles *et al.* (2005)
1984-1993	1.5	8	Meredith *et al.* (1997)
Mean	7.0		

† Approximate range of years cultivars in the tests were released. Adapted from Schwartz (2005).

OBSOLETE VS. MODERN APPROACHES

Dry Matter Partitioning

Wells and Meredith (1984a) examined twelve cultivars covering seven decades of breeding effort, six each from the Deltapine and Stoneville breeding programs. They found that modern cultivars partition greater dry matter into reproductive growth than vegetative growth (Fig. 2). At the last harvest date the new, intermediate and old cultivars displayed a mean reproductive-to-vegetative ratio of 1.0, 0.78, and 0.70 kg reproductive weight/kg vegetative weight, respectively. The modern cultivars generally produced a maximal vegetative dry weightwhich was smaller and occurred earlier chronologically than their previously released counterparts. The same trend was found in flowering, with the modern cultivars producing more early white flowers than the obsolete cultivars, which continued producing a greater number of flowers later in the season (Fig. 3). Modern cultivars also generally produced a greater number of smaller bolls with a higher lint percentage (Wells and Meredith, 1984b). In a subsequent study, five obsolete cultivars, five popular and high yielding cultivars from five cotton breeding businesses, and 15 advanced lines from five cotton breeding establishments were examined for growth and yield (Meredith and Wells, 1989). The obsolete cultivars yielded 24% less than the twenty modern genotypes. As before, the obsolete cultivars produced more vegetative mass with respect to reproductive mass with the obsolete, current, and future genotypes displaying reproductive-to-vegetative ratios of 0.80, 1.14, and 1.17, respectively. Regression analysis of the relationship between yield and either boll weight/total dry weight or reproductive/vegetative ratio for the 20 modern cultivars were positive and significant, indicating that alterations in dry matter partitioning continued in ongoing breeding programs. These alterations in dry matter partitioning in more modern genotypes are due to indirect effects from selection for higher yield since no breeding programs use harvest index as a selection criterion.

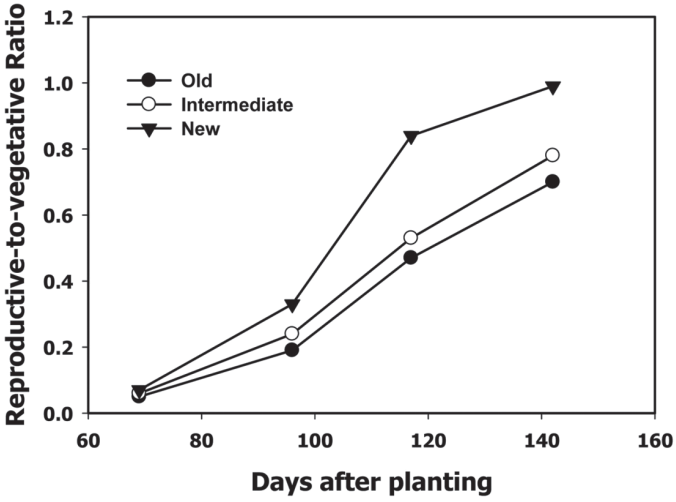

Figure 2. Mean reproductive-to-vegetative ratio occurring at different dates for the cultivars grouped by their year of release and planted on 26 Apr. 1982 (new = 1950 to present; intermediate = 1920 to 1950; old = 1900 to 1920). Adapted from Table 6 of Wells and Meredith (1984b).

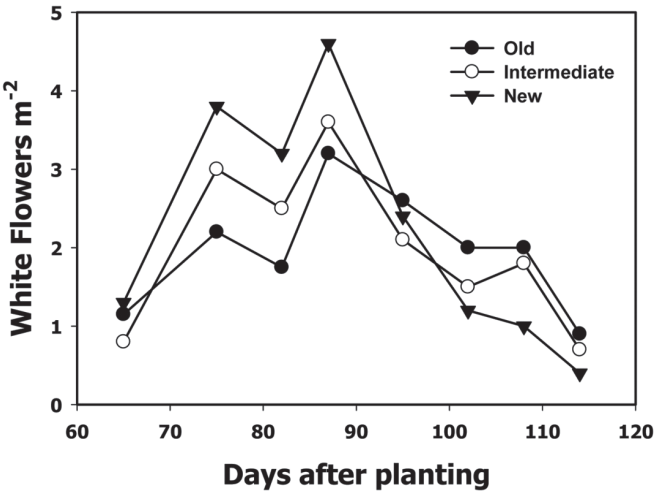

Figure 3. Mean white flower counts determined at various dates for the cultivars grouped by their year of release and planted on 26 April 1982 (new = 1950 to present; intermediate = 1920 to 1950; old = 1900 to 1920). Adapted from Figure 2 of Wells and Meredith (1984a).

Effect of Plant Density

Schwartz and Smith (2008) examined nine current and obsolete cultivars grown at five plant densities. The plants were spaced 3 by 3 m, 2 by 2 m, 1 by 1 m, 1 by 0.3 m, and 1 m by 0.07 to 0.1 m (commercial density). Genetic gain was highest in the commercial, 1 x 0.3m, and 1 x 1m spacing with gains of 8.7, 8.2, and 7.1 kg ha^{-1} yr^{-1}. Lower gains were made in the two largest and least competitive plant interplant distances. They suggest that gain in yield may be due to an increased tolerance to interplant competition and not only yield alone. Their data also support the idea that yield increases have been primarily due to increased boll retention and less from increased lint percentage.

EARLINESS

Movement of the boll weevil (*Amthonomus grandis* Boheman) into the USA occurred in the late 19th century. The goal of cotton management was to hasten crop maturation before boll weevil pressure became too severe (Buie, 1928). Prior to this time, the need for earlier maturity or earliness of cotton was little appreciated or desired. Earliness in a physiological sense has been defined as the percentage of the total fiber yield that is produced by the first harvest. Alternatively, Munro (1971) defined it as the shortest time required to produce an adequate yield. Both definitions are extremely vague on providing an exact length of time for maturation. More recent studies have defined measures of earliness as time to first square, time to first flower, time taken to mature the node of the first boll, and the plant region of greatest fiber production. Bange and Milroy (2004) reported that the attainment of crop maturity (60% open bolls) was related to when the fruit growth rate per unit area was equivalent to the crop growth rate (total dry weight per area per day). They suggested that a key trait related to earliness is the timing of the start of reproductive growth tied with the subsequent development of the demand for dry matter. In another study, they found that earlier maturing cultivars produced more dry matter due to great

radiation use efficiency and light interception (Bange and Milroy, 2000). In addition, the greater photosynthetically active radiation (PAR) interception was due to larger canopies and not altered canopy light extinction coefficients.

The industry convention is that each flowering interval occurs at 3 days vertically within the plant and at 6 days horizontally along branches as reported by McClelland (1916). Bednarz and Nichols (2005) found these same respective intervals to be 2.5 and 3.8 days when averaged over nine cultivars and three years. The horizontal flowering interval (HFI) was 2 days shorter than previous reports and different between earlier and later maturing cultivars, suggesting that this attribute has been influenced through the quest for early crop maturity. There was also a difference in boll maturation period and the percentage of bolls produced at lower main-stem nodes. Jenkins *et al.* (1990) reported that newer, earlier maturing cultivars had significantly greater fiber located at nodes 6 through 8 than the older Stoneville 213. Figure 4 is a graph of an early cultivar (STV 213), a more modern cultivar (DPL 50) and the cultivar with the highest yield (DES 119). Both DPL 50 and DES 119 showed greater fiber at lower nodes than STV 213. In addition, DES 119 maintained greater fiber yield through node 13 when compared to STV 213.

Bednarz and Nichols (2005) suggested that the three most viable strategies to attain earlier maturity useful in breeding programs are lessening the horizontal flowering interval, shortening the boll maturation period, and selecting for longer sympodial branches at lower main-stem nodes. Similarly, Hood (1984) found four key components as having an effect on the time required to attain maturity. These were a combination of morphological characteristics affecting plant stature and the initiation of reproductive development, genetic potential for enhanced flowering rate, shorter boll maturation period, and earlier attainment of crop maturity.

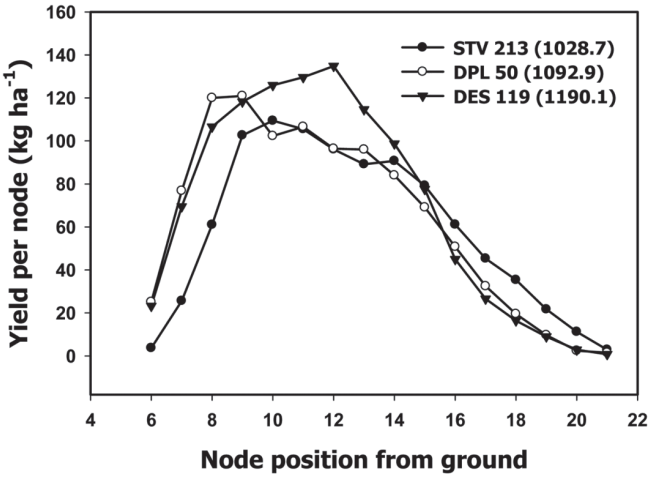

Figure 4. Fiber yield per node for Stoneville 213 (STV 213), Deltapine 50 (DPL 50), and Delta Experiment Station 119 (DES 119) averaged over two years. Adapted from Table 1 of Jenkins *et al.* (1990).

Genetics of Earliness

Godoy and Palomo (1999a) investigated the inheritance of certain phenological and morphological variables contributing to earliness of seven early genotypes. Days to first flower (DFF), days to first open boll (DFOB), node of first fruiting branch (NFFB) and plant height were negatively correlated to vertical flowering index (VFI), total blooms, maturity index, and the percentage of seedcotton at both the first and second harvests (Table 3). Significant dominance effects were noted for NFFB, plant height, VFI, and HFI. Additive effects were greater than dominance effects only for DFF, DFOB, and HFI. The strength of additivity is reflected in the heritability estimates, of which only plant height, DFF, DFOB, and HFI exceeded 0.25. Fiber yield was not correlated with early season measurements of earliness. The general trend found was as maturity became earlier, fiber yield decreased (Godoy and Palomo, 1999b).

The data of Bridge and Meredith (1983) do not show the same trend. Plotting the yield and percent lint in the first harvest from their Table 1 results in the relationships found in Figure 5. The relationship between yield and year of cultivar release showed a 9.46 kg ha^{-1} increase per year. In addition, percent lint at the first harvest was significantly related to year of cultivar release with a 0.18% increase per year ($r^2 = 0.47$; $P = 0.003$). In fact, the relationship between fiber yield and percent of lint in the first harvest was highly significant with nearly 30 kg ha^{-1} increase in yield for every percent increase in first harvest lint ($Y = -1609 + 29.6(X)$; $r^2 = 0.60$**). Guo *et al.*, (2008) utilized molecular markers to locate quantitative trait loci (QTLs) for node of first fruiting branch (NFFB). They found three significant QTLs mapped to chromosomes 16, 21 and 25. Four markers accounted for 33% of the variation in NFB.

Table 3. Phenotypic correlation among various earliness components examined by Godoy and Palomo (1999).

Component	DFF	DFOB	NFFB	Plant height
Days to first square	0.85**	0.71**	0.23**	0.30**
Days to first flower (DFF)	-	0.77**	0.26**	0.37**
Days to first open boll (DFOB)	-	-	0.31**	0.38**
Vertical flowering index	-0.48**	-0.29**	-0.11	-0.30**
Horizontal flowering index	0.08	0.13	0.04	0.26**
Total blooms	-0.59**	-0.52**	-0.27**	-0.28
Maturity index	-0.46**	-0.60**	-0.29**	-0.24**
Boll maturation period	0.20*	0.34	0.17	0.13
Node of First fruiting branch (NFFB)	0.26*	0.31**	-	0.34**
Plant height	0.37*	0.38**	0.34**	-
Percent harvest 1st	-0.58**	-0.72**	-0.34**	-0.27**
Percent harvest 2nd	-0.49**	-0.61**	-0.24**	-0.21**

*,** Significant at 0.05 and 0.01 probability levels, respectively.

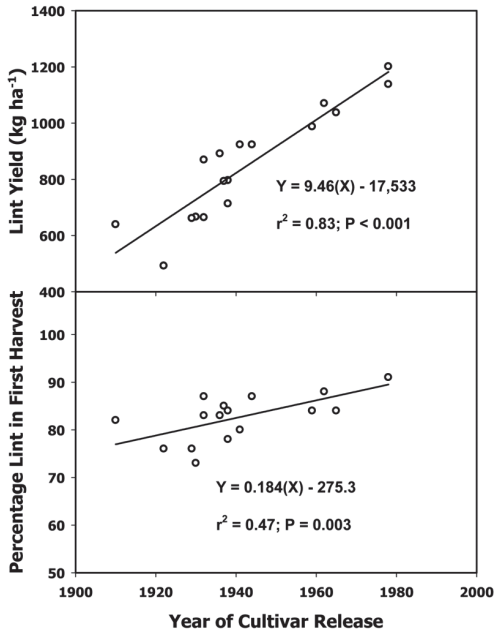

Figure 5. Fiber yield and percentage of fiber present at the first harvest for obsolete and modern cultivars grown in the Mississippi Delta in 1978 and 1979. Adapted from Table 1 of Bridge and Meredith (1983).

PHOTOPERIODISM

While modern cultivars have little or no photoperiod response, any discussion of genetic differences in fruiting would be remiss without mention of its existence. Stephens (1975) found no primitive forms of *G. barbendense* L. or *G. hirsutum* L. that flowered during the long days of summer of temperate latitudes through nearly twenty years of research. In 1960, a collection of perennial forms of *G. hirsutum* L. lines were grown near Cienfuegos, Cuba. Most accessions did not flower until November, when the daylengths were 11.5 h long. Only 18 of 169 accessions flowered before 15 October. The bulk of the accessions showed a classic "short day" flowering response. A majority of the accessions in the USDA cotton germplasm collection are short-day, photoperiodic plants and present a major obstacle to their use in breeding programs.

Zhong *et al.* (2002) used four generations of backcrossed, selected day-neutral flowering plants and compared them with the day-neutral parent and the photoperiodic parent. More of the amplified fragment length polymorphism molecular markers were found from the day-neutral parent than were found from the photoperiodic parent. Day-neutral flowering was developed in the crosses. Their data suggested that after one backcross to the accession parents, day-neutral populations could be selected for use to gain greater genetic diversity in breeding programs. Guo *et al.* (2009) conducted quantitative trait loci (QTL) analysis in two F_2 populations from

crosses between day-neutral Deltapine 61 and two photoperiod sensitive *G. hirsutum* L. accessions. Each population had one or two QTLs that explained 15.9 to 63.5% of the phenotypic variation for node of the first fruiting branch (NFB). For photoperiod sensitive accessions in which low NFB is controlled by a relatively small number of QTLs, introgression of day-neutral genes into them should be fairly easy. They did observe linkage drag and suggested that it could be due to the complex nature of flowering initiation. Zhong *et al.* (2002) found that day-neutral derivatives of photoperiodic accessions carried more alleles from the day-neutral parent than from the accession parents.

LEAF MORPHOLOGY AND FRUITING

An extensive genetic survey of New World cottons by Stephens (1945) showed that leaf shape is controlled by a single allelomorphic series having a minimum of four members namely, super okra (L^S), okra (L^O), Sea-Island (L^E) and normal leaf (l). Meredith (1983, 1984, and 1985) reported between 0 and 4% loss in fiber yield due to the okra-leaf trait. Wilson and George (1982) found an 8% reduction due to okra leaf. Landivar *et al.* (1983) reported a 5% yield reduction due to okra leaf and suggested that okra-leaf genotypes do well in more optimal growth environments but perform less well than normal leaf in adverse environments. These lower yields are despite the greater reproductive structures that are found with okra-leaf as compared with normal-leaf (Wells and Meredith, 1986). At peak flowering, the okra-leaf had 48 and 81% more white flowers than the normal-leaf in an early and late planting date, respectively (Fig. 6). There was a positive curvilinear relationship between the maximum number of immature bolls and the percent boll abscission. While no significant leaf type effect was found, the data indicated that producing greater boll numbers led to greater abscission.

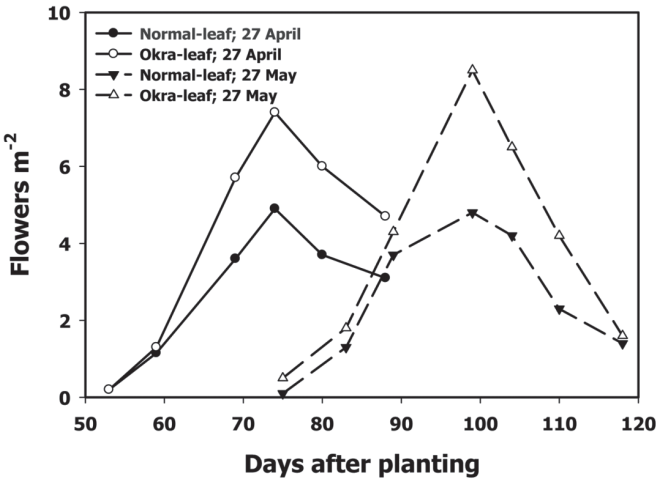

Figure 6. White flowers counted at various dates of normal- and okra-leaf genotypes planted on either 27 April or 27 May 1983

Thomson (1994) reported success of okra leaf commercial cultivars grown in Australia. He utilized a system of breeding involving a wide genetic base with frequent crossing to different types of cotton. His efforts resulted in about 50% of total Australian cotton plantings for the eight-year period of 1986 to 1993. These results indicate that for each mutant trait, there is an identifiable niche where a particular trait has an advantage and can be very successful. When grown in the USA, the Australian okra cultivars were acceptable but not as much as in the Australian management-environment. Meredith (unpublished data) found that the Australian okra-leaf cultivars' leaf area was the same as that for Mid-South cultivars.

Since most okra-leaf cotton genotypes are produced using the backcross method, Meredith and Wells (1986) used a direct selection technique to create okra-leaf and normal-leaf populations. Results from three separate experiments implied that in certain populations there was genetic potential to produce okra-leaf lines with superior yield ability than their normal-leaf counterparts. Similarly, sub-okra lines were shown to yield higher than normal-leaf by 4.8 (Meredith, 1984) and 3.0% (Meredith and Wells, 1987), especially in better growth environments that allow greater plant stature. The sub-okra trait is not expressed at Stoneville, MS until the onset of flowering (~node 10-12). Therefore, during the period prior to canopy closure the normal leaves would be present and lend to greater light interception. Thereafter, sub-okra leaves would be produced and allow greater light penetration to lower canopy positions but nearly attenuated at ground level. Wells *et al.* (1986) found that integrated canopy photosynthesis was negatively associated with light penetration to ground level measure on five different dates over two years. Sub-okra canopies had photosynthesis rates comparable to normal leaf over both years.

YIELD AND YIELD COMPONENTS

Genetic association of yield with yield components and fiber traits has long been described to be due to linkage or pleiotropy (physiology). If linkage was the cause of genetic association, frequent crossing within segregating populations and selection should reduce the negative association. In a review of earlier cotton quantitative genetics, Meredith (1984) reported on four genetic studies where genetic correlations were estimated. More recently, Meredith (unpublished data) determined the genetic correlation involved in 56 year-location environments and 98 genotypes. These tests were conducted from 2001 through 2007. The genetic correlation of yield with lint percentage, fiber length, fiber strength, and micronaire for these two studies may be found in Table 4. Considering that the two studies involve different genotypes grown in different management-time periods, the trends of genetic correlations are very similar. Fiber length and strength are still negatively correlated with yield. The positive genetic correlation for micronaire is not generally desired in the Mid-South as higher micronaire has large discounts. In other regions, such as Texas, higher micronaire is desired as the cotton in that area is frequently discounted for being too low. The search goes on to determine the physiological and genetic casual factors for the negative yield-fiber quality relationships.

Table 4. Genetic correlation of yield with lint percent and three fiber traits.

Fiber trait	Meredith (1984)	Meredith *et al.* (2011)
Lint Percent	0.81	0.587
Fiber Length	-0.25	-0.556
Fiber Strength	-0.51	-0.526
Fiber Micronaire	0.50	0.464

SUMMARY

To the casual eye, today's Upland cotton cultivars probably do not seem too far removed from those of a century ago. The truth is the new cultivars are very different in a number of ways. They yield much more, with new cultivars producing approximately 700 kg/ha more fiber than the cultivars in the 1930s. These new cultivars have realized this increase through earlier crop maturity, greater reproductive dry matter partitioning, and smaller but more efficient vegetative canopies than found in obsolete cultivars. During this period fiber quality has remained high despite negative yield-fiber quality relationships. Photoperiodism has been removed and different leaf morphologies may lead to enhanced light utilization throughout the canopy profile. The question is, what will the next century of crop improvement bring to the new cottons of the future?

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Chapter 3

PHOTOSYNTHESIS AND CARBON PARTITIONING / SOURCE-SINK RELATIONSHIPS

William T. Pettigrew
USDA-ARS, Crop Production Systems Research Unit, Stoneville, MS

INTRODUCTION

Photosynthesis is one of the principle biochemical processes underpinning plant growth and development. Because of its basic nature, it is intimately involved with reproductive growth and determining crop yields. Photosynthesis of a crop canopy can be broken down into three components: 1) leaf area development, 2) photosynthetic rate per leaf area, and 3) partitioning assimilates between vegetative and reproductive growth, or source-to-sink relationships (Krieg, 1983). The leaf surface area intercepts the solar radiation and allows for the photosynthetic conversion of that radiant energy into chemical energy. This production of chemical energy and the subsequent use of that chemical energy use to fix CO_2 into photosynthetic carbon assimilates constitutes the source side of yield development. The fruiting buds, flowers, and fruit development constitute the reproductive sink side of the yield equation, although other vegetative growing points can operate as secondary sinks. This interplay between the vegetative source and the reproductive sink can influence crop photosynthesis because the capacity for carbon assimilation can be somewhat regulated by the utilization of those photoassimilates in many crops (Krieg, 1983). The scope of this review chapter is to examine the intimate but complex relationship cotton photosynthesis (source) has with flowering and yield development (sink). The complexity occurs because major interactions change as the boll load increases with day length, temperature, and water availability decreasing as the season progress.

LEAF PHYSIOLOGY

The physiological changes a cotton leaf undergoes as it unfurls and expands have been well documented. Similar patterns of peak CO_2-exchange rates (CER) during leaf development were reported by Constable and Rawson (1980a) with the peak CER occurring between 13 to 15 days after leaf unfurling, and Wullschleger and Oosterhuis (1990a) who reported peak CER between 16 to 20 days after unfurling. This peak leaf CER occurred just before the leaf had become fully expanded (Constable and Rawson, 1980a). Both studies demonstrated that once this peak CER was achieved, it was then sustained for a few days before gradually declining as the leaf aged. Pettigrew and Vaughn (1998) also demonstrated that peak chlorophyll fluorescence variable to maximum ratios (Fv/Fm), an estimate of the photosystem two (PS II)

activity, and leaf chlorophyll concentrations occurred during a similar time frame to that seen for the occurrence of peak CER. The slight differences in the timing of the events may have to do with the fact that field plants were utilized by Wullschleger and Oosterhuis (1990a) and Pettigrew and Vaughn (1998), while Constable and Rawson (1980a) utilized plants grown in the controlled environment of a greenhouse. The use of different cotton genotypes in these various studies could also somewhat contribute to these subtle timing differences reported. These observations have led to photosynthetic measurements in most subsequent studies generally being collected on the youngest fully expanded leaf of cotton plant to hit the period when the peak CER was occurring. Many researchers have generally wanted to hit the period of peak leaf CER with their photosynthetic measurement because they are often working under the assumption that any differences they were wanting to detect with their leaf CER measurements would be maximized during the period of peak CER. Measuring the youngest fully expanded leaf would also minimize the confounding factor of leaf age when assessing treatment effects on CER.

CER, LEAF AGE, AND GENETICS

The peak CER is not only determined by leaf age but also by genetics of the cotton. El-Sharkawy *et al.* (1965) reported leaf photosynthetic differences among numerous species of *Gossypium*. Beyond these species differences, a next logical place to look for photosynthetic variation is within a species, and particularly, among genotypes that possess different types of leaves. Across multiple studies leaf-type isolines of okra and super okra have exhibited increased lobing and reduced area of the leaves compared to the normal leaf-type (Wells *et al.*, 1986), but they have not shown consistent photosynthetic differences. Leaf-type isolines did not differ in $^{14}CO_2$ fixation (Kerby *et al.*, 1980; Karami *et al.*, 1980) or canopy CER when that photosynthetic rate was expressed on a leaf area basis (Pegelow *et al.*, 1977). However, there was an overall trend in these studies for the okra- and super okra- lines to have numerically higher photosynthetic rates. Wells *et al.*, (1986) reported that super okra and okra leaf-type isolines had reduced integrated canopy photosynthesis when expressed on a ground area basis due to their reduced overall leaf area production and canopy light interception when compared to the normal leaf-type isoline. Peng and Krieg (1991) also reported reduced late season canopy photosynthesis on a ground area basis for okra leaf cotton, but in contrast, they reported elevated canopy photosynthesis when expressed on a leaf area basis for okra leaf lines compared to normal leaf. Perry *et al.* (1983) also reported both okra and super okra leaf-types to have greater leaf CER than normal leaf-type. Similarly, Pettigrew *et al.* (1993b) found that the leaf CER of an okra leaf-type isoline was 22% greater than that of the normal leaf-type isoline counterpart. The leaf CER of super okra leaf-type isoline was also 24% greater than the normal leaf-type. Furthermore, Pettigrew *et al.* (1993b) reported the okra leaf-type line to have greater specific leaf weights (SLW), thicker leaves, and increased leaf chlorophyll concentrations than the normal leaf isoline. These aspects led them to conclude that the okra leaf-type isoline had a greater concentration of the photosynthetic apparatus per unit leaf area than the normal leaf-type isoline.

VARIATION IN PHOTOSYNTHESIS IN NORMAL LEAF-TYPE GERMPLASM

Genetic variation in photosynthesis can also exist within the normal leaf-type germplasm cotton lines. Rosenthal and Gerik (1991) reported that Upland (*Gossypium hirsutum* L.) cultivar Acala SJ-2 had a higher radiation use efficiency than either DPL 50 or Tamcot CD3H. Normal leaf-type genotypes that differed in their source-to-sink ratio were also found to differ in leaf CER by Quisenberry *et al.* (1994). They reported that leaf photosynthesis increased as the source-to-sink ratio was decreased. Furthermore, using a collection of 18 diverse normal leaf-type genotypes, Pettigrew and Meredith (1994) documented significant genotypic variation in leaf CER. Pettigrew and Meredith (1994) also observed a significant positive correlation between leaf CER and leaf chlorophyll concentration (0.768) and SLW (0.568). Beyond the *hirsutum* material, genetic variation in leaf CER also exists in the Pima (*Gossypium barbadense*) germplasm. Cornish *et al.* (1991) demonstrated that modern Pima lines had higher CER and increased stomatal conductance (g_s) than older, more primitive lines. They attributed the yield increases seen with the modern lines to the increased CER and g_s. In follow-up studies, Radin *et al.* (1994), Lu *et al.* (1994), and Lu and Zeiger (1994) indicated that yield improvements in modern Pima lines were associated with improved heat tolerance due to superior g_s and smaller leaf size.

VARIABILITY IN CER MEASUREMENTS

Another factor that can cause variability among leaf CER measurements is the decline in photosynthesis when measured on leaves during the afternoon compared to morning photosynthetic rates, even when measured under comparable sunlight conditions. A handful of studies have documented lower afternoon photosynthetic rates in both Upland (Pettigrew, 2004; Pettigrew and Turley, 1998; Pettigrew *et al.*, 1990) and Pima (Cornish *et al.*, 1991) cotton. Physiological reasons for this afternoon decline are still being debated. Processes that have been implicated in this afternoon decline for various plant species include: 1) high temperature stress (Baldocchi *et al.*, 1981; Perry *et al.*, 1983), 2) photoinhibition after exposure to the intense solar radiation encountered at solar noon resulting in damage to the photosynthetic apparatus (Powles, 1984), 3) a "down-regulation" of the photosynthesis in response to intense light conditions without damaging the photosynthetic structure by dissipating the excess absorbed photons as heat (Baker and Ort (1992), 4) end-product inhibition of the photosynthetic process due to the buildup of large carbohydrate levels during the afternoon hours (Nafziger and Koller, 1976; Peet and Kramer, 1980), 5) stomatal closure in response to an increasing H_2O vapor pressure deficit in afternoon hours (Bunce, 1982, 1983; Farquhar *et al.*, 1980; Pettigrew et at., 1990), and 6) nonstomatal photosynthetic inhibition caused by transient and localized water-deficit stress in response to high afternoon transpiration demand (Sharkey, 1984). The complexity of this phenomenon dictate that any or all of the aforementioned processes could be contributing to the afternoon photosynthetic decline observed at any particular time. Furthermore, when the photosynthetic decline actually begins during the day and the degree to which it inhibits photosynthesis is difficult to

predict. For instance, this phenomenon has been observed to be more pronounced under moisture deficit conditions than under well-watered conditions (Pettigrew, 2004). Nonetheless, this afternoon photosynthetic decline must be taken into account whenever making photosynthetic measurements in any study. Researchers would be wise to complete their CER measurements prior to solar noon on most days to avoid this confounding effect.

LEAF CER AND YIELD

Even though theoretically there is an obvious connection between photosynthesis and reproductive growth, it has not always been easy to demonstrate the connection between leaf CER and yield production. Part of the problem lies in the fact that one would be using an individual leaf measurement to mimic a canopy phenomenon. Another problem is that you would be using an instantaneous measurement to mimic what happens during the entire growing season (Elmore, 1980). The perennial nature of cotton further complicates the issue because there has to be appropriate partitioning of the increased photosynthate into reproductive growth rather than vegetative growth to impact yield. Wells and Meredith (1984) were able to demonstrate that improved reproductive partitioning was responsible for much of the yield improvements in newer cotton varieties. Confirmation of this photosynthesis-yield relationship has also come from a series of source-to-sink manipulation studies (Pettigrew, 1994). Increasing the amount of sunlight intercepted by canopy leaves through either opening the canopy by pulling back adjacent rows or by placing reflective strips between the rows increased the lint yield by 17% and 6%, respectively. Furthermore, covering the plants with 30% shade cloth reduced the yield by 20%. Eaton and Ergle (1954) and Guinn (1974) also noted that lowering the light intensity resulted in increased boll and square shedding and also lower yields. Shading not only reduced yield but also reduced the fiber strength and micronaire of the fiber compared to the fiber produced under full sunlight conditions (Eaton and Ergle, 1954; Pettigrew, 1995). Increasing the photoassimilate supply by growing the cotton plants under elevated CO_2 conditions also has been shown to increase yields (Krizek, 1986). So yields can be increased whenever the total pool of photosynthetic assimilates is increased, either by increasing the amount of solar radiation that is intercepted by the canopy or by elevating the level of CO_2 the plants are exposed to during growth. By averaging the leaf CER over multiple measurements during the boll development period, Pettigrew and Meredith (1994) were able to demonstrate a direct connection between leaf CER and yield development for a diverse group of 18 genotypes. While increased photosynthesis can be a component of yield increases, it does not, in and of itself, guarantee a yield increase. The extra photo-assimilates produced with elevated photosynthesis must be directed to the reproductive structures rather than any of the potentially competing vegetative sinks, such as roots, stems, branches, or new leaf growth.

SINK STRENGTH AND PHOTOSYNTHETIC PERFORMANCE

Documenting the effect that sink strength can exert upon photosynthetic performance for cotton is a complex issue. This relationship is generally defined as a function of the source activity (photosynthesizing area × photosynthetic rate) and sink activity (number of actively growing sinks X the dry matter incorporation rate) (Krieg, 1983). It is simple to believe that as the strength of the to-

tal sink activity increases that there might also be a concurrent increase in the total source activity to feed the growing sink demand. Finding evidence to support this assumption has proven difficult. The results from multiple studies can be confusing to interpret and at times appear contradictory in nature. On the one hand, Quisenberry *et al.* (1994) reported that single-leaf photosynthesis increased as the source-to-sink ratio decreased, suggesting that the lower amount of source tissue was having to increase production to meet an increased sink demand. The altered source-to-sink ratios in the Quisenberry *et al.* (1994) study were accomplished with genotypes of maturity ranging from very early to very late and by also including a photoperiod sensitive line that would not flower during the growing season at that latitude. In contrast to Quisenberry *et al.* (1994), when fruit removal was utilized to increase the source-to-sink ratio, either leaf photosynthesis was unaffected (Pettigrew *et al.*, 1993a) or the radiation use efficiency was increased (Sadras, 1996). Using insect predation to implement their fruit loss, Holman and Oosterhuis (1999) reported the both leaf and canopy CER were increased when the plants suffered damage from tarnished plant bugs (*Lygus lineolaris* Palisot de Beauvois) and bollworms (*Helicoverpa zea* Boddic). They concluded that the improved canopy CER was due to greater light penetration into the canopy also produced by the insect infestation. Growth regulators can also be used to manipulate the source and sink relationships. Gwathmey and Clement (2010) reported that the growth regulator mepiquat chloride reduced leaf area per plant, while also increasing the number of bolls per leaf area. Reduced leaf area development from mepiquat chloride application was also reported by Hodges *et al.* (1991), but they also found higher gross photosynthesis for the mepiquat chloride-treated plants. Results from Hodges *et al.* (1991) tend to lend support to the Quisenberry *et al.* (1994) findings of increased photosynthesis with decreased source-to-sink ratios. It was primarily a reduction of the source material that decreased the source-to-sink ratios in the Quisenberry *et al.* (1994) work, because the sink size (boll weight plant^{-1}) was not consistently affected by the mepiquat treatments.

SYNCHRONIZATION OF PHOTOSYNTHESIS AND ASSIMILATE DEMAND

One of the reasons for the inconsistency in describing the relationship between photosynthesis and sink size or strength is that the temporal pattern of photosynthetic production for an individual leaf is not totally synchronized with the development and assimilate demand patterns of the nearby fruiting structure. Constable and Rawson (1980a) demonstrated the peak photosynthesis for a main-stem leaf occurred several days prior to anthesis of a 1st position bloom on the adjacent sympodial branch. During the filling and development of the boll, the photosynthesis of that leaf had already decreased substantially. The subtending leaf to that flower was in better synchronization as its peak photosynthesis occurred near anthesis, however, but it was not able to supply all the carbon needs of that developing boll by itself (Ashley, 1972; Constable and Rawson, 1980b). Utilizing a carbon budget model, (Constable and Rawson, 1980b) were able to calculate that considerable remobilization and movement of carbon between nodes and into and out of storage pools was needed to support the developing boll load. Wullschleger and Oosterhuis (1990b) confirmed this out of step timing between leaf photosynthesis and boll demand for assimilates with field grown plants. Constable and Rawson (1980b) further predicted that as the cotton canopy approaches cutout, a period of slowing vegeta-

tive growth occurs due to increased assimilate demand from the developing bolls, thus, the overall canopy photosynthesis would decline due to the aging and declining photosynthetic capacity of the individual leaves. This prediction was confirmed by the research of Wells *et al.* (1986) and Peng and Krieg (1991) who demonstrated that the canopy photosynthesis declined as the canopy aged. Unfortunately, this declining canopy photosynthesis is occurring during a period of greatest assimilate demand from the developing boll load. These results further confirm that the lack of synchronicity between assimilate demand of an individual developing fruit and the assimilate production from the nearby leaves ultimately causes the demand for photoassimilates by the developing boll load to be out of phase with the level of assimilate production by the cotton canopy.

REASONS FOR LACK OF SYNCHRONY

Part of the reason for the lack of synchrony between leaf photosynthetic potential and boll carbon assimilate demand is that the plant essentially cannibalizes its leaves by remobilizing its N-based components to feed the N needs of the growing bolls. Wells (1988) was able to clearly demonstrate this property by showing that, for all but the upper most leaves, the concentrations of chlorophyll, soluble protein, and ribulose bisphosphate carboxylase/oxygenase (Rubisco) decreased as the leaves advanced in age. Furthermore, he also demonstrated that leaves emerging during vegetative growth had higher levels of the N components when the leaves first reached full expansion than leaves that emerged during fruit development, when the reproductive sink for N assimilates is growing. By assessing the photosynthetic performance on leaves of plants from different planting dates on a mid-August measurement date, Pettigrew *et al.* (2000) were able to demonstrate that leaves from early planted plants had reduced leaf CER, soluble protein, and Rubisco activity compared to leaves from late planted cotton on that date. In contrast, they found that neither leaf chlorophyll concentration nor the chlorophyll fluorescence variable to maximum ratio (Fv/Fm) were altered by planting date. They concluded that N remobilization from the leaves to feed the developing boll load initially targets the proteins in the carbon fixation half of the photosynthetic equation and then later goes after the proteins and chlorophyll involved in light harvesting and the conversion of that sunlight into chemical energy.

IDIOSYNCRASIES OF PHOTOSYNTHESIS

Many of the idiosyncrasies intertwined with photosynthesis and crop yield in cotton are related to the fact that cotton is a perennial crop that is grown as an annual. While this perennial nature and indeterminate flowering pattern provides cotton with some flexibility in enduring short-term unfavorable environmental conditions, it also leads to the out of sync fruit development pattern with regards to photosynthetic production and further complicates the defoliation and harvesting process. Maintaining crop canopy photosynthesis for a longer duration might be a desirable pursuit because it could help to increase overall yield production since the level of photosynthetic production would be better timed to fulfill the current reproductive demand. Additional N fertilization has been suggested as a possible venue to maintain canopy photosynthesis because the remobilization of N out of the leaves to support the developing boll load was identified as a component involved in the photosynthetic decline observed late in the

growing season. Bondada *et al.* (1996) were able to demonstrate higher rates of N fertilization increased canopy photosynthesis, delayed cutout, extended the duration of the cotton canopy and increased yields. Unfortunately, N fertilization in cotton can be a complex issue. In the Mississippi Delta, for instance, fertilization rates above 112 kg N ha^{-1} rarely elicit a yield response, but in California with its higher yield potential rates, higher than 112 kg N ha^{-1}, often produce yield increases. The nitrogen requirement for optimal yields is complicated because of the indeterminate growth habit of cotton and the complexity of N cycling in the soil. (Gerik *et al.*, 1998). While higher N fertilization may extend the duration of cotton canopy photosynthesis, it can also have negative consequences because it can create a lusher canopy that is more attractive to insects and cause complications for the defoliation process. Clearly any bolls lost to insect predation would create a disconnect between the higher canopy photosynthesis after additional N fertilization and yield production.

SUMMARY

Logically it would seem to be beneficial if cotton's photosynthetic production was better synchronized the with yield development. At the present time, however, there doesn't appear to be any obvious production technique to improve the timing of photosynthesis and yield production. The perennial aspect of cotton creates additional assimilate sinks and storage pools to ensure sufficient reserves will be available for new growth during subsequent seasons if the crop is not cultured as an annual and dies in the off season. This lack of synchrony between photosynthetic source production and reproductive sink demand creates the need for some assimilates from these secondary sinks and storage pools to be remobilized to support the growing fruit load. Because of this partial dependence on remobilized assimilate, a timing / distribution bottleneck could potentially occur in the assimilate supply / demand function during critical phases of reproductive growth and theoretically limit overall yield production. Conversion of cotton from a perennial plant to an annual plant could theoretically minimize the size or totally eliminate some of these secondary sinks and storage pools, and in the process free up some of those assimilates for further reproductive growth. There would still have to be adequate partitioning of these "freed-up" assimilates to reproductive growth to see any improvement on the yield front. Perhaps in the future, our friends in molecular biology and plant genomics could devote a portion of their efforts toward producing a truly annual cotton plant for the physiologists and agronomists to utilize.

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Chapter 4

NUTRITIONAL REQUIREMENTS OF COTTON DURING FLOWERING AND FRUITING

Ian J. Rochester[1], Greg A. Constable[1], Derrick M. Oosterhuis[2] and Meredith Errington[3]
[1]CSIRO Plant Industry, Narrabri, Australia
[2]University of Arkansas, Fayetteville, AR
[3]University of Sydney, NSW, Australia

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) yields may be limited unless adequate amounts of all required nutrients are accumulated in the plant during its growth. Most soils where cotton is grown commonly have deficiencies of at least one nutrient (e.g. N, P or K) that requires addition of fertilizers to optimise production. There have been some excellent reviews of cotton nutrition (Braud, 1974; Hearn, 1981; Mullins and Burmester, 2010). However, some of those reviews were from studies at relatively low yield levels (<1,500 kg lint/ha) and as such those experiments were conducted under conditions where nutrition was not necessarily the most limiting factor. Those older studies may have been relevant for the cropping system at that particular time and environment, but not necessarily helpful for understanding of nutrition principles under high yield. If water stress, pest damage, disease, soil constraints, unfavorable temperatures, etc are limiting, then real nutritional demands cannot necessarily be developed, particularly considering the importance of redistribution from vegetative to reproductive material during boll development. In this chapter we aim to concentrate on nutrition/physiology during flowering for cotton crops at yields >1,500 kg lint/ha. Under these circumstances the efficiency of nutrient use is improved (Mullins and Burmester, 2010) and the true demands of the crop and fruit can be determined.

Being of indeterminate growth habit, cotton is able to take advantage of conditions when they are favorable, so it is tempting to suggest that cotton might be more tolerant to nutrient stress than determinate species and so nutrition management could be more flexible. But under commercial production, cotton crop nutritional stress costs efficiency and yield to the producer, so a professional approach to cotton nutrition should be one that minimizes deficiencies and toxicities. Thus at high yield levels, managing nutrition is critical for high yield and efficiency as much for cotton as for other crops.

Uptake of nutrients via the roots is governed by nutrient transport to the root surface and absorbed with the water as part of the transpiration stream; or become concentrated in the xylem sap due to a facilitated (protein transporter) or an active uptake process that requires metabolic energy to overcome a concentration gradient (Bassirirad, 2000). Nutrient uptake by cotton is driven by the demand for nutrients from the developing crop, which is regulated by the supply of nutrients from the soil.

Nutrient Uptake by a Cotton Crop

Nutrients are taken up throughout the growing season and in proportion with the demand for nutrients as dictated by the developing crop biomass and boll load. The rates of nutrient uptake increase at flowering through fruiting, and then slow as the bolls mature (Mullins and Burmester, 2010). Nitrogen and K are taken up in greatest amounts – at least 200 kg/ha (Hodges, 1992), while micronutrients such as Cu may have less than 30 g/ha taken up in a season (Fig. 1; Table 1). Using typical uptake curves as depicted in Figure 1, daily rates of nutrient uptake can be calculated (Table 1). While the amounts of each nutrient taken up vary widely, the patterns of accumulation are similar for most nutrients with the timing of peak uptake ranging from day 101 for S to 130 for Fe (mid-flowering stage) (Table 1; Figure 1) and tend to follow the pattern of crop growth, although with higher concentration of nutrients in younger plants (reviewed by Mullins and Burmester 2010).

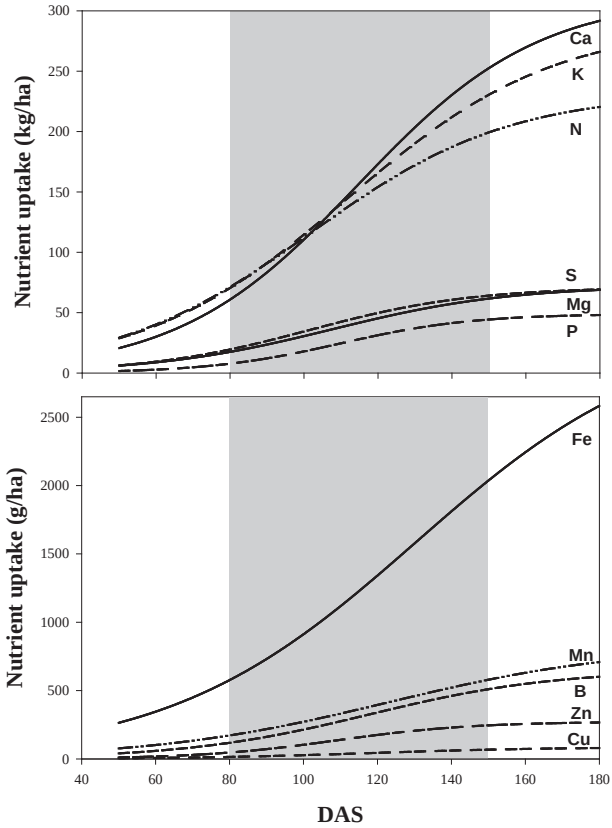

Figure 1. The pattern of nutrient uptake during the growth of an irrigated cotton crop that yielded 2250 kg lint/ha in Narrabri, Australia.

Table 1. Maximum nutrient uptake, rates and timing uptake of nutrients in whole crop (kg for N, P, K, Ca, Mg and S; g for Fe, Zn, B, Cu and Mn). Values are calculated from Figure 1.

	Maximum uptake (per ha)	Maximum uptake rate (per day)	Time of maximum uptake (days from sowing)	Percentage taken up during flowering
Nitrogen	232	2.1	102	55
Phosphorus	49	0.7	110	75
Potassium	312	3.2	115	61
Sulfur	71	0.8	101	63
Calcium	289	2.6	112	55
Magnesium	72	0.7	108	61
Iron	2592	24.0	130	46
Manganese	829	6.5	123	49
Boron	652	6.5	118	60
Copper	77	0.9	119	61
Zinc	272	3.7	109	73

Nutrient Accumulation in Cotton Leaves

Thompson *et al.* (1976) showed that individual leaf N concentration dropped from 6% to 2% as the leaf aged; with the reduction from day 40 to 60 in leaf age being due to redistribution. For a typical leaf the amount exported would be about 50% of leaf N; a similar amount was found by Zhu and Oosterhuis (1992).

Typical declines in the concentrations of most nutrients during the flowering and fruiting periods for high-yielding cotton in Australia are shown in Figure 2. Nitrogen, P, K, Fe, Cu and Zn levels normally decline in leaf tissue as the crop ages (as they are either more mobile or marginal in availability), whereas Ca, Mg, Na, Mn, S and B increase (i.e., not as mobile or in luxury supply). Figure 2 also demonstrates that a critical level of each nutrient can be devised indicating sufficiency or deficiency for each period of crop growth. Declines in the leaf concentrations of N, P, K, Fe, Cu, and Zn (Fig. 2) may indicate redistribution of nutrients from foliage to the developing bolls. Leaf K concentration declines from 2-3% at peak flower to 1% at harvest (Halevy, 1976). The very mobile nature of K, coupled with the ability of many physiological parameters and growth conditions to influence K tissue concentrations, has led to inconsistent reports of critical leaf K values (Kerby and Adams, 1985; Reddy and Zhao, 2005). During flowering, critical leaf K concentrations which impact plant growth, physiology and yield range from 0.60-2.45% (Hsu *et al.*, 1978; Oosterhuis *et al.*, 2003), although narrower ranges have been reported (Baker *et al.*, 1992; Bednarz and Oosterhuis, 1996; Reddy and Zhao, 2005).

Crop modelers use the concept of minimum and maximum concentration of nutrients in plant parts (e.g., Seligman *et al.*, 1975) and use that range to simulate organ growth and development. For cotton leaves, the minimum N leaf concentration for old leaves on a mature crop is about 1.8%; the maximum for young leaves on young plants is about 6.2% (Boquet and Breitenbeck, 2000). Field leaf samples are usually intermediate (Fig. 2).

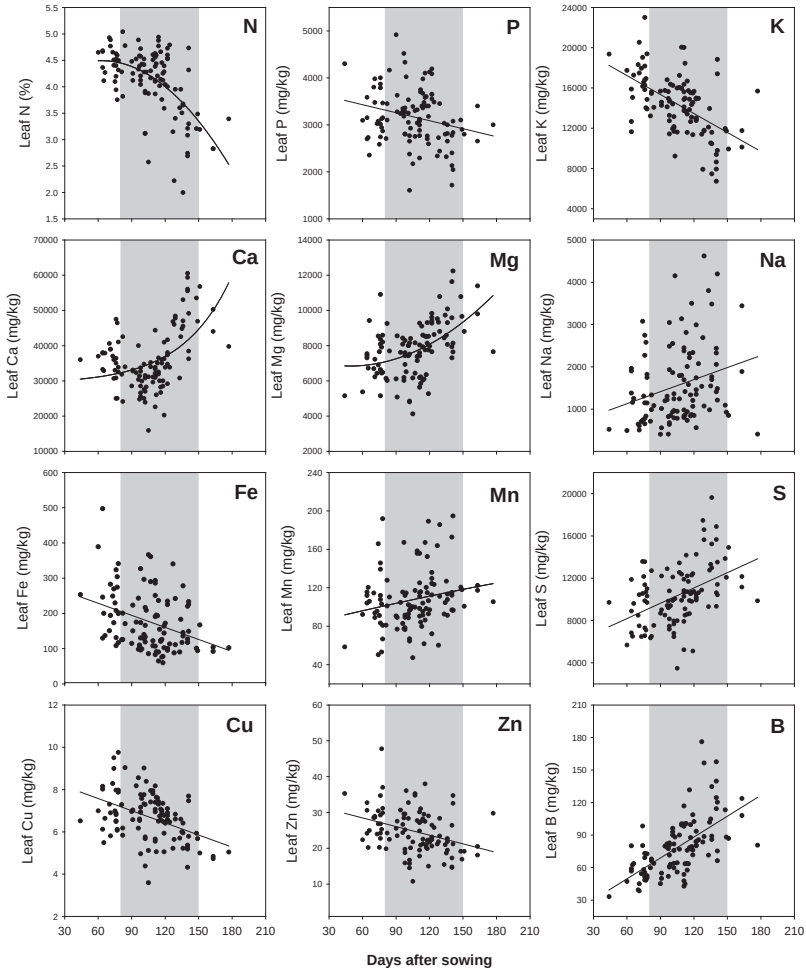

Figure 2. Change in leaf nutrient status during the growth of high-yielding irrigated cotton crops in Australia.

Nutrient Requirement during Flowering/Anthesis

The flower represents a central part of the plant's reproductive growth in which anthesis, pollination, and fertilization occur (Oosterhuis and Jernstedt, 1999). It has been speculated that nutrient imbalances in the flower may cause lower yield and unpredictable year-to-year yield variability (Oosterhuis *et al.*, 2008). These authors documented the mineral composition of cotton flowers, and showed that flower N increased with increasing N fertilizer rates, while P, K, and B decreased. The effect of environmental stresses on flower nutrient content is not known.

Calcium in the pistil of numerous plant species is essential in promoting directional pollen germination and tube growth (e.g. Ge *et al.*, 2009) and ovule fertilization (Faure *et al.*, 1994; Digonnet *et al.*, 1997). During heat stress, potentially damaging reactive oxygen species (ROS) accumulate in plant tissues (Tang *et al.*, 2006), and cytosolic Ca levels have been shown to increase in vegetative tissues (Gong *et al.*, 1998; Jiang and Huang, 2001). Calcium is essential in enhancing the antioxidant enzyme activity required to protect the plant under oxidative stress conditions via ROS scavenging (Gong *et al.*, 1998; Jiang and Huang, 2001), and exogenous Ca application has been shown to enhance antioxidant protection in heat-stressed leaves (Jiang and Huang, 2001). In contrast with antioxidant enzymes, nicotinamide adenine dinucleotide phosphate oxidase produces O_2^- in a Ca-augmented fashion, and is needed to soften cell walls and promote cell expansion during pollen tube growth (Potocky *et al.*, 2007). In a study of high temperature stress on cotton, Snider *et al.* (2010) found that exogenous Ca application had no effect on reproductive thermostability due to poor Ca uptake under high temperature. Under 30/20° C day/ night temperature conditions, exogenous $CaCl_2$ application resulted in an 11% increase in total calcium concentrations, but under the 38/20° C temperature regime, there was no response of total pistil calcium to exogenous $CaCl_2$ application. However, pre-stress Ca content (together with higher antioxidant enzyme activity) in the pistil was higher in thermo-tolerant compared to thermo-sensitive cultivars. They concluded that pre-stress Ca content, together with antioxidant enzyme activity and adenosine triphosphate (ATP) of the pistil may be associated with reproductive thermo-tolerance in cotton.

Nutrient Accumulation in Cotton Bolls

Nutrient accumulation within a single boll shows steady increases in most macronutrients in phase with the increase in boll weight (Fig. 3; also see Leffler and Tubertini, 1976; Constable *et al.*, 1988). Daily rates and timing of accumulation of nutrients into bolls are shown in Table 2. Notably N, P, K, B, Cu and Zn are taken up relatively early but Ca, Mg, S, Fe and Mn tend to accumulate as the boll matures.

Table 2. Accumulation and timing of nutrients in a boll (mg for N, P, K, Ca, Mg and S and ug for Fe, Zn, B, Cu and Mn). Values are calculated from Figure 3.

Nutrient	Maximum uptake per boll	Maximum uptake per day	Time of maximum uptake (days from anthesis)
Nitrogen	111	3.6	19
Phosphorus	21.4	0.71	19
Potassium	103	3.2	19
Sulfur	17.5	0.37	26
Calcium	31.0	0.82	27
Magnesium	17.2	0.45	21
Iron	221	5.6	24
Manganese	111	2.5	22
Boron	118	3.8	18
Copper	30	0.91	19
Zinc	104	3.0	18

Nutrients do not accumulate in the mature lint – it contains 80 to 95% cellulose (Meinert and Delmer, 1977; Beasley, 1979). The boll wall contains the highest amount of K in the boll, between 32 and 60% (Bassett *et al.*, 1970; Kerby and Adams, 1985; Mullins and Burmester, 2010). This is thought to act as a storage although large proportions of the K and Ca (more than 70%) remain in the boll walls and do not move to the seed. Most of the N, P, Mg, Fe, Cu and Zn in bolls end up in the seed (over 60%) rather than boll walls, while Mn and B tend to remain in boll walls. The seed N concentration equates to 3.5% when cotton is fertilized for economic optimum N use-efficiency (Egelkraut *et al.,* 2004; Rochester, 2012).

Cotton is reputed to be less efficient for potassium uptake from soil than other crops (Cope, 1981; Kerby and Adams, 1985; Cassman *et al.*, 1989) although these reports were from soils that had high K-fixation. That characteristic may be due to soil types and climate in *Gossypium hirsutum's* original area of evolution. Additionally, K is required for maintaining cotton fiber cell turgor pressure and so facilitating cell growth (fiber elongation) in cotton - a critical aspect. Dhindsa *et al.* (1975) showed that K and malate could account for 55% of total osmotic potential during cotton fiber elongation. If K is in limited supply during active fiber growth, there would be a reduction in the turgor pressure of the fiber resulting in less cell elongation and shorter fibers at maturity. Also, seen as K is associated with transport of sugars, a likely implication of a K deficiency is an effect on secondary cell wall deposition in fibers affecting fiber strength and micronaire. Indeed, Pettigrew *et al.* (1996) reported reductions in fiber elongation and other fiber quality properties in response to K deficiency. However, few field studies demonstrate that this critical role is reflected in fiber properties: Kerby and Adams (1985) noted that the literature on K deficiency shows relatively small effects on fiber length (e.g. Bennett *et al.*, 1965; Cassman *et al.*, 1990; Pettigrew *et al.*, 1996; Pettigrew, 1999) or no effects (Read *et al.*, 2006). It is likely that a severe K deficiency is needed before an effect on fiber quality is observed. It is possible that K deficiency reduces fruit production to ensure sufficient K for surviving bolls.

Removal of Nutrients

In measurements from high-yielding irrigated cotton in Australia, removal of nutrients in seed cotton is a function of crop yield and differs between nutrients (Table 3). There is good agreement between boll data (Fig. 3) and crop data (Table 3) in that larger proportions of the N, P Mg, Zn and Cu taken up by the crop into vegetative material are redistributed to the developing bolls and removed at harvest (also see Rochester, 2007). The values for crop nutrient uptake removal in Table 3 are a good guide to replace nutrients and therefore to maintain soil chemical fertility.

Table 3. The ranges of each nutrient taken up and the proportion of each nutrient exported relative to that taken up by the crop at three yield levels (from Rochester 2007).

Lint yield (kg/ha)	----------nutrient uptake----------			----------% exported----------		
	1000	**1800**	**2400**	**1000**	**1800**	**2400**
Nitrogen (kg/ha)	63	175	290	66	52	46
Phosphorus (kg/ha)	13	27	41	82	69	60
Potassium (kg/ha)	77	167	250	21	17	15
Sulfur (kg/ha)	10	39	62	42	21	18
Calcium (kg/ha)	71	94	155	3	3	2
Magnesium (kg/ha)	16	36	63	45	34	25
Iron (g/ha)	227	820	1620	40	17	11
Manganese (g/ha)	152	355	655	5	3	2
Boron (g/ha)	75	320	560	22	13	11
Copper (g/ha)	25	52	81	51	38	31
Zinc (g/ha)	58	119	203	99	73	61

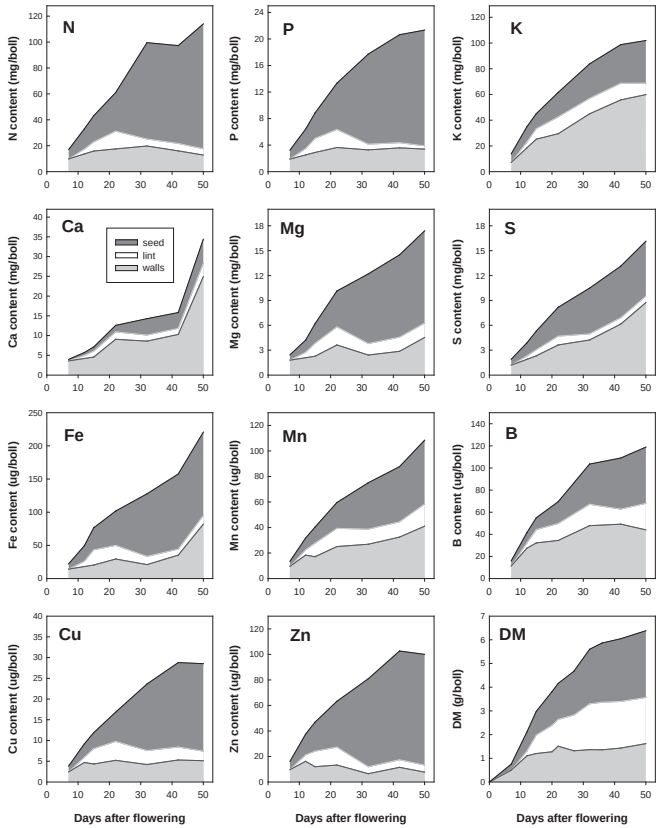

Figure 3. The accumulation of nutrients in bolls that flowered 97 days after sowing at first position on the tenth node from the base of the plant. Narrabri, Australia.

Redistribution of Nutrients - Supply and Demand

Redistribution of nutrients from vegetative to reproductive plant parts is common in crop plants. Determinate grain crops redistribute nutrients during grain filling. For example, Pan *et al.* (1986) showed 50% of the N in maize grain was from N taken up before silking. Dekhuijzen and Verkerke (1984) showed that 83% of N in *Vicia faba* seeds at maturity came from redistribution from vegetative parts present at flowering.

The importance of nutrient redistribution in cotton can be gauged by using the data contained in Tables 1 and 2. Comparing the relative requirements of bolls with total uptake, N and P would be most reliant on remobilization, more so than Ca, Mg, Fe, Mn, B, and S, when nutrient requirement is not limited by soil nutrient supply. Potassium and Zn were intermediate.

In a cotton field study, Zhu and Oosterhuis (1992) found that for a main-stem node segment consisting of the main-stem leaf, three sympodial leaves, the branch and three bolls, about 300 mg N were contained in the three bolls 60 days from appearance of the subtending main-stem leaf (first position boll 29 days after anthesis). The reduction on total leaf N on this branch was about 30 mg N, the majority of which came from the main-stem leaf which lost half its N from day 20 to day 60. Thus, the nominal redistribution of N in this branch segment over that time period was about 10% of boll requirement. Given that canopy boll retention is about 60% (Kerby *et al.*, 1987; Constable, 1991), this data would suggest only 17% of total plant boll N requirement was supplied by redistribution from leaves alone. However other studies show higher redistribution.

Rosolem and Mikkelsen (1989) in a glasshouse ^{15}N study found that leaves were the main source of N after first square. The total leaf N at 120 to 150 days dropped by 200 mg; at the same time the total boll N increased by about 500 mg. Similar distributions of 30-40% N from the vegetative components to the bolls have been reported (Halevy, 1976; Oosterhuis *et al.*, 1983). This would indicate the redistribution accounted for 30- 40% of boll requirement, particularly later in crop development. The proportion of N redistributed was, therefore, not necessarily constant: the N for early bolls could be supplied by current N uptake from the soil; the N for later bolls was more likely to be supplied from redistribution from leaves. By first open boll, most of the N in seeds was from redistribution. Similar results were found by Hocking and Steer (1995) with sunflower.

Although stem reserves can also supply some N through redistribution (Rosolem and Mikkelsen, 1989) and there is high redistribution of N from the capsule wall to seeds (also Leffler and Tubertini, 1976), particularly at the top of the plant (45%), the absolute amounts of N from the capsule wall in particular was small. As N is one of the more mobile nutrients (Fig. 3), other nutrients may have a lesser proportion redistributed.

Redistribution will obviously be affected by nutrient status: a crop deficient in N will not be able to mobilize as much N as a crop with high N status. Leffler and Tubertini (1976) hypothesized that there was a physiological continuum among boll components during development whereby the nutrient content declines throughout the plant and the seeds alone acted as net sinks. Errington *et al.* (2009) showed that the decline in N content of the leaves, stems, petioles, boll walls, bracts and lint may equated to 2/3 the N found in the seed at maturity. Seeds reached

peak dry weight and size at 45 days after flowering but did not reach peak N content until 60 days after flowering (Leffler, 1986).

In cotton, even if redistribution of N accounted for 50% of boll requirements, continued nutrient uptake from the soil is required to develop those bolls. Under high yield levels, the total amount of N required would be substantial, indicating that available soil N and good plant health are required to enable that soil N to be taken up through the boll filling period.

Rochester (2007) indicated large proportions of the N, P and Zn taken up be the crop were redistributed into the developing bolls and removed in seed cotton (Table 3).

Nutrition in Practice - Applying Nutrition Physiology

Mineral nutrient deficiencies can limit the growth and yield of cotton, particularly when they occur during the reproductive phase. For this reason, cotton producers should aim to eliminate the chance of mineral nutrients becoming limiting during the flowering and fruiting period. This can be achieved by analyzing the soil before sowing to assess the limitations of nutrient supply from the soil and to plan the amounts and timing of fertilizer application. During the growth of the crop, leaf tissue analysis can indicate whether the crop is taking up sufficient amounts of nutrients to be corrected by side dressing or foliar application if necessary. Nutrients applied after cutout will not necessarily be utilized effectively by the crop.

Assessing In-Season Nutrient Status

Assessing crop nutrient status with plant tissue testing is becoming more commonplace. While petiole testing for nitrate and potassium concentration has been used for several decades (Tucker, 1965; Kerby and Adams, 1985; Miley and Maples, 1988; Constable *et al.*, 1991), leaf blade testing has the advantage of being used throughout the growing season (Fig. 2). Petiole testing is more dynamic and nutrient concentrations fluctuate in accordance with prevailing weather conditions, making it less reliable with unfavorable weather conditions (i.e., cold shock or heat stress, drought or water logging). Furthermore, petiole testing has been reported to be too variable and not correlated to yield (Oosterhuis and Morris, 1973). Petiole nitrate (Constable *et al.*, 1991) and K concentrations decline quickly, making these analyses less useful through the late-flowering and fruiting period than leaf blade testing. While the smaller decline in tissue nutrient concentrations may make the detection of changes difficult, the concentration of each nutrient can help growers make informed fertilizer management decisions when the ideal level is known for each nutrient at that stage of crop growth.

Other techniques for determining in-season cotton nitrogen status include chlorophyll meters (Wood *et al.*, 1992; Wu *et al.*, 1998) and leaf/canopy reflectance sensors (Raper, 2011; Zhao *et al.*, 2005). Moderate correlations between chlorophyll meter readings and N content have suggested these instruments can determine in-season N status without destructive tissue sampling (Wood *et al.*, 1992; Wu *et al.*, 1998). Techniques for determining in-season nutrient status based on spectral characteristics of deficiencies have received much interest in cotton due to success noted in other crops (Samborski *et al.*, 2009). Research characterizing spectral shifts in cotton

reflectance due to N deficiencies has suggested leaf and canopy sensors have the potential to determine N status in real time (Raper, 2011; Zhao *et al.*, 2005). However, determination of nutrient status from spectral characteristics is not production-ready and few studies have examined cotton spectral reflectance shifts of nutrients other than N (Fridgen and Varco, 2004). Although most spectral research has focused on the spectral characteristics of N deficiencies, the spectral characteristics of other nutrients are beginning to be explored (Fridgen and Varco, 2004).

Fertilizers

A strategy to plan the timing, rates and placement, as well as form of each nutrient will ensure that the nutrient demand of the cotton crop can be met. This plan can be moderated where in-season tissue testing indicates a nutrient is deficient (requiring remediation) or sufficient, thereby possibly reducing the need to apply the fertilizer that was planned. Timing of fertilizer application needs to take account of crop nutrient demand (Figs. 1 and 3) as well as the delay in availability or uptake of the particular form of fertilizer.

Where micronutrients are deficient, in-season foliar applications may satisfy the nutrient requirement if those nutrients have not been applied to the soil pre-sowing. The timing of foliar applications should ensure that peak demands as shown in Table 1 are met. Care is required to avoid damaging leaves with high nutrient/salt concentrations; foliar fertilizers are best applied when the air temperature is low and humidity high such as early morning (Zhu, 1989).

Slow-release fertilizers and nitrification or urease inhibitors do not generally provide any substantial improvement in cotton nutrition, at least in some soil types, but may help reduce losses of applied nutrients (Chen *et al.*, 2007; Rochester *et al.*, 1994, 1996). Oosterhuis and Howard (2008) showed some advantage of slow release N and K fertilizers through improving nutrient use efficiency by maintaining yield at reduced fertilizer rates. There is a recent report of urease inhibitors increasing cotton yields (Kawakami *et al.*, 2011).

Nutrient Use-Efficiency

Crop nutrient use efficiency can be expressed as kg lint/ kg nutrient uptake, but this efficiency measure may change with yield level (*see* review by Mullins and Burmester, 2010). Little research has been published on the use-efficiency of any nutrient other than N (Bronson, 2008; Rochester, 2011).

Newer cultivars tend to accumulate greater quantities of nutrients than older cultivars, while producing higher yields, but nutrient use efficiency tends to increase over time (Rochester, unpublished). There is little evidence that transgenic traits affect nutrient concentrations tissues or the uptake of nutrients in cotton (Rochester, 2006). However, where those traits increase the yield due to reduced insect pest damage in the case of Bt technology or weed competition in the case of herbicide resistance, more nutrients may be removed from the system in seed cotton. Thus higher levels of nutrient replacement would be required to optimize nutrition of future crops and avoid soil fertility decline. The expression of Bt proteins in cotton can be reduced by crop N deficiency (Rochester, 2006). Deficiencies of other nutrients may also reduce the expression of transgenes by restricted growth and poor crop health.

Opportunities for Improved Cotton Nutrition

Salinity, acidity/alkalinity and sodicity each pose nutritional stress on cotton crops by restricting the availability and uptake of some nutrients. Selection of genotypes bred under specific conditions that may restrict crop production (e.g., soil type or extreme temperatures) may perform better than those bred under normal conditions (Rochester and Constable, 2003). Hostile soils, and particularly subsoils, pose severe restrictions to root growth and nutrient uptake.

Changes to the cropping system can afford improved crop nutrition. Choice of rotation crops for cotton can greatly impact nutrient supply, e.g., growing legume crops can supply N to subsequent crops and improve the condition of clay soils in particular (Rochester and Peoples, 2005). Cotton has evolved a mutualistic relationship with mycorrhizal fungi. The more widespread use to reduced tillage provides a better environment for the growth of roots and mycorrhizal fungi that promote nutrient acquisition, such that greater nutrient uptake may be seen in cotton grown under these conditions (Rich and Bird, 1974; Pearson and Jakobsen, 1993). Hence, Zn and P deficiency may be rarer in minimum-tilled sites (Mehravan, 2001).

Multiple Deficiencies and Nutritional Syndromes

Potassium deficiency syndrome in the US (Ashworth *et al.*, 1982; Oosterhuis, 1994) and premature senescence in Australia (Wright, 1999) have been associated with K deficiency, as well as phosphorus deficiency and high levels of sodium uptake (Rochester, 2010). These syndromes are more commonly seen at the end of the flowering period when the demand for nutrients may exceed supply and result in foliar symptoms typical of P and K deficiency (Table 1).

SUMMARY

Cotton has high demand for nutrients such as N and K in excess of 200 kg/ha and daily accumulation rates of up to 4 kg/ha/day, whereas trace nutrient needs are more easily met (up to 1 kg/ha) with fertilizer application where appropriate. Understandably, N has been more thoroughly studied than other nutrients, as N is most commonly deficient in agricultural systems without fertilizer addition. Redistribution of nutrients from vegetative to reproductive plant parts is a vital component of cotton plant nutrition, particularly for N and P.

Research gaps in this subject area include (a) nutrient deficiency effects on events in the flower, i.e. during anthesis for pollen tube growth and fertilization leading to early seed formation, and early partitioning in the seed between oil, protein and fiber; (b) amelioration of stress effects on nutrient imbalance/deficiency in the seed and developing boll; and (c) redistribution, removal and replacement of all nutrients, particularly those approaching deficiency in older cropping systems.

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Chapter 5

WATER STRESS AND REPRODUCTIVE DEVELOPMENT IN COTTON

Dimitra A. Loka and Derrick M. Oosterhuis
Department of Crop, Soil, and Environmental Sciences
University of Arkansas, Fayetteville, AR 72704

INTRODUCTION

Water is the most limiting factor for plant growth and crop productivity (Kramer, 1983), and water-deficit stress adversely affects crop growth and yield throughout the world (Boyer, 1982). Crop yields are being reduced by drought and the increasing scarcity of water for irrigation, and changing world climatic trends may increase the severity of the problem (Le Houerou, 1996). Water availability and quality affect the growth and physiological processes of all plants, since water is the primary component of actively growing plants, ranging from 70-90% of plant fresh mass (Gardner *et al*., 1984).

Due to its predominant role in plant nutrient transport, chemical and enzymatic reactions, cell expansion, and transpiration, water-deficit stress alters how plants grow, their morphology, and the biochemical processes that occur in them (Hsiao, 1973; Kramer, 1980). In general, plant water-deficit stress is defined as the condition where a plant's water potential and turgor are decreased sufficiently to inhibit normal plant function (Hsiao 1973). The effects of water stress depend on the severity and duration of the stress, the growth stage at which stress is imposed, and the genotype of the plant (Kramer, 1983). The effect of water-deficit stress on the growth, physiology and yield of cotton was recently reviewed by Loka *et al.* (2011). This review discusses the effects of water-deficit stress on reproductive development of cotton (*Gossypium hirsutum* L.).

Sensitivity of Cotton to Water-deficit Stress

Crop sensitivity to water deficit varies by growth stage and is crop-dependent (Doorenbos and Pruitt, 1977; Saini and Westgate, 2000). In many crops, reproductive development is the most sensitive period to drought stress following seed germination and seedling establishment (Saini, 1997), and cotton appears to follow this pattern, as well (Loka, 2012). Cotton is sensitive to water deficit during both flowering and boll development (Constable and Hearn, 1981; Cull *et al.*, 1981a,b; Turner *et al.*, 1986). Recent research has shown that the developing pollen (Burke *et al.*, 2002) and pollen tube growth (Snider *et al.*, 2011) are highly sensitive to environmental stress.

The perennial nature and indeterminate growth pattern of cotton results in the simultaneous occurrence of several stages of flowering and fruiting. This ambiguity has contributed to conflicting reports on which stage of crop reproductive development is most sensitive to water deficit (Loka *et al.*, 2011). According to Reddell *et al.* (1987), the early flowering period in cot-

ton is the most sensitive to water stress, whereas Orgaz *et al.* (1992) concluded that water stress during peak flowering had the most detrimental effects on cotton yield.

On the other hand, a number of reports (Radin *et al.*, 1992; Plaut *et al.*, 1992; de Cock *et al.*, 1993) state that boll development, particularly well after the end of effective flowering, is the most water-deficit-sensitive period for cotton. Additionally, in an earlier experiment, Harris and Hawkins (1942) reported that delaying irrigation at fruiting could increase yield by inhibiting excessive vegetative growth, a result reinforced by Singh (1972), who reported increased number of flowers and bolls per plant as well as increased yield when cotton plants were stressed during the pre-flowering season.

Conversely, Stockton *et al.* (1961) and Lashing *et al.* (1970) observed that increased irrigation resulted in increased flowering. Guinn *et al.* (1981) concluded that a moderate water-deficit stress early in the season could be beneficial to the plants by slowing vegetative growth, but that the risk of negative results meant that these practices should be approached with caution.

The effects of water deficit on different plant physiological processes are complex and interrelated. Cellular water content largely controls stomatal aperture, and stomatal conductance directly affects CO_2 diffusion and photosynthetic carbon fixation, which in turn affects carbohydrates and metabolic functions such as translocation, respiration and available energy. However, for ease of discussing these physiological functions, we have addressed the effects of water deficit pre-flower, during flowering and after flowering on boll development.

Water-deficit Stress Prior to Flowering

As stated by Grimes *et al.* (1969), cotton yield is positively correlated to the number of bolls produced. Initiation of floral buds, however, occurs 35-40 days before anthesis, while the number of carpels and anthers is determined 30-35 days before anthesis (Stewart, 1986). Because fiber production is based on the number of ovules contained in a boll, and that the number of ovules is determined 15-25 days before anthesis, pre-flowering is a critical period for yield determination. Hence, environmental conditions that occur during this period and cotton's responses are extremely interesting. Nevertheless, little information exists on the effect of water-deficit stress during pre-flowering.

As early as 1932, Beckett and Hubbard conducted field experiments with Upland cultivars and reported that limited water supply before flowering had minimal influence on the number of carpels per flower. Similar results were observed by Leding and Lytton (1933), who found that water stress decreased the number of carpels in the flower, albeit not significantly. Singh (1975) reported increased numbers of flowers and bolls per plant accompanied by yield increase when irrigation was withheld at the preflowering stage, and similar results were reported by El-Zik *et al.* (1977) and Mauney *et al.* (1980). Young squares, however, appeared more prone to abscise when the plant was subjected to lower than optimal moisture (McMichael, 1979), with their most sensitive period being the first week after visibility (Ungar *et al.*, 1989). In view of these observations, Rijks (1965) reported that limited supply of water before flowering increased fruit retention, but reduced nodes, fruiting branches, and fruiting sites. Similar results were reported by Stockton *et al.* (1971), whereas increased flowering rates with increased irrigation were

reported by Bruce and Romkens (1965) and Lashin *et al.* (1971). Krieg (2000) concluded that inhibition of flowering site initiation rather than square shedding was the reason for decreased fruiting sites due to water-deficit stress prior to flowering.

Zhao and Oosterhuis (1997) reported that in growth chamber experiments, dry weight of water-stressed floral buds was significantly lower compared to the control. Tarpley and Sassenrath (2006) monitored carbohydrate concentrations of floral buds starting from 10 days before anthesis until 2 days after anthesis under water-sufficient conditions. They reported that carbohydrate (glucose, fructose, sucrose and starch) concentrations and water content of flower buds were relatively stable until the day of anthesis, when they showed a significant increase in both carbohydrate and water content. Guinn *et al.* (1990) reported that 3 days before anthesis, flower buds contained higher concentrations of indoleacetic acid (IAA) compared to the control, while abscisic acid (ABA) concentrations remained unaffected.

Water-deficit Stress during Flowering

Cotton white flowers have not been reported to abscise, but to actually sustain expansion under extreme water-deficit conditions, even after leaf emergence and expansion have been arrested. However, significant reductions in yield are observed when water-deficit stress occurs during flowering. Redell *et al.* (1987) reported that conditions of limited water supply during early flowering can result in significantly decreased yields, while Orgaz *et al.* (1992) argued that peak flowering is the most sensitive stage of cotton development to water-deficit stress.

Trolinder *et al.* (1993) reported that in field studies where plants were subjected to mild and severe limited water conditions, water stress resulted in petal water potentials that were significantly higher than leaf water potential. In addition, it was observed that even though petal water potential varied in accordance with plant water status, due to the direct vascular connection between the petals and the plant stem, the water potential gradient the petals required for their expansion did not exist. They speculated that this inverted gradient could be attributed to metabolic reasons, such as rapid solute breakdown. However, further investigation under conditions that restrained metabolic activity resulted in the same inverted gradient (Trolinder *et al.*, 1993). Loka and Oosterhuis (unpublished data) observed significantly higher carbohydrate concentrations in petals than leaves under both well-watered and water-stressed conditions. In addition, Loka and Oosterhuis (2011) reported that water potential of the ovary and the style of white flowers were significantly higher compared to the leaves under both well-watered and water-stressed conditions.

Guinn *et al.* (1990) conducted field experiments where plants were subjected to two cycles of water stress and flowers were collected the day of anthesis in order to investigate the effect of limited water supply on ABA and IAA concentrations. ABA levels of water stressed flowers were increased compared to the control, while after irrigation its levels decreased. Conversely, water-deficit stress had a minimal effect on IAA concentrations, resulting in increased levels of conjugated IAA in water-stressed flowers, whereas free IAA concentrations of water-stressed flowers were similar to those of control. The authors speculated that the lack of an effect of water-deficit stress on the levels of free IAA was due to the small increase in ABA levels of the flowers.

Water-deficit Stress during Boll Development

Cotton bolls appear to be less sensitive to water-deficit stress than the leaves since they are significantly resistant to water loss and are considered essentially non-transpiring (McMichael and Elmore, 1976; Radin and Sell, 1975; Wullschleger and Oosterhuis, 1990; Trolinder *et al.*, 1993; Van Iersel and Oosterhuis, 1994; 1996). A number of researchers however, have reported that limited supply of water during boll development can result in significantly lower yields (Radin *et al.*, 1992; Plaut *et al.*, 1992; de Cock *et al.*, 1993). In support of these observations, McMichael *et al.* (1973) observed that if water stress occurs during the first fourteen days after anthesis, young bolls generally abscise. However, after that period, bolls are retained.

Wullschleger and Oosterhuis (1990) conducted growth chamber experiments where bract and capsule wall water potential of 5-, 20-, and 30-day old bolls was monitored along with leaf water potential under a moderate and a severe water stress regime. They reported that mild water stress had no effect on bract and capsule wall water potentials while leaf water potentials were significantly decreased. A similar pattern was observed under severe water stress conditions with the exception of the dark respiration rates of the capsule wall that were significantly decreased under water-deficit stress conditions. Trolinder *et al.* (1993) reported that the inverted water potential gradient that was observed for the petals was also present in 20-day after anthesis bolls. Van Iersel and Oosterhuis (1995, 1996) investigated water relations of cotton fruits in field as well as growth chamber experiments. Water and osmotic potential of bracts and subtending to the bolls leaves compared to the bolls. This was attributed to the xylem connections of the fruits being immature and, hence non-functional, until three weeks post anthesis, and it was concluded that since the water potential gradient is directed from the fruits to the leaves, the main entrance of water in cotton bolls is through the phloem.

However the apoplastic isolation of the bolls and their independence from the water status of the plant, cotton boll hormonal balance appears to be significantly affected by water-deficit stress. Guinn (1976) observed that ethylene evolution rates of 3-day old bolls were significantly increased under conditions of limited water supply. Similarly, free and conjugated ABA levels of 3-day old water-stressed bolls as well as their abscission zones were reported to be significantly higher compared to the levels of well watered plants (Guinn and Brummett, 1988). Free and conjugated IAA of 3-day old bolls followed a differential pattern with free IAA decreasing when soil moisture became limiting while conjugated IAA significantly increased in both water-stressed bolls and their abscission zones (Guinn and Brummett, 1988).

Despite the differences in ABA, IAA and ethylene, no effect of water-deficit stress was observed on the carbohydrate content of 3-day old bolls (Guinn and Brummett, 1988). Krieg and Sung (1986) conducted translocation experiments with ^{14}C and reported that direction of the photosynthate flow was not affected by the water-deficit stress treatment while no differences in dry weights were observed between water-stressed and well watered bolls. Further research by Krieg (2000) concluded that if water-deficit stress occurs after flowering young fruits are more likely to abort due to decreased carbon and nitrogen supply as well as perturbations in hormone metabolism.

SUMMARY

Water-deficit stress has a significant effect on cotton's growth and development. The effects of water stress depend on the severity and duration of the stress, the growth stage at which stress is imposed, and the genotype of the plant. The cotton crop is sensitive to water shortage at all growth stages, but particularly reproductive development is the most sensitive period to drought stress following seed germination and seedling establishment. In cotton, water sensitivity during flowering and boll development has been well established. Recent research has shown that the developing pollen and pollen tube growth are highly sensitive to environmental stress. However, the exact physiological metabolic processes responsible for this sensitivity remains to be elucidated.

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Chapter 6

HEAT STRESS AND POLLEN-PISTIL INTERACTIONS

John L. Snider[1] and Derrick M. Oosterhuis[2]
[1]Department of Crop and Soil Sciences, University of Georgia, Tifton, GA
[2]Department of Crop, Soil, and Environmental Sciences,
University of Arkansas, Fayetteville, AR

INTRODUCTION

For a number of plant species, sexual reproduction is substantially more sensitive to heat stress than vegetative processes (Zinn *et al.*, 2010). Consequently, the yield of crops with valuable reproductive structures used for food (i.e. grain crops and horticultural crops) and fiber (i.e. cotton) are especially sensitive to moderately elevated temperatures projected to result from global climate change (Peng *et al.*, 2004; Reddy *et al.*, 2002). Other than concerns over global climate change, another important consideration for many cotton producers is the extreme year-to-year variability in yields caused by temperature extremes. For example, a negative correlation has been reported between average maximum temperature during flowering and lint yields in cotton (*Gossypium hirsutum*; Oosterhuis, 2002). There is no exact identification of the most heat-sensitive aspect of the reproductive process in cotton, but Reddy *et al.* (1996) concluded that there was a short period associated with flowering when reproduction was most vulnerable to average daily temperatures above 32.8° C to 34.4° C, and high temperature has been shown to substantially limit *in vivo* fertilization in thermosensitive cultivars (Snider *et al.*, 2009, 2011c; Fig. 1). Because a number of reproductive processes must occur in a highly concerted fashion during flowering for fertilization to occur, sexual reproduction is only as tolerant to heat stress as the most thermosensitive process (Hedhly *et al.*, 2009; Zinn *et al.*, 2010). Depending upon the timing, duration and severity, heat stress could limit fertilization by inhibiting male (Jain *et al.*, 2007) and female (Saini *et al.*, 1983; Snider *et al.*, 2009) gametophyte development, inhibiting pollen germination (Burke *et al.*, 2004; Kakani *et al.*, 2005; Jain *et al.*, 2007), limiting pollen tube growth (Burke *et al.*, 2004; Kakani *et al.*, 2005; Hedhly *et al.*, 2004; Snider *et al.*, 2011a), or by altering the development of tissues required to carry out reproductive processes (i.e. anther and pistil tissues; Zinn *et al.*, 2010). Although literature concerning heat stress and reproductive development in sexual plants is extensive, the approaches used by various investigators to elucidate plant reproductive responses to high temperature vary substantially from study to study. Consequently, it is the aim of this review to characterize the impact of timing, duration and severity of heat stress on sexual processes occurring during the progamic phase. A special emphasis is placed on the biochemical response of the pistil to moderately high temperature and the resultant influence on *in vivo* pollen performance and fertilization. Although this chapter will focus on heat stress effects in cotton, inferences will also be drawn from work conducted with other species where information is lacking for cotton.

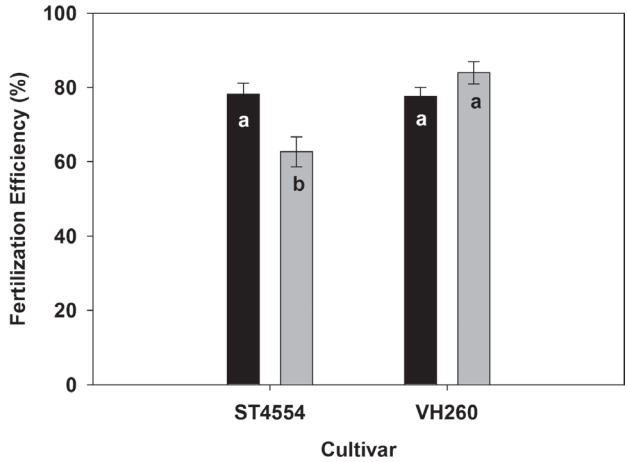

Figure 1: Fertilization efficiencies (B) of ST4554 and VH260 under a 30/20° C day/night temperature regime (black vertical bars; 30) and 38/20° C day/night temperature regime (gray vertical bars; 38). All values are means ± standard error (n = 9), and values not sharing a common letter are significantly different (LSD; $P < 0.05$). (Adapted from Snider *et al.*, 2011b)

SEXUAL REPRODUCTION IN COTTON

Sexual reproduction in flowering plants occurs in essentially three stages: gametophyte development (from meiosis to pollination), the progamic phase (from pollination to zygote formation), and embryo development (from zygote to seed; Herrero and Hormaza, 1996). During the progamic phase a number of reproductive processes must occur in a highly concerted fashion for successful fertilization to occur. 1) Anther dehiscence allows mature pollen grains to be transferred to a receptive stigmatic surface; 2) pollen grains germinate and pollen tubes penetrate the stigmatic surface of the pistil; 3) pollen tubes grow through the transmitting tissue of the style and towards a sexually competent ovule; 4) finally, double fertilization produces a zygote and its associated endosperm. The precise timing and coordination of events during the progamic phase is to a large extent genetically predetermined and varies substantially between plant species (Lankinen *et al.*, 2007; Williams, 2008). For example, *Quercus rubra* has a progamic phase lasting 57 weeks, whereas *Cichorium intybus* completes the progamic phase in 15 to 20 min (Williams, 2008).

There is a wealth of literature available on the pattern of reproductive development in cotton (e.g. Beasley, 1975; Stewart, 1986), and the sequence and timing of events during sexual reproduction have been well defined. For example, the first flower is produced approximately 8 weeks after plant emergence, and due to the indeterminate growth habit of cotton, flowers are continually produced in a 3 day vertical flowering interval and a 6 day horizontal flowering interval throughout the growing season (Oosterhuis, 1990). The day of anthesis is a critical event in the overall reproductive development of cotton, where a white flower opens at dawn (Stewart, 1986), pollination occurs approximately between 0700 and 1100 h (Pundir, 1972) and pollen germination within 30 minutes after pollination (Stewart, 1986). The pollen tube extends

through the transmitting tissue of the style and fertilization occurs between 12 and 24 h after pollination (Stewart, 1986). Successful *in vivo* pollen tube growth and subsequent fertilization of the ovule is a prerequisite for seed formation in cotton, and seeds with their associated fibers are the basic components of yield. Although events during the progamic phase occur in a relatively consistent manner for cotton, the temperatures encountered either before or during the progamic phase also exert considerable control over the fertilization process (Snider *et al.*, 2009; Oosterhuis and Snider, 2011; Snider *et al.*, 2011b), and can strongly influence yield (Oosterhuis, 2002; Pettigrew, 2008; Oosterhuis and Snider, 2011).

HEAT STRESS AND YIELD

Final yield has been shown to be strongly influenced by temperature in cotton (Wanjura *et al.*, 1969) and a negative correlation between cotton lint yield and high temperature was reported for the Mississippi Delta (Oosterhuis, 2002). Year-to-year variation in cotton yields, a major concern of cotton producers, has been associated with unpredictable variation in seasonal temperatures (Oosterhuis, 1999). Oosterhuis (unpublished) compared final lint yields with average maximum temperatures weekly after flowering for cotton in eastern Arkansas, and showed a significant decline in yield when average maximum temperatures exceeded 32° C during the flowering period. Reddy *et al.* (1996) reported a sharp decline in fruit efficiency (boll weight per total dry weight produced) when temperatures exceeded about 29° C. It is interesting that as long ago as ninety years, Balls (1919) reported that cotton in Egypt seemed to grow and yield best around 32° C, and that prolonged temperatures above 35° C were harmful. High, above average, temperatures during the day can decrease photosynthesis and carbohydrate production (Bibi et al, 2008), and high night temperatures will increase respiration and further decrease available carbohydrates (Gipson and Joham, 1968; Loka and Oosterhuis, 2010), resulting in decreased seed set, reduced boll size and decreased number of seeds per boll, and the number of fibers per seed (Arevalo et al, 2008).

Boll retention has been shown to decrease significantly under high temperature (Reddy *et al.* 1991; Reddy *et al.* 1992b; Reddy *et al.* 1995; Reddy *et al.* 1999; Zhao *et al.* 2005) and was reported to be the most heat sensitive yield component of cotton. For example, Reddy *et al.* (1991) observed that temperatures in excess of a 30/20° C day/night temperature regime resulted in significantly lower boll retention due to enhanced abortion of squares and young bolls. Subsequently, Reddy *et al.* (1992a) and Reddy *et al.* (1992b) observed declines in boll retention at temperatures in excess of a 30/22° C day/night temperature regime for both Pima and Upland cotton. An additional study showed even greater sensitivity of boll retention to increasing temperatures, where boll retention was negatively impacted at day temperatures in excess of 26.6° C (Reddy *et al.* 1995). Recently, Zhao *et al.* (2005) found that cotton plants exposed to a 36/28° C day/night growth temperature regime retained approximately 70% fewer bolls than plants grown under a 30/22° C day/night temperature regime. It is also important to note that a large proportion of the ovules available in a given ovary must be fertilized to ensure boll retention (Stewart, 1986). Consequently, limitations to the fertilization process could be at least partially responsible for poor boll retention under high temperature conditions.

The number of seeds per boll is an important basic component of cotton yield. Groves (2009) emphasized the importance of seed number in determining yield by reporting that the number of seeds per acre accounted for more than 80% of total yield variability in cotton. Seed number is a function of the number of locules (carpels) per boll and the number of ovules per locule (Stewart, 1986). Several factors such as the lack of fertilization, post-fertilization termination of embryo growth, cultivar and environment can also contribute to variation in the number of seeds per boll (Turner *et al.*, 1977). Researchers have shown that high temperature stress is a major factor negatively impacting seed development. For example, Reddy *et al.* (1999) showed that temperatures higher than 26.0° C increased short fiber mote frequency in Upland cotton and suggested that either fertilization had been negatively impacted due to insufficient pollen/ovule development or that fertilized ovules aborted soon after the fertilization event had occurred. Pettigrew (2008) reported that slight elevations in temperature (approximately 1° C above control temperatures) under field conditions were not sufficient to cause a decline in seed weight but were sufficient to cause a significant decline in seed number per boll, which was the primary cause of reduced yield under high temperature conditions. Similarly, Lewis (2000) compared a cool year 1990 in the Mid-south (mean maximum daily temperature of 32.2° C for July) with a hot year 1996 (mean maximum daily temperature of 36.6° C for July) and showed that the number of seeds decreased in the hot year from 2.987 to 2.093 million per hectare. This was associated with a lower average number of seeds per boll, i.e. 23.6 seeds/boll in the hot year compared to 28 seeds/boll in the cool year. Lewis (2000) concluded that about 99 percent of the variation in number of seeds per hectare in his three year study was explained by changes in the mean maximum July temperatures.

HEAT STRESS AND GAMETOPHYTE DEVELOPMENT

Poor fertilization has been cited as a likely cause of reduced yields and seed production in cotton exposed to high temperature during reproductive development (Pettigrew *et al.*, 2008; Snider *et al.* 2009, 2011b). Although a number of elegant studies have been conducted to specifically identify the most thermosensitive stage of reproduction in other species, no consensus currently exists regarding the most heat-sensitive stage of reproduction leading up to fertilization in cotton. In an early study with cotton, Meyer (1966) reported a positive correlation between anther sterility and the maximum temperatures at 15 and 16 days prior to anthesis, suggesting that microgametophyte development was exceptionally sensitive to high temperature immediately after meiosis of the microspore mother cells. Studies with other species utilizing moderately high temperature exposure at different reproductive stages of development have implicated early pollen development as the most heat-sensitive process in plant sexual reproduction (Peet *et al.*, 1998; Porch and Jahn, 2001; Sato *et al.*, 2002). For example, Peet *et al.* (1998) showed that reproductive output (i.e. fruit and seed production) could be completely abolished when pistils of male sterile tomato plants grown under an optimal day/night temperature regime (28/22° C) were pollinated with pollen that had developed under a high (32/26° C) day/night temperature regime. In contrast, when male sterile pistils exposed to the high day/night temperature regime were pollinated with pollen that had developed under optimal conditions, fruit set and seed per fruit were maintained at 40 and 87% of the levels observed when both pollen and pistils were kept at the optimal growth temperature.

Based on the aforementioned reports and those of other researchers, the meiotic phase of pollen development has been widely regarded as an exceptionally thermosensitive stage of the reproductive process (Ahmed *et al.*, 1992; Porch and Jahn, 2001; Erickson and Markhart, 2002; Pressman *et al.*, 2002; Sato *et al.*, 2002). High temperature exposure during this stage of pollen development can limit fertilization and subsequent seed development by 1) decreasing the number of mature pollen grains available for pollination (Ahmed *et al.*, 1992; Porch and Jahn, 2001; Pressman *et al.*, 2002); 2) causing abnormal pollen development, resulting in decreased viability and germinability of available pollen grains (Ahmed *et al.*, 1992; Porch and Jahn, 2001; Erickson and Markhart, 2002; Sato *et al.*, 2002); and 3) resulting in abnormal anther morphology, thereby limiting anther dehiscence at anthesis (Ahmed *et al.*, 1992; Porch and Jahn, 2001; Erickson and Markhart, 2002; Sato *et al.*, 2002). Under moderately elevated temperature exposure extending from microsporogenesis to anthesis, altered carbohydrate metabolism of developing anthers and pollen grains prevents the accumulation of carbohydrates needed to drive the initial, autotrophic phase of pollen tube growth and accounts for poor pollen viability at anthesis (Aloni *et al.*, 2001; Pressman *et al.*, 2002; Firon *et al.*, 2006; Sato *et al.*, 2006; Jain *et al.*, 2007).

Although the effects of heat stress on male reproductive development have been well documented in a number of species, including cotton (Oosterhuis and Snider, 2011), comparably little is known about the sensitivity of female gametophyte development. Snider *et al.* (2009) reported declines in ovule number and fertilization efficiency when cotton plants were exposed to a 38/20° C temperature regime prior to flowering; the declines in fertilization efficiency observed in that study could have been explained by limitations to *in vivo* pollen performance or by decreased pollen tube guidance due to a higher proportion of defective ovules. For example, Saini *et al.* (1983) reported that exposing wheat plants to high temperature (30° C) for three days during the meiotic stage of pollen and megaspore mother cell meiosis did not alter pollen germinability, but pollen tube guidance to the ovules was prevented due to an increase in ovule abnormalities and a decrease in the proportion of functional ovules.

HEAT STRESS EFFECTS ON ISOLATED POLLEN GRAINS

Many of the available reports investigating the effects of high temperature on plant sexual reproduction have been *in vitro* studies of pollen performance (i.e. pollen germination and pollen tube growth) and have allowed researchers to identify the temperature sensitivity of the fully mature male gametophyte in isolation from either parental or female reproductive tissues. In contrast with vegetative and pistil tissues, mature pollen does not exhibit acquired thermotolerance via a typical heat shock response and is extremely sensitive to high temperature exposure (Frova *et al.*, 1989; van Herpen *et al.*, 1989; Dupuis and Dumas, 1990; Hopf *et al.*, 1992). For example, Dupuis and Dumas (1990) reported that pre-exposure of mature maize pollen to high temperature (40° C) for 4 h prior to pollination abolished *in vitro* fertilization even when pollination was performed on spikelets maintained at 28° C throughout the experiment. In contrast, when spikelets, previously exposed to 40° C for 4 h, were pollinated with unstressed pollen, a 43% fertilization rate was obtained (Dupuis and Dumas, 1990). Because of this study and other reports (reviewed in Zinn *et al.*, 2010; Hedhly *et al.*, 2009), it has been proposed that mature pollen grains on the exposed surface of the stigma would be more sensitive to high temperature than the more deeply seated ovules (Kakani *et al.*, 2005), and

recent studies with cotton have focused on pollen germination and tube growth responses to high temperature using *in vitro* systems (Burke *et al.*, 2004; Kakani *et al.*, 2005; Liu *et al.*, 2006).

Data from *in vitro* studies have shown that the optimal temperature range for cotton pollen germination is between 28 and 37° C (Burke *et al.*, 2004; Kakani *et al.*, 2005). Burke *et al.* (2004) and Kakani *et al.* (2005) showed that the optimal temperature across a range of *G. hirsutum* cultivars for pollen tube growth was from 28 to 32° C (Fig. 2). Liu *et al.* (2006) reported a 27.8° C temperature optimum for pollen tube growth and showed a strong correlation between maximum pollen tube growth and boll retention in *G. hirsutum*. Recently, Burke (2011) showed that the germinability of otherwise viable cotton pollen exposed to 39° C decreased to ~40% of the levels observed under control temperature conditions (28° C). In a previous study, Barrow (1983) compared techniques to evaluate the response of cotton pollen to high temperature, including pollen viability staining, pollen germination, pollen tube penetration of the stigma, penetration to the base of the style, and penetration of the ovules. This author showed that viability, and germinability were unaffected by pre-treating pollen with temperatures as high as 40° C. However, penetration of the stigma, style, and ovules was negatively impacted at 33° C and above, where cotton pollen exposed to temperatures ≥ 35° C for 15 h prior to anthesis was unable to penetrate the ovules. These findings suggested that pollen fertility under high temperature could not be directly inferred from pollen viability and germination measurements (Barrow, 1983). Using style penetration by the pollen tubes as a criterion for pollen fertility, Rodriguez-Garay and Barrow (1988) showed that heat tolerance could be genetically transferred to heat-sensitive lines by performing crosses with pollen that had been exposed to temperatures ≥ 35° C for 15 h, thereby only pollinating with pollen that survived the high temperature treatment. The maximum daily temperatures experienced by cotton plants during the flowering period often exceed the optimal temperature for successful pollen tube growth, and negative impacts on the male gametophyte can be expected.

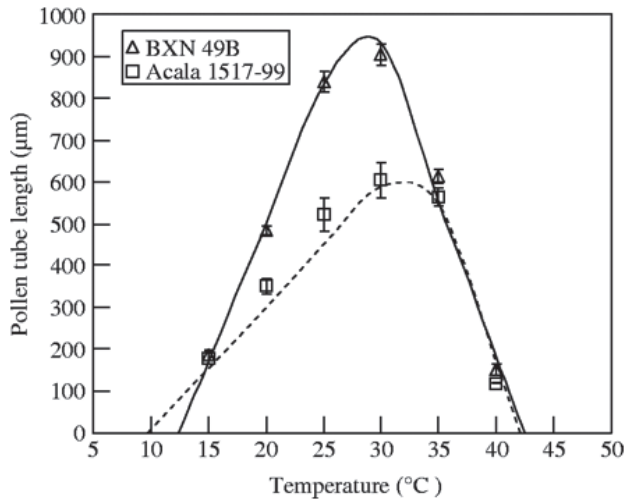

Figure 2: Pollen tube length responses to temperature for two cotton cultivars (BXN 49B and Acala 1517-99). Error bars indicate ± s.e. (Adapted from Kakani *et al.*, 2005)

HEAT STRESS AND POLLEN-PISTIL INTERACTIONS

Although pollen development and function are considered exceptionally heat-sensitive processes, it is also important to note that under natural conditions, high temperature will simultaneously impact male and female reproductive tissues, resulting in a synergistic effect on reproductive output (Reviewed in Zinn *et al.*, 2010; Oosterhuis and Snider, 2011; Snider and Oosterhuis, 2011). Furthermore, it has been reported that *in vivo* pollen performance under high temperature can be modulated by the pistil tissue through which the pollen tubes grow (Pundir, 1972; Hedhly *et al.*, 2005). For example, Pundir (1972) reported that pollen tube growth rates could be increased in *G. hirsutum* (♀) x *G. arboreum* (♂) crosses or slowed in *G. arboreum* (♀) x *G. hirsutum* (♂) crosses, relative to the rates typically obtained by the pollen donor. Given these results, Stewart (1986) suggested that the nutritional or hormonal balance of the transmitting tissue of the style was important for *in vivo* pollen tube growth. Several researchers have since confirmed in a variety of species that a number of complex physical and biochemical pollen-pistil interactions are required for successful pollen tube growth from the stigma to the ovules (Herrero and Arbeloa, 1989; Gonzalez *et al.*, 1996; Herrero and Hormaza, 1996; Lord and Russell, 2002; Lord, 2003). As a result, heat-induced changes in the pistil can be expected to exert considerable control over pollen performance *in vivo*.

Because mature pollen grains lacking a heat shock response and exposed on the stigmatic surface of the pistil are considered more vulnerable to heat stress than the deeply seated ovules, many authors have utilized *in vitro* pollen germination and tube growth assays to screen for heat tolerant genotypes in a number of species (Kakani *et al.*, 2002; Kakani *et al.*, 2005; Liu *et al.*, 2006; Reddy and Kakani, 2007; Salem *et al.*, 2007). However, it has been reported that *in vitro* pollen germination and tube growth responses to high temperature are not necessarily predictive of *in vivo* pollen performance under elevated temperature (Hedhly *et al.*, 2005a; Barrow, 1983; Young *et al.* 2004), and high temperature can also limit pollen germination through loss of stigmatic receptivity (Hedhly *et al.*, 2005b). Furthermore, under field conditions, it was recently reported that the diurnal pattern of pollen tube growth was strongly altered by moderately elevated temperature in *G. hirsutum*, where pollination occurred earlier in the day under higher diurnal temperatures (Snider *et al.*, 2011a; Fig. 3C). Interestingly, pistil and air temperatures were comparable during the estimated time of pollination, and pollen germination was unaffected by high temperature (Snider *et al.*, 2011a; Fig. 3A-B). It is interesting to speculate that temperature-dependent anther dehiscence may have allowed for pollen to be deposited on the stigmatic surface when temperatures were favorable for pollen germination in *G. hirsutum*.

In vivo pollen tube growth is sensitive to high temperature, where above optimal temperatures accelerate tube growth in some species (Buchholz and Blakeslee, 1927; Pasonen *et al.*, 2002; Hedhly *et al.*, 2004) and slow tube growth in others (Gawel and Robacker 1986; Snider *et al.*, 2011a). Given the importance of pollen-pistil interactions in determining successful pollen tube growth to the ovules, biochemical responses of the pistil to high temperature will necessarily influence pollen tube growth and fertilization. For example, Snider *et al.* (2009) reported that heat-induced declines in fertilization efficiency for *G. hirsutum* were associated with increased oxidative stress in the pistil, declines in the soluble carbohydrate and ATP content of the pistil, and decreased subtending leaf photosynthesisunder high temperature (38/20° C). Subsequent investigations have identified biochemical parameters of the pistil and subtending leaf that significantly impact pollen tube growth and fertilization under high temperature in *G. hirsutum*.

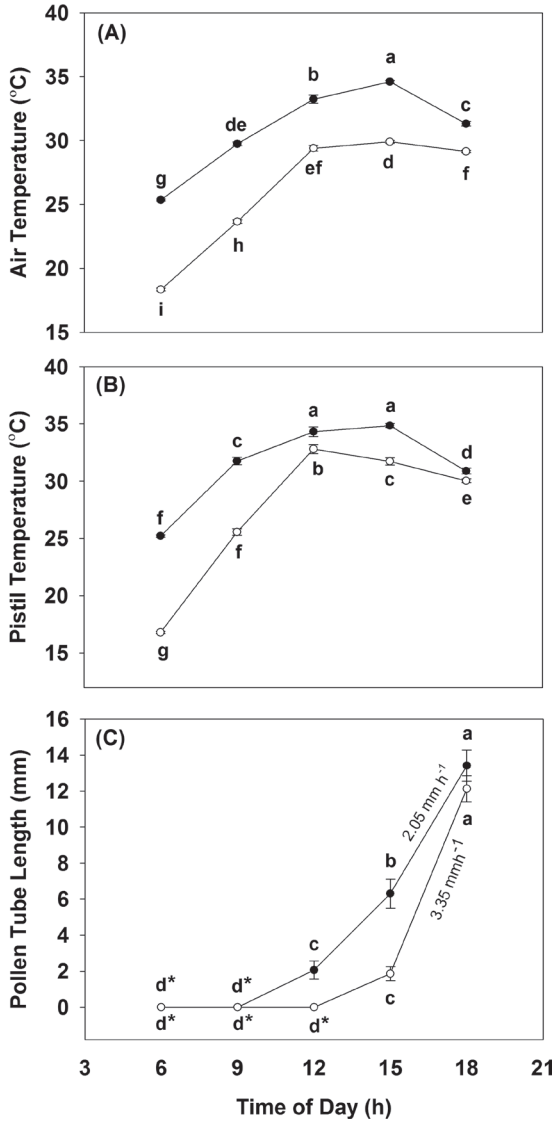

Figure 3: Diurnal air temperature (A) pistil temperature (B) and *in vivo* pollen tube growth (C) under optimal (T_{max} = 29.9° C; open circles) and high (T_{max} = 34.6° C; closed circles) air temperature conditions from 06:00 to 18:00 h in 3 h increments. An asterisk next to a data point indicates that no pollen grains were present on the stigmatic surface at that time of day (pollen tube length = 0). All values are means ± standard error (n = 6), and values not sharing a common letter are significantly different (LSD; $P < 0.05$). Pollen tube growth rates (in mm h^{-1}) under optimal and high temperature conditions are shown adjacent to the corresponding line. (Adapted from Snider *et al.*, 2011a)

The Subtending Leaf

Because the carbohydrate balance of reproductive tissues strongly influences reproductive success in cotton (Zhao *et al.*, 2005; Snider *et al.*, 2009), it is important to discuss the influence of high temperature on source strength. In *G. hirsutum*, most of the carbohydrate required for boll development is obtained from leaves subtending the reproductive unit (Ashley, 1972; Wullschleger and Oosterhuis, 1990). The importance of the subtending leaf in maintaining carbohydrate supply in the pistil was also demonstrated by Pettigrew (2001) who showed that exposure of cotton plants to shaded conditions (~70% of full sunlight) resulted in significant declines in nonstructural carbohydratecontents of both subtending leaves and ovules on the day of anthesis. The relationship between source leaf thermostability and reproductive success was recently demonstrated in a report showing that arabidopsis mutants exhibiting thermostable photosynthesis also yield more seeds under high temperature than thermosensitive variants (Kurek *et al.*, 2007). For cotton, Snider *et al.* (2009) reported that poor fertilization efficiency was associated with lower soluble carbohydrate and ATP content in the pistil under heat stress and lower rates, lower quantum yield, and lower total chlorophyll content in the subtending leaves. Subsequently, Snider *et al.* (2010) evaluated the subtending leaf photosynthetic response of two cotton cultivars known to exhibit differences in reproductive thermal stability: VH260 (thermotolerant) and ST4554 (thermosensitive). Although photosynthesis was significantly lower for ST4554 exposed to a 38/20° C day/night temperature regime relative to a 30/20° C day/night temperature regime, subtending leaf photosynthesis was unaffected by high temperature in VH260 (Snider *et al.*, 2010). Using rapid leaf temperature changes and quantum efficiency measurements at a range of temperatures (15-50° C), these authors further reported a 7.5° C higher optimal temperature (T_{opt}) and a 5.5° C higher threshold temperaturefor quantum efficiency ($T_{15\Phi PSII}$) of VH260 subtending leaves relative to ST4554 subtending leaves (Snider *et al.*, 2010). These findings suggest that genotypic differences in reproductive thermotolerance are closely associated with the thermal stability of the subtending leaf.

Calcium, Antioxidants, and ROS

Another factor essential for reproductive success is calcium. For example, calcium is known to promote pollen germination *in vitro* (Brewbaker and Kwack, 1963), and accumulation of high levels of loosely bound calcium in the transmitting tissue of the style prior to the passage of the pollen tube through that tissue is thought to promote pollen tube growth through the style in cotton (Zhang *et al.*, 1997) and other species (Zhao *et al.*, 2004; Ge *et al.*, 2009) because calcium uptake by pollen tube tips *in vitro* is required for pollen tube growth by promoting vesicle fusion at the tip of the elongating tube (Pierson *et al.*, 1996). Furthermore, calcium is known to promote fertilization (Faure *et al.*, 1994; Tian and Russell, 1997) and egg activation (Digonnet *et al.*, 1997). During heat stress, potentially damaging reactive oxygen species (ROS) accumulate in plant tissues (Foyer and Noctor, 2005; Tang *et al.*, 2006) along with a concomitant increase in cytosolic calcium (Gong *et al.*, 1998; Jiang and Huang, 2001). Calcium is essential in enhancing the antioxidant enzyme activity required to protect the plant under oxidative stress conditions via ROS scavenging (Gong *et al.*, 1998; Jiang and Huang, 2001). In contrast with

antioxidant enzymes, NADPH oxidase (NOX) produces O_2^- in a calcium-augmented fashion, which is needed to soften cell walls and promote cell expansion during pollen tube growth (Potocky *et al.*, 2007).

Snider *et al.* (2009) recently reported increases in the water soluble calcium concentration and glutathione reductase activity of heat-stressed cotton pistils, but a decline in NOX activity of pistils exposed to high day temperature. These authors suggested that a calcium-augmented antioxidant response to high temperature interfered with NOX activity required for successful pollen tube growth *in vivo*. Further research has shown that cotton pistils from a cultivar with known reproductive thermotolerance (VH260), as evidenced by higher fertilization efficiencies under high temperature (Fig. 1), also had significantly higher levels of total and water soluble calcium content than a more sensitive cultivar (ST4554 B2RF), and genotypic thermotolerance was associated with higher antioxidant enzyme (superoxide dismutase and glutathione reductase) activity in the pistil under optimal growth temperatures (Snider *et al.*, 2011b; Fig. 4). These findings suggest that calcium content and pre-stress antioxidant enzyme activity of the pistil may be important criteria for identifying thermotolerant cultivars. Additionally, the genotypic differences in subtending leaf thermostability discussed previously were shown to be dependent upon pre-stress antioxidant enzyme activity, where the thermotolerant cultivar had significantly higher levels of pre-stress antioxidant enzyme activity in the subtending leaf than the thermosensitive cultivar (Snider *et al.*, 2010).

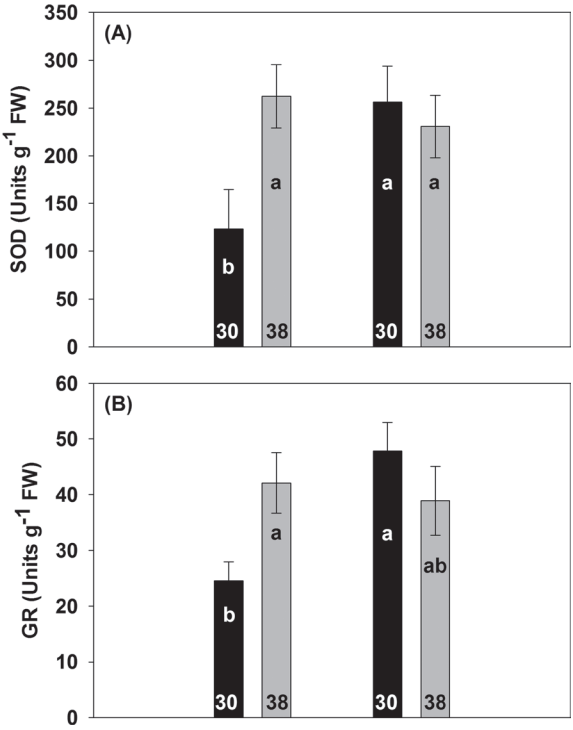

Figure 4: The effect of cultivar and temperature regime on superoxide dismutase (SOD) activity (A), and glutathione reductase (GR) activity (B) in *Gossypium hirsutum* pistils. For VH260, both SOD and GR activities were no different under the 38/20° C day/night temperature regime (gray bars; 38) when compared with the 30/20° C day/night temperature regime (black bars; 30), whereas ST4554 had significantly higher SOD and GR activities under high day temperature when compared with control temperature pistils. All values are means ± standard error (n = 10 for SOD and 16 for GR). Values not sharing a common letter are significantly different (LSD; $P < 0.05$). (Adapted from Snider *et al.*, 2011b)

Carbohydrates and ATP

A readily available supply of carbohydrates in the pistil is essential in promoting a number of key events during plant reproductive development, including gametophyte development (Rodrigo and Herrero, 1998; Castro and Clemente, 2007; Jain *et al.*, 2007), pollen germination (Jain *et al.*, 2007), pollen tube growth (Herrero and Arbeloa, 1989; Gonzalez *et al.*, 1996), and fertilization (Snider *et al.*, 2009). However, heat stress results in substantial alterations in the carbohydrate balance of reproductive tissues, causing poor reproductive success under high temperature. For example, Zhao *et al.* (2005) reported that high temperature conditions resulted in significantly lower levels of nonstructural carbohydrates in one day old cotton bolls and significantly higher abscission rates of young bolls; abscission rates were negatively correlated with the nonstructural carbohydrate content of the young boll. Some authors have shown that heat-tolerant cultivars of tomato (defined as cultivars with greater seed set under high temperatures) retain higher carbohydrate concentrations in the pollen grains and anther walls following chronic heat stress than do less heat tolerant cultivars (Pressman *et al.*, 2002; Firon *et al.*, 2006). Additionally, Jain *et al.* (2007) reported that season-long high temperature in grain sorghum resulted in poor pollen germination and reduced seed set concomitant with non-detectable levels of sucrose and 50% reductions in starch content of microspores during late developmental stages relative to optimal temperature conditions.

For cotton, Snider *et al.* (2009) recently reported that soluble carbohydrate and ATP concentrations in pistils exposed to high ambient temperature conditions (38/20° C) one week prior to flowering were approximately 20.3 and 55% lower, respectively, on the day of anthesis than under control temperature conditions (30/20° C). Because the decline in energy reserves occurred concomitantly with a decline in fertilization efficiency, these authors concluded that the energy demands for proper gametophyte development or pollen tube growth were insufficient and thereby limited the fertilization process.

Subsequent research has shown that a cotton cultivar known to exhibit reproductive thermotolerance (VH260), as evidenced by good boll retention and stable fertilization efficiency under high temperature, also had higher pistil ATP concentration than a conventional cultivar (ST4554 B2RF) widely utilized by cotton farmers in the Mississippi River Delta in 2008 (Snider *et al.*, 2011b). These findings suggest that the energetic status of the pistil may be a strong determinant of reproductive thermotolerance in cotton.

Under field conditions and much more moderate high temperature exposure (T_{max} = 34.6° C), diurnal pollen tube growth rates were significantly slowed in *G. hirsutum* pistils (Snider *et al.*, 2011a; Fig. 3) without any alterations pollen germination, or fertilization, suggesting that *in vivo* pollen tube growth may be more sensitive than the other reproductive processes. Subtending leaf photosynthesis, pistil oxidative status, and pistil ATP content (Snider *et al.*, 2011a, 2011c) were also unaffected by the moderately elevated temperatures observed under field conditions. In contrast, high temperature significantly decreased soluble carbohydrate supply in the pistil during pollen tube growth through the style (Snider *et al.*, 2011c; Fig. 5), and pollen tube growth rates were highly correlated (r^2 = 0.932) with the soluble carbohydrate

content of the pistil during pollen tube growth (Fig. 6). It is well established that pollen tube growth transitions from an autotrophic growth phase (utilizing carbohydrate reserves preexisting within the pollen grain at the time of anthesis) to a heterotrophic growth phase (utilizing carbohydrate reserves within the transmitting tissue of the style) (Herrero and Hormaza, 1996; Herrero and Arbeloa, 1989). Given that the energy requirements of actively growing pollen tubes are approximately 10 fold higher than those of vegetative tissues (Tadege and Kuhlemeier, 1997), it is to be expected that heat-induced declines in pistil carbohydrate supply should have a pronounced affect on *in vivo* pollen tube growth. Consequently, the carbohydrate balance of the pistil should be strongly influenced by the moderate temperature increases projected to result from global climate change.

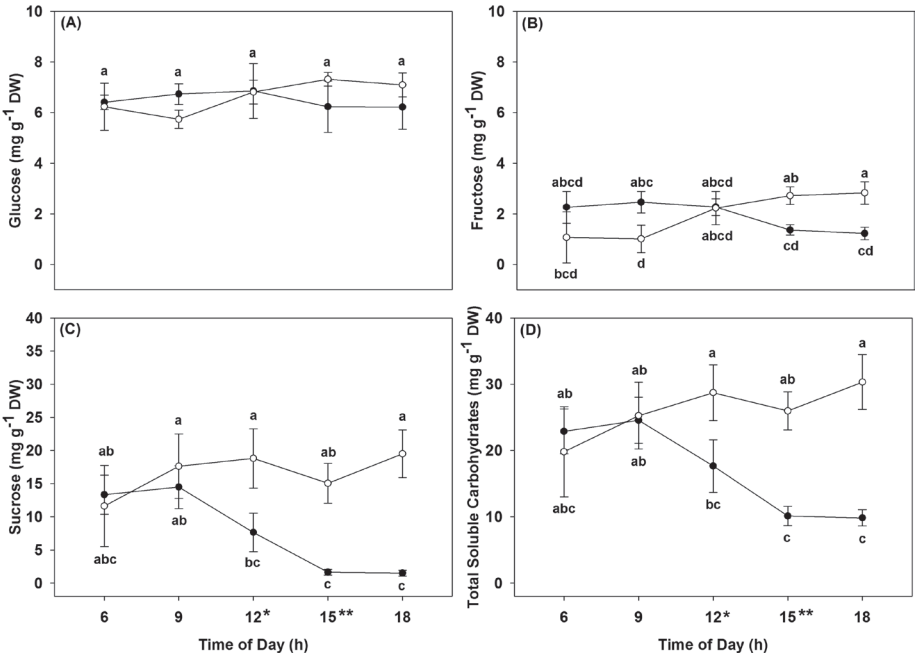

Figure 5: Diurnal levels of glucose (A), fructose (B), sucrose (C), and total soluble carbohydrates (D) for *Gossypium hirsutum* pistils sampled on August 4 (closed circles) and August 14, 2009 (open circles) from 0600 to 1800 h in 3 h increments. All values are means ± standard error (n = 12 for 0600 and 0900 h, 10 for 1200 and 1800 h, and 8 for 1500 h on August 4; n = 12 for 1500 h, 10 for 1800 h, 8 for 0900 and 1200 h, 4 for 0600 h on August 14), and values not sharing a common letter are significantly different (LSD; $P < 0.05$). Single or double asterisks indicate the sample time at which pollen tubes were first observed in the style on August 4 and 14, respectively. (Adapted from Snider *et al.*, 2011c)

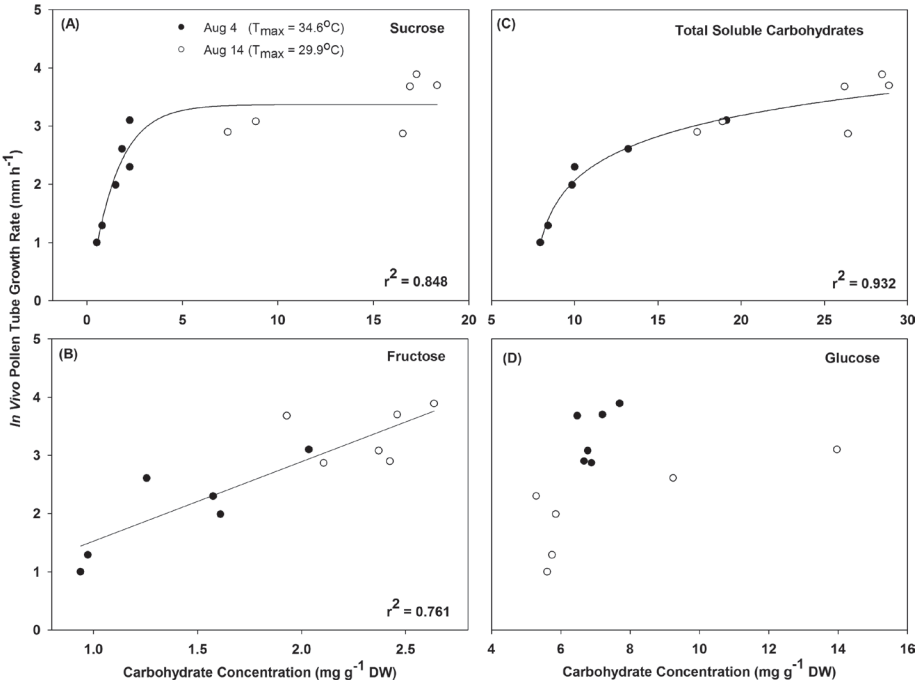

Figure 6: The relationship between pistil sucrose (A), fructose (B), total soluble carbohydrate (C), and glucose (D) concentrations and pollen tube growth rate in *Gossypium hirsutum* pistils sampled on August 4 (closed circles) and August 14, 2009 (open circles) between 1500 and 1800 h. (Adapted from Snider *et al.*, 2011c)

SUMMARY

For a number of plant species, sexual reproduction is substantially more sensitive to heat stress than vegetative processes, resulting in negative implications for the yield of agronomic crops with reproductive structures of economic importance. For example, a negative correlation has been reported between average maximum temperature during flowering and lint yields in cotton. Sexual reproduction in flowering plants occurs in essentially three stages: gametophyte development (from meiosis to pollination), the progamic phase (from pollination to zygote formation), and embryo development (from zygote to seed). For cotton, on the day of anthesis, the progamic phase lasts 12 to 24 h, and a number of events must occur in highly concerted fashion for successful fertilization and lint production to occur. Consequently, poor fertilization and seed set has been proposed as the likely cause of heat-induced yield reductions in cotton.

Depending upon the duration, timing and severity of the stress, fertilization could be limited by poor gametophyte development, decreased pollen germination, and limited pollen tube growth. Based on the existing literature for a number of species, high temperature exposure

either during early pollen development or during the progamic phase of pollen development will negatively impact pollen performance and reproductive output, where both phases of pollen development are considered exceptionally sensitive to moderate heat stress. However, moderately elevated temperatures either before or during the progamic phase can limit fertilization by negatively impacting important pollen-pistil interactions required for successful pollen tube growth toward the ovules. In cotton, heat stress (38/20° C) has been shown to limit *in vivo* fertilization concomitant with decreases in subtending leaf photosynthesis, declines in pistil ATP and carbohydrates, increases in oxidative stress in the pistil, and alterations in pistil calcium concentration. Having higher pistil concentrations of ATP and calcium and having elevated pre-stress antioxidant enzyme activity in cotton pistils and subtending leaves has been related to genotypic fertilization thermostability. Under field conditions, diurnal pollen tube growth rate is more sensitive to moderately high temperatures (34.6° C) than either pollen germination or fertilization, where elevated temperature significantly slows pollen tube growth rates. Additionally, the same ambient temperature conditions (34.6° C) significantly decrease the soluble carbohydrate content of the pistil without influencing other biochemical parameters, and pollen tube growth rates are strongly and positively correlated with the soluble carbohydrate content of the pistil ($r^2 = 0.932$). Consequently, the carbohydrate balance of the pistil should be strongly influenced by the moderate temperature increases projected to result from global climate change.

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Chapter 7

COTTON FLOWERING AND FRUITING: CONTROL AND MODIFICATION WITH PLANT GROWTH REGULATORS

Vladimir A. da Costa[1] and J. Tom Cothren[2,3]
[1]Dow AgroSciences LLC, Sloughhouse, CA 95683
[2]Soil and Crop Sciences Dept., Texas A&M University,
College Station, TX 77843

INTRODUCTION

Cotton (*Gossypium hirsutum* L.), by nature, is a perennial woody shrub that possesses an indeterminate growth habit. Through breeding and selection, cotton has been adapted to an annual production system and is currently grown under both semi-arid and humid conditions. As such, the crop is often subjected to environmental extremes and exposed to various stresses that impact its yield. The crop may be more vulnerable to these stresses at key developmental stages, such as flower initiation and boll filling.

At present, cotton is not genetically limited for yield, but the ability to retain and mature the fruit that are produced remains a challenge. Because of the indeterminate growth habit, cotton produces fruit over an extended fruiting period. Thus, these fruit are developed under varying moisture, temperature, and light regimes. The fruiting habit of the crop normally proceeds from fruit production commencing at around the sixth node and proceeding upward and out on fruiting branches until it reaches a stage of development referred to as cutout. Each fruiting branch that is produced normally initiates from 1 to 4 fruiting sites, with fruiting continuing upward until around the eighteenth mainstem node. Previous research indicates that the majority of yield is produced from the first and second fruiting sites on main-stem nodes 9 through 14. Reports indicate that as much as 80% of the yield originates at these sites. The obvious question is, "why is this the case." A major contributor to this occurrence is that of source and sink. The first position fruit on a node constitutes a stronger demand for assimilates and if supplies are limited, the subsequent fruit produced on the fruiting branch suffer the consequences.

Because of these growth characteristics, ways to modify and control the flowering/fruiting of the cotton plants are often desirable. The alterations may be accomplished through the use of plant growth regulators (PGRs). An organic substance is considered a plant growth regulator if in low concentrations it promotes, inhibits, or modifies plant growth and development, eliciting responses similar to the ones observed from endogenous plant hormones. However, interactions with the environment and differences in cultural practices are mainly responsible for the complex responses generated by crops to PGRs. Lack of consistency in performance, and the fact that PGRs may not be economically beneficial are some of the limitations for PGR usage.

[3] Mention of proprietary products does not constitute an endorsement by Texas AgriLife Research or Dow AgroSciences LLC, nor are products mentioned inclusive of all plant growth regulators. Specific products are mentioned as examples of physiological potential for cotton growth and modification.

Since most control and modification of cotton flowering and fruiting processes are regulated by natural plant hormones, these processes may be manipulated either by modifying the hormonal concentrations within the plant or by altering the natural way plants sense/respond to their hormones. PGRs, diverse in both their chemistry and use, are part of the management tools that can be used to ensure efficient cotton production system.

PGRS AND COTTON MANAGEMENT

Management of cotton with plant growth regulators (PGRs) is a season-long process. A successful PGR program encompasses a systems approach that includes many crucial decisions. Because we cannot predict weather with 100% accuracy, it is important to minimize factors that contribute to stress as much as possible. Fine-tuning fertility programs, water management, and pest control are key in optimizing lint production. There are no substitutes for sound cultural practices. One way that producers can supplement these inputs is by judicious use of plant growth regulators. These compounds are not meant to be used as a salvage or rescue operation, but should be used to more efficiently manage the crop to adjust plant growth and to improve lint yield and quality. This can best be achieved through the use of well-adapted, high-yielding cultivars. Again, there is no substitute for genetics, but even the "best" cultivar cannot be expected to provide higher yields under all circumstances due to inconsistencies in the environment. PGRs and other stress management practices can be used in an effort to consistently produce higher yields.

Yield of the cotton plant is determined by a combination of factors: boll number, boll size, seed number per boll, and fiber/seed. These parameters are influenced by the physiological activity of the plant and its interaction with the environment. Due to the perennial nature of cotton, fruiting continues during its maturation, thus impacting any or all of these parameters. According to Mauney (1986), three nodes on each sympodium (fruiting branch) are most likely to mature. His scenario is that about 50 prime squares will be produced by the presence of 18 sympodia (3 squares/sympodium X 18 sympodia/plant = 54 squares/plant). If 50, 30, and 10% of the squares at fruiting position 1, 2, and 3, respectively, mature into open bolls with 1.5 grams of lint per boll, a population of 30,000 plants per acre would yield 1500 pounds of lint/acre. This data is reflective of work reported by Mauney (1986) for studies conducted at various locations in the U.S. from 1940 through 1982. A major portion of this yield is contributed by nodes 9 through 14 (Jenkins et al., 1990), depending on length of the growing season and other cultural inputs. According to the nutritional balance theory of fruiting, the cotton plant will set as many bolls as it can produce substrate for and maintain maximum growth (Guinn, 1976a). Seed number, mass and surface area, and lint mass, as well as fiber number, were recorded for the first fruiting position bolls located from nodes 9 and 14 in an irrigation by plant density study (Feng et al., 2010). In this study, individual seed surface area and mass increased with increases in irrigation and decreases in plant density. Seeds per locule responded in a likewise manner. Fiber number per unit seed surface, however, were not affected by any of the treatments suggesting this component was likely heritable.

Temperature

Two of the major abiotic stresses impacting cotton growth and development, and thus PGR usage are temperature and water stress. Temperature purportedly has only a small effect on canopy photosynthesis, but strongly influences vegetative growth and development, light capture during the vegetative period, and light conversion during much of the boll-filling period (Reddy and Hodges, 2006). Previ-

ous studies show that the minimum temperatures for cotton growth is about 15° C and the optimum is about 28° C (Reddy et al., 1992), which is well below commonly occurring air temperatures in most cotton growing areas. We know that cotton is capable of much higher productivity than typically observed even under the best management practices. Although photosynthesis is an important component of yield, it generally correlates poorly with dry matter production or harvestable yields because of the multiplicity of factors limiting yield (Evans, 1993) and the fact that this conclusion was drawn from instantaneous measurements (often on single leaves) conducted under standardized conditions rather than from seasonal measurements on canopy photosynthesis in the field (Zelitch, 1982). However, Cornish et al. (1991) reported that genetic advances in cultivated cotton types (Gossypium barbadense L.) were closely associated with increasing single-leaf photosynthesis rate and stomatal conductance, when grown under greenhouse conditions. One would assume that higher stomatal conductance increases CO2 diffusion into the leaf that would favor higher photosynthetic rates. In chambers with twice atmospheric [CO2] (720 µmol CO2 mol-¹ air) maximum photosynthetic rates were about 6 mg CO2 m-² s-¹ compared to maximum rates of about 4 mg CO2 m-² s-¹ at 360 µmol CO2 mol-¹ air (Reddy and Hodges, 2006). If these higher photosynthetic rates are sustained they could in turn favor higher crop yield. Previous work with advanced Pima cotton lines showed a higher photosynthetic capacity than older, low-yielding cultivars, but use of the same leaves used to measure stomatal conductance showed that photosynthetic rates in these same leaves were not positively correlated with yields (Radin, 1994). Therefore, it appears that higher stomatal conductance favors higher yields by a mechanism not directly related to photosynthesis. Studies by Lu et al. (1998) pointed out that selection for higher yields in irrigated crops at high temperature indirectly imposed selection pressure for higher stomatal conductance than lowered leaf temperature. Subsequently the deleterious effects of heat stress on critical flowering and fruiting stages were reduced, thus leading to higher crop yields.

Hodges et al. (1993) showed that high temperatures strongly influence numbers of vegetative and reproductive branches in cotton. Vegetative branches increased and fruiting branches decreased with high temperatures. In this study, number of fruiting sites increased by 50% as temperature was increased from 30 to 40˚ C; however, number of squares and bolls decreased dramatically above 35˚ C to a value of zero at 40˚ C. Later work by Bibi et al. (2008) and Snider et al. (2009) indicated that photosynthesis in cotton is highly sensitive to temperatures above 35˚ C which detrimentally affects quantum efficiency of the photosynthetic apparatus and decreases chlorophyll content (Snider et. al., 2010). The temperature effect is especially important with respect to rubisco activase, which is necessary for activation of ribulose-1, 5-bisphophate carboxylase/oxygenase (Rubisco) (Crafts-Brandner and Salvucci, 2000). As temperatures increased, the rate of Rubisco deactivation exceeded the capacity of activase to promote activation. This results from activase being inhibited by a lower temperature than that for Rubisco. Rubisco activation decreased when leaf temperature exceeded 35˚ C, whereas activities of isolated activase and Rubisco were highest at 42˚ C and >50˚ C. Studies in Arkansas using membrane leakage and fluorescence as techniques for determining tolerance of cotton germplasm to high temperature failed in most instances to show significant differences, but a few lines were identified with appreciable temperature tolerance (Oosterhuis et. al., 2009). Similar measurements were made in a multi-level determination of heat tolerance in cotton under field conditions by Cottee et. al. (2010).The most rapid and reliable screens for heat tolerance in this study with high yielding cotton included electron transport rate, membrane integrity, and enzyme viability. Kawakami et. al. (2010) showed that 1-methylcyclopropene treatment to cotton at first flower and first-flower plus two weeks significantly increased seed cotton and lint yield in a two-year study compared to the untreated control. In both years of this study, maximum temperatures were well above the optimum 30˚ C temperature for cotton at the study location, indicating that cotton was under heat stress. Therefore, PGRs

have shown potential for increased yield under stress conditions. Efforts to identify increased thermotolerance and tolerance to water limitation remain high priorities for improving yields in cotton.

Water-Deficit Stress

Temperature and water stress often occur simultaneously in cotton producing areas, and the ability to identify crop response to either one in solo is difficult. Wanjura et al. (1984) investigated the use of canopy and air temperature differences to compute a crop water stress index (CWSI) for assessing plant water status using cotton crop canopies that fully or partially covered the ground. The results showed that the slope of the non-stressed baseline of the CWSI for a cotton crop with a canopy which had about 50% ground cover was approximately one-half of that reported for full canopies. The study emphasized the importance of complete canopy for calculating CWSI values by either "theoretical" or "empirical" procedures. CWSI calculated under complete canopy condition agreed more closely for the two procedures than when they were calculated under a partial canopy situation. Since the effective use of plant growth regulators is often stress based, we continue to search for methods that serve as more reliable triggers of stress. Similar to the work of Wanjura et al. (1984), Howell et al. (1982) previously found that canopy temperature in cotton was a sensitive indicator of water stress caused by either soil water deficit or soil osmotic stresses. Recent work (Conaty et al., 2012) indicated that a plant-based thermal optimum approach to irrigation scheduling provides potential benefits when water applications are scheduled on basis of plant response to water stress. The ability of the plant to maintain its optimum temperature (Topt) range uses the principle that plant performance is maximized when a plant is maintained at this temperature. Methods of achieving continuous measurement of plant canopy temperature in agricultural settings can be achieved with a low-cost wireless temperature monitoring system compared to that of higher-cost industrial-grading sensors, thus potentially making them a viable alternative in many agricultural settings (Mahan et al., 2010). Mahan et al. (2010) stated there are conditions where plants cannot evaporate fast enough to maintain their temperature below the Topt, regardless of how well they are supplied with water. Although cotton is considered to be a drought-tolerant crop, sensitivity varies greatly among genotypes (Igbal et al., 2011) Moreover, for successful breeding of cotton cultivars to drought, there must be significant variability to water stress and this variation must be genetically controlled (Mitra, 2001). The occurrence of variation for drought tolerance within G. hirsutum has been shown (Pettigrew, 2004a; Basal et al., 2005), but less is known about the genetic mechanism that controls this variation in drought tolerance of G. hirsutum (Singh and Singh, 2004). These factors further complicate achieving consistent responses to PGR use, but PGRs have shown a potential to partially alleviate the detrimental effects of water stress on specific physiological activities of cotton growing under growth chamber conditions (Fernandez et al., 1992; Zhao and Oosterhuis, 1997). The more we understand of the complex interaction of the different plant stresses with PGRs, especially water and temperature, the more successful we can become in knowing when and in what quantities PGRs can be used to effectively reduce these stresses.

Plant Hormones

Plant hormones are plant-made (endogenous) growth regulators that alter growth and development. According to Davies (2010), the concept of plant hormones was first defined in a 1937 publication based on animal physiology. Plant hormone was described as an organic compound that was synthesized in one part of a plant and translocated to another part, where it caused a physiological response. Currently, the concept based on animal physiology clearly no longer applies to the definition of a plant hormone,

since the synthesis of hormones in plants can either be localized as in animals or occur in a wide range of tissues. The site of action may or may not coincide with the site of synthesis. Ethylene, the only plant hormone that is a gas, causes physiological changes at the site of synthesis, and has no need of being transported. Transport is only required if the hormone sites of action and synthesis do not coincide.

Plant hormones serve as chemical messengers to coordinate growth, development, differentiation and environmental responses. Plant hormones at very low concentrations are able to cause responses through a signal transduction pathway that produces a cascade effect. The five classical major plant hormones are auxins, gibberellins, cytokinins, abscisic acid and ethylene (Gaspar et al., 1996). The search to identify new plant hormones is ongoing, resulting in the recognition of five additional new compounds as plant hormones: brassinosteroids, jasmonates, salicylic acid, polyamines, and peptides (Davies, 2010). In reality, plant hormones do not perform alone, but rather in conjunction or opposition to each other resulting in plant growth/development changes. The following presents an abbreviated summation of the major plant hormones and their physiological roles in crop growth and development.

Auxins

Decapitated grass coleoptiles were recognized as being able to bend after having their growth stimulated by agar blocks saturated with diffused substances from the tips of grass coleoptiles (Went, 1926). The substance that was diffused to the agar blocks was later named auxin, constituting the first plant hormone ever discovered (Davies, 2010). It was subsequently demonstrated that auxin was synthesized in the tips of grass coleoptiles and moved basipetally, i.e. from the tip to the base (Wildman, 1997). The most important representative in the auxin group is indole-3-acetic acid (IAA) since it is one of the main auxin molecules present in the majority of plants (Davies, 2010). The IAA molecule is not only found in its original form, but is also present in plants in various conjugated forms. Auxin has little transport in xylem and phloem. Auxin transport is mainly polar and proceeds from the apex to the base (basipetally) in the shoots. In roots, besides having a basipetal transport, auxins are also transported acropetally (base to tip). The leaf apex, young leaves, as well as developing seeds, serve as the primary sites for auxin biosynthesis, which can occur from tryptophan-dependent or tryptophan-independent pathways (Bartel, 1997). Genetic and biochemical studies, however, have indicated that tryptophan is the main precursor for IAA in plants (Woodward and Bartel, 2005; Zhao, 2010). Auxins promote cell enlargement, stem elongation, apical dominance (repressing growth of lateral buds), vascular differentiation (xylem and phloem), tropistic responses (root and shoot response to light and gravity), and growth of flower parts, as well as delaying senescence. Auxins induce fruit set and growth in some fruit, delay fruit ripening, and stimulate flowering in bromeliads. At high concentrations, this plant hormone inhibits root growth (Chadwick and Burg, 1967).

Cell elongation as well as cell wall loosening (plastic nature of walls from cells) is caused by auxins (Salisbury and Ross, 1992). The most popular mechanism that explains cell wall loosening is the acid-growth hypothesis (Ray, 1987) This hypothesis proposes that auxins cause receptor cells in stem sections to secrete H+ into their surroundings. Secreted H+ ions eventually reduce the pH, presumably allowing activation of certain cell wall-degrading enzymes which are inactive at a higher pH. The activity of these enzymes breaks bonds in the wall polysaccharides resulting in wall loosening and accelerated growth through increased cell turgor pressure.

Gibberellins

Investigations in plant diseases led researchers to the discovery of gibberellins (GAs). The name gibberellin originated from the fungus Gibberella fujikuroi, from which culture filtrates allowed

scientists to gain chemical identification of this group of plant hormones (Davies, 2010; Wildman, 1997). Gibberellins contain over 136 compounds identified in various fungi and plants (MacMillan, 2002), with all containing the ent-gibberellane structure (Davies, 2010). Among these already identified GA compounds, gibberellic acid (GA3), a fungal product, is the one most widely available, with GA1 being the most important GA in plants. The vast majority of the GAs are precursors or inactivation products of the biologically growth-active form of GA1.

Gibberellins are mainly synthesized in young seeds and tissues of the shoot tissues from glyceraldehyde-3-phosphate via isopentenyl bisphosphate taking place initially in chloroplasts followed by activities in the cytoplasm (Davies, 2010). Gibberellins exhibit many physiological effects, suggesting that they have more than one primary site of action. More specifically, GA1 stimulates cell elongation and division in the stem, which together with cell turgor pressure, causes its elongation (Davies, 2010). Gibberellin causes bolting in long-day plants such as cabbage, germination in seeds that require cold/light to break dormancy, production of enzymes such as amylase that is required during seed germination, and fruit setting and growth as in grapes treated with GA (Taiz and Zeiger, 2010). Gibberellins also are linked to changes in juvenility and flower sexuality through induction of maleness in dioecious flowers, and are known to increase leaf size of a number of different plants (Taiz and Zeiger, 2010). The most important bioactive agents for vegetative growth and development are probably GA1 and GA4. In most species that have been investigated, GA1 is the predominant bioactive (Hedders, 1999).

Cytokinins

Tissue culture experimentation led scientists to the discovery of cytokinins (Davies, 2010; Wildman, 1997). The chemical identification of this group of plant hormones was made possible with the use of autoclaved herring sperm DNA (Davies, 2010). Cell division is known as cytokinesis, which has resulted in the term cytokinin being assigned to substances that typically stimulate cell division. Cytokinin synthesis derivates from adenine, a purine base found in RNA/DNA, and is most abundant in the young, rapidly dividing cells of the shoot and root apical meristems. Cytokinin transport is mainly through the xylem system. Besides being involved in cell division (either in tissue culture in the presence of auxin, or in crown gall tumors, or in actively dividing tissues), cytokinins participate in seed and chloroplast development (exogenous application of cytokinins leads to accumulation of chlorophyll and conversion of etioplasts to chloroplasts) , chloroplast maturation, leaf senescence delay and expansion, cell enlargement, and embryo development (Davies, 2010; Hare et al., 1997). Cytokinins stimulate the synthesis of specific chloroplast proteins that are encoded by nuclear genes and synthesized by cytoplasmic ribosomes (Binns, 1994; Taiz and Zeiger, 2010).

Abscisic Acid

Studies on abscission control and dormancy led to the finding of abscisic acid (ABA) (Davies, 2010; Wildman, 1997) which is a single compound isolated from cotton fruits in the early 1960s. Addicott and colleagues first identified ABA while studying compounds related to cotton fruit abscission (Ohkuma et al., 1963). Since it was believed that ABA was involved in abscission, the compound was then named abscisic acid (Addicott et al., 1968). Nowadays, it is known that in fact ABA has little effect on abscission which is mainly driven by ethylene. ABA is synthesized via isopentenyl diphosphate and carotenoids from glyceraldehyde-3-phosphate in almost all cells that contain plastids in roots and mature leaves (Davies, 2010). ABA, which is classified as a growth inhibitor (since exogenous applications do

inhibit growth in plants) (Davies, 2010), inhibits auxin-induced cell growth by preventing cell loosening (Zeevaart and Creelman, 1988). ABA also inhibits growth by interfering with nucleic acid synthesis, reducing the rate of cell enlargement, and reducing the rate of cell division. ABA is a promoter of bud and seed dormancy, and mutants that are deficient in ABA are viviparous (Pilet and Barlow, 1987). In addition, ABA is considered the plant's signal for water stress. The root synthesizes more ABA under water stress which is translocated to the shoot. ABA levels of the leaf can increase 50-fold during water stress (Christmann et al., 2005). The increased concentration of ABA in turn induces the closure of the guard cells of the stomata because high concentrations of ABA cause potassium and other ions to leave the guard cell. After the ions leave the guard cell, the guard cell loses turgidity and the stomata close (MacRobbie, 1997). In addition to closing stomata, ABA increases hydraulic conductivity of the root and increases the root:shoot ratio at low water potentials (Taiz and Zeiger, 2010).

Ethylene

The burning of the gas used for public and private illumination during the 19th century led to the discovery of ethylene (Davies, 2010; Wildman, 1997). Chemical identification of ethylene was possible through study of this illumination gas (Davies, 2010). Ethylene is a gaseous plant hormone involved in a wide range of physiological processes that range from seed germination to apoptosis (cell death). Although its concentration in plants is normally low, levels are greatly increased during particular physiological processes such as leaf and flower abscission, fruit ripening, as well as in responses to a wide range of biotic and abiotic stimuli (Lin et al., 2009). The capability of higher plants to produce ethylene is evident in all tissues. The rate at which ethylene is synthesized varies among plant tissues and is affected by the age of the respective tissue (Mattoo and Suttle, 1991).

Ethylene Synthesis

Two key enzymes are involved in the ethylene synthesis pathway. The first enzyme ACC-synthase (ACS) converts S-adenosylmethionine (SAM), which originates in the methionine cycle, to 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is then oxidized to ethylene by ACC-oxidase (ACO) (Chaves and Mello-Farias, 2006; Kende, 1993; Zarembinski and Theologis, 1994). Tissues that do not produce significant levels of ethylene have low ACS activity, but upon stimulation ACS activity can be quickly induced (Chae et al., 2003). Both ACS and ACO can be induced upon stress (Morgan and Drew, 1997). Unlike ACS, ACO has a constitutive activity present in most tissues. Thus, one of the major steps during ethylene induction is ACS, which is a rate-limiting enzyme (Chae et al., 2003). The ACS6 gene encodes for one of the ACS proteins and is part of a multi-gene family (Fluhr and Mattoo, 1996; Kende, 1993; Tsuchisaka and Theologis, 2004) in which all genes are independently regulated (Fluhr and Mattoo, 1996). *ACO2* also belongs to a multi-gene family encoding ACO proteins (Barry et al., 1996; Kende, 1993). Ethylene perception occurs when the plant hormone binds to an ethylene receptor (ETR). ETRs are a family of membrane receptors (Chang et al., 1993), and the ETR5 gene encodes for a membrane protein which is part of this multi-gene family. Ethylene perception and its signal transduction pathway that follows are feedback regulated (Urao et al., 2000).

It is desirable to protect yield by preventing fruit loss induced by the peak in ethylene prior to abscission. Thus the need for alternatives that could reduce or prevent abortion of cotton bolls under stress is worthwhile. Heitholt et al. (1993) suggested that preventing loss of flowers and young fruit is essential in cotton yield enhancement; thus ethylene inhibitors may provide an alternative for reducing the loss of reproductive structures in an effort to improve cotton yield.

Water-Deficit Stress and Ethylene

In recent years drought stress tolerance has become one of the main points of interest to agronomic research since major crops such as cotton are experiencing drier years than normal due to changes in weather patterns (Gowda et al., 2007; Pettigrew, 2004a). As a result, declining irrigation reserves are occurring together with an increase in costs associated with irrigation (Gowda et al., 2007). This is due to dwindling water supplies from aquifers that have had less recharge (Howell et al., 2004). Water-deficit stress detrimentally impacts cotton production (Howell et al., 2004; Mooney et al., 1991; Pettigrew, 2004b). Although cotton is able to maintain a leaf turgor potential by osmotic adjustment while facing moisture deficit (Oosterhuis and Wullschleger, 1987), it eventually faces a reduction in leaf water potential under dry conditions (Nepomuceno et al., 1998; Turner et al., 1986). In response to drought, stomata tend to close reducing their conductance which consequently affects leaf photosynthesis (Ephrath et al., 1990; Faver et al., 1996; Genty et al., 1987). Under water-deficit stress, the overall dry matter accumulation in cotton plants decreases (Mooney et al., 1991) and expansion of leaf blades and plant growth is reduced, thus promoting stunted growth (Ball et al., 1994; Gerik et al., 1996). Limited water availability causes cotton plants to generate fewer flowers resulting in reduced boll production (Guinn and Mauney, 1984).

The variable which contributes most to lint yield is the number of bolls per unit area (Boquet et al., 2004; Worley et al., 1974; Wu et al., 2005). Therefore, increased boll abortion in plants under severe stress during their reproductive development consequently reduces lint yield (Gerik et al., 1996; Pettigrew, 2004a; Turner et al., 1986). One of the factors interacting with stress is hormones. A burst in ethylene synthesis that lasted four days was observed prior to occurrence of boll abscission (Morgan et al., 1992). The authors suggested that this peak in ethylene synthesis may be the signal necessary to initiate cell wall hydrolysis in the abscission zone followed by abscission of that particular structure.

Diverging opinions exist on the impact of water deficit on ethylene synthesis. Reports of increased ethylene synthesis due to water stress were based on detached plant parts being subjected under a rapid dry down period and then stored in closed chambers while air samples were collected for ethylene measurements (Adato and Gazit, 1974; Aharoni, 1978; Apelbaum and Yang, 1981; Ben-Yehoshua and Aloni, 1974; Bergner and Teichmann, 1993; Hoffman et al., 1983; Huberman et al., 1993; McKeon et al., 1982; McMichael et al., 1972; Michelozzi et al., 1995; Narayana et al., 1991; Tudela and Primo-Millo, 1992; Wright, 1977; Wright, 1981). On the other hand, ethylene emission studies which exposed plants to a gradual dry down period by terminating watering and collecting air samples from intact plants or plant parts placed in closed chambers, with or without constant air flow, indicated that water-deficit stress did not increase ethylene production (Ben-Yehoshua and Aloni, 1974; Eklund et al., 1992; Feng and Barker, 1992; Hubick et al., 1986; Morgan et al., 1990; Narayana et al., 1991).

Brassinosteroids

Brassinosteroids were discovered in Brassica pollen extracts. Their properties in plant growth were demonstrated through bioassay in bean petioles (Davies, 2010). Brassinosteroids include over 60 steroidal compounds (Davies, 1995) that are classified as growth promoting substances accelerating cell division and cell elongation (Adam and Marquardt, 1986; Clouse and Sasse, 1998). In addition, brassinosteriods are also involved in light-regulated development, and brassinosteroid-induced cell growth is light dependent (Li et al., 1996). Kasukabe et al. (1999) filed a patent on the production of cotton fibers with improved fiber characteristics by treatment with brassinosteroids. Subsequent work by Sun et al. (2005) showed that exogenous applications of the brassinosteroid brassinolide (BL)

promoted fiber elongation while treatment with brassinazole (Brz), a brassinosteroid biosynthesis inhibitor, inhibited fiber development. When cotton floral buds were treated with Brz, fiber differentiation was completely absent. In addition, expression of fiber genes associated with cell elongation increased in ovules treated with BL and was suppressed by Brz treatment, establishing a correlation between brassinosteroid-regulted gene expression and fiber elongation (Sun et al., 2005).

Jasmonates

Jasmonic acid and its methyl ester, substrates of the biosynthesis of jasmonates, are considered powerful senescence-promoting substances (Gross and Parthier, 1994; Ueda et al., 1991). Jasmonates accelerate senescence by reductions in chlorophyll content and degradation of chloroplast proteins, especially Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) (Beltrano et al., 1998; Creelman and Mullet, 1995). They are also known to promote tuber formation, fruit ripening, pigment formation, and tendril coiling while inhibiting growth and seed germination (Davies, 1995; Gross and Parthier, 1994). Recent work has focused on the role of jasmonate in promoting abscission. Jasmonic acid and its methyl ester affect sugar metabolism in the abscission zone in bean petioles through an increase in cellulase activity involved in the degradation of cell wall polysaccharides (Ueda et al., 1991). In addition, jasmonates are involved in the plant's defense against water stress, wounding, insect attack, and pathogen attack (Baron and Zambryski, 1995; Creelman and Mullet, 1995; Creelman and Mullet, 1997). Jasmonates also induce ethylene formation (van Loon et al., 1998).

Salicylic Acid

Salicylic acid, which is chemically related to aspirin, belongs to a diverse group of plant phenolics (Raskin, 1995). Like jasmonates, salicylic acid may be involved in the resistance to pathogens because it induces the production of pathogenesis-related proteins. However, the salicylic acid defense pathway is independent of the jasmonate defense pathway (van Loon et al., 1998). Pathogenesis-related proteins are protein compounds with antimicrobial and antifungal activities; eleven pathogenesis related protein families have been characterized (Sticher et al., 1997). Transgenic tobacco plants lacking the ability to produce salicylic acid were unable to induce a resistance mechanism, called systemic acquired resistance, to certain plant diseases (Baker et al., 1997; Delaney et al., 1994; Hammerschmidt and Becker, 1997; Ryals et al., 1996). Salicylic acid also enhances flower longevity, inhibits ethylene biosynthesis, inhibits seed germination, blocks the wound response, and reverses the effects of ABA (Davies, 1995; Gross and Parthier, 1994).

Salicylic acid serves as a trigger for increasing the activity of alternative respiration (Kapulnik et al., 1992). Alternative respiration refers to a minor respiratory pathway in plants that is not sensitive to cyanide, unlike conventional respiration. Alternative respiration represents approximately 27 to 30% of the electron flow through the electron transport chain (Lennon et al., 1997; Ordentlich et al., 1991). Because alternative respiration does not produce much energy in the form of ATP, most of the energy produced in alternative respiration is released as heat. The heating of plant tissue caused by an increase in alternative respiration is coined thermogenecity. Thermogenecity plays a key role in increasing the temperature of Araceae (Arum family) inflorescences by as much as 25° C. Increasing the temperature of the inflorescence volatilizes amine compounds, and the odor given off from the amines attracts insect pollinators (Taiz and Zeiger, 2010). The alternative oxidase associated with alternative respiration has been implicated in various biochemical processes including a role in lowering mitochondrial reactive oxygen production in tobacco cells (Ribas-Carbo et al., 2000) to alleviate stress, as

an avenue for improving tolerance to chilling injury, and as a means for improving resistance to various pests. Bi et al. (1997a) had previously indicated that insect herbivory on cotton induced resistance to the cotton bollworm (Helicoverpa zea). Since abundant evidence had accumulated showing that salicylic acid plays a key role in coordinating the expression of systemic acquired resistance against phytopathogens (Vernoolj et al., 1994), Bi et al. (1997b) investigated whether herbivory impacted production of foliar salicylic acid and hydrogen peroxide, a frequently observed response following pathogenesis. In cotton, herbivory enhanced foliar catalase and ascorbate peroxidase activities, but the application of salicylic acid or methyl salicylate to cotton plants did not affect foliar resistance to H. zea. Studies by Heitholt et al. (2001) also failed to show a response to exogenously applied salicylic acid relative to flower production, boll retention, and yield.

Polyamines

Polyamines are not only crucial components in DNA, but are also vital substances present in all forms of life. Polyamines are classified as a plant hormone group since they promote plant growth and development at small concentrations (Davies, 2010). Polyamines are generally antagonistic to abscisic acid and are indispensable to plants at the time of flowering, as well as at the time of early fruit development (Kloareg et al., 1986). Thus, a deficiency of polyamines during flowering and early fruit development causes direct negative effects on the reproductive development of plants. Bibi et al. (2010) examined the effect of putrescine, one of the most common polyamines, on ovary development and seed set of cotton under high temperature stress (day/night temperature of 38/20° C) and controlled environmental conditions. Putrescine was applied to floral buds of cotton 24 hours prior to anthesis. Increased temperature in this study decreased seed set in cotton flowers which was ameliorated by the exogenous application of putrescine. Putrescine application led to increased levels of putrescine in cotton flowers, which was associated with increased seed set despite the negative effect of increased temperature.

Peptides

To date, four peptide signal molecules have been discovered in plants: systemin, endo40, cyi1a, and sulfokine (Franssen, 1998), although plants appear to possess the receptors for a plethora of peptide signals (Schaller, 2001). Plant peptides are active in the nanomolar to picomolar range (Van de Sande, 1996). The peptide signal molecule systemin behaves as an active factor that is transported out of the wounds of wounded tomato plants to distal tissues inducing the expression of two well characterized wound-inducible proteinase inhibitor encoding genes (Pearce et al., 1991). As such, these genes are systemically induced in tomato plants as part of the inducible defense repertoire of the plant. Endo40 was reportedly isolated as a gene that is activated during root nodule formation on legumes as a result of the interaction of these plants with soil-borne Rhizobium bacteria (Yang et al., 1993). Additional studies suggest that the function of endo40 is not restricted to nodule formation (Franssen, 1998), but that it may also play a role in cell proliferation, which in most cases is controlled by an auxin-cytokinin balance. Miklashevichs et al. (1997) suggest that cyi1a encodes a peptide which participates in the events downstream of a junction point of cytokinin and auxin action that leads to cell division. Three of the four peptides that have been isolated thus far appear to have a role in cell division and proliferation, these being enod40, sulfokins, and cyi1a. Of these three, enod40 and cyi1a seem to interact with the activity of the classical hormones auxin and cytokinins; sulfokins, however, work independently of these hormones (Franssen, 1998).

PLANT GROWTH REGULATORS

Several plant growth regulators (PGRs) are available in the market for cotton production systems. These growth regulators are organized into groups based on the stages of development at which they trigger a response: germination, seedling, vegetative, reproductive developments, and harvest aids. Our discussion of the PGRs will focus mainly on vegetative and reproductive stages and cotton flowering and fruiting aspects to positively influence cotton production.

Gibberellins and Auxins

In a study with four concentrations (50, 100, 150, 200 mg/l) of gibberellic acid (GA3), cotton sprayed just before anthesis (five-week-old plants) produced a significantly greater number of flowers (at the two highest concentrations) than did the control (Mathur and Mittal, 1964). Although all four concentrations of gibberellic acid increased the number of flowers shed during the eight-week-period, the highest concentration still retained more flowers than the untreated control. The potential for increasing yield through increased flower production is more importantly reflected in the components of the mature boll (boll size, boll weight, ovules (seed)/boll, fibers/seed, and weight per fiber). Yield may be increased through a single increase in a given input or by a multiple additive effect. Miller and Rawlings (1967) reported that as yield increased by selection, lint percentage and seeds per boll increased while boll and seed size decreased. If boll size is decreased, the number of bolls produced per acre and the total surface area of seed therein becomes more important. Experiments by Giavalis and Seagull (2001) demonstrated changes induced by hormone application can increase fiber initiation. Significant increases in fiber production, relative to untreated controls, were found with an exogenous application of either indole-3-acetic acid or gibberellic acid. The largest increase in fiber initiation was realized with a pre-anthesis treatment of indole-3-acetic acid. These authors suggested 1) that manipulation of the hormone level might cause an increase in the proportion of epidermal cells that differentiated as fibers or 2) that hormone treatments might induce cell division, resulting in more epidermal cells that could potentially lead to a greater number of fiber cells. Berlin (1986) reported that fiber number per ovule varied among species and cultivars. Examination of ovule surfaces on the day of anthesis by scanning electron microscopy revealed about 60,000 cells per ovule regardless of the cotton type. This number increased from about 1,000 epidermal cells at 23 days preanthesis to the 60,000 at anthesis and to nearly 350,000 cells at 6 days postanthesis. Initial ovule fiber cell members were controlled primarily by additive gene effects in a study by Bowman et al. (2001) suggesting that positive combining ability of some cultivars for that trait would make them good parents in a breeding program for improving fiber cell numbers. Reports of the proportion of epidermal cells that develop into fibers vary from 10% (Ryser, 1999) to 25% (Beasley, 1975). The good news is that the development cues for fiber production appear to be present over a considerable time frame, so there may be a long “window of opportunity” over which development can be manipulated to increase fiber production (Seagull and Giavalis, 2004).

Although the data for the timing of fiber initiation is varied, most reports indicate that initiation begins several hours to several days before anthesis (Berlin, 1986; Joshi et al., 1967; Stewart, 1975). Chen and Guan (2011) reported that increasing auxin levels at the right time and place during ovule and fiber development improves the yield and quality of cotton fibers. Their contention is that biotechnology succeeded in increasing cotton yields through introduction of transgenes for herbicides and insecticides. However, the ability to improve cotton quality has not been possible without penalty in fiber yield or seed size or number. Simultaneous improvement in yield and quality of cotton fiber was

obtained by over-expression of a gene responsible for the synthesis of the auxin indole-3-acetic acid (Chen and Guan, 2011). The question is whether these same responses can be elicited by exogenous foliar applications of auxins.

Chaperone

Chaperone is a PGR containing the nitrophenolates sodium 5-nitroguaiacolate, sodium ortho-nitrophenolate, and sodium para-nitrophenolate. These active ingredients, termed nitrophenolates, are found naturally in plants and have been shown to stimulate plant growth by altering the activity of specific antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) (Djanaguirman et al., 2004). These enzymes are involved with scavenging reactive oxygen species, such as superoxide ions, hydrogen peroxide (H2O2), hydroxyl (OH-), and singlet oxygen (O2-). Reactive oxygen species can attack proteins, nucleic acids, and polysaccharides (Matysik et al., 2002) and are increased in response to plant stress (Apel and Hirt, 2004; Gill and Tuteja, 2010). As a result of their overproduction, more are produced than are metabolized and oxidative stress occurs (Dhindsa et al., 1981). Therefore, the ability to lessen the impact of ROS on the physiology and yield of crop species is desirable. As early as 1995, Guo and Oosterhuis (1995) stated that Atonik, the former name of Chaperone, may enhance cotton growth and yield through increased assimilation of nutrients, nitrate reduction, and photosynthesis, as well as improved translocation, cytoplasmic streaming, and increased cell integrity. Guo and Oosterhuis (1995) also reported that Atonik hastened cotton maturity by 7 days compared to the non-treated cotton, but failed to show differences in lint yield. In years such as the 2001 growing season in Arkansas, which was a favorable year for cotton production, Atonik failed to show significant differences between treatments for yield or components of yield when applied alone or in combination with mepiquat chloride (Oosterhuis et al., 2001). Accordingly, spray application of other PGRs also failed to show significant yield responses. Subsequent work by Oosterhuis and Brown (2003) suggested that Chaperone may be a viable means for enhancing lint yields in cotton through enhancement of plant protein levels with concomitant increase in endotoxin levels. Increases in bollworm mortality were recorded for growth-chamber and field studies; all Chaperone treatments showed increased bollworm mortality that was increased with increasing rates of Chaperone. The results of two field studies (Study 1 - 2004 and 2005 in 28 locations in Texas at the commercially recommended rate of 0.43 g ai ha-1 and Study 2 - 8 locations from 2001-2005 in Burleson County, Texas at three rates: 0.43 g ai ha-1, 0.86 g ai ha-1, and 1.72 g ai ha-1) showed no differences in lint yield in Study 1 between Chaperone treated and the untreated control. However, in Study 2, across all experiments, Chaperone at 1.72 g ai ha-1 increased lint yield by 7.5% over the untreated control (Bynum et al., 2007). Results from this study did not support the use of Chaperone in cotton at its current recommended rate. Field studies were conducted from 2002 to 2005 in India (Djanaguiraman et al., 2010) to evaluate foliar spray of Atonik on cotton boll abscission rate by monitoring various reactive oxygen species (ROS) contents, antioxidant content, and antioxidant enzyme activity from 1 to 9 days after anthesis. This work suggested that the nitrophenolate (Atonik) sprayed plants counteracted deleterious effects of ROS by a peroxide/phenolics/ascorbate system, resulting in reduced boll abscission and increased yield. Yield responses to the nitrophenolate PGR have been inconsistent in tomato (Lycopersicon esulantum L.) and strawberry (Fragaria X ananassa) plants (Djanaguiraman et al., 2004; Zurawicz, 2004). Investigations with peanut (Arachis hypogaea L.) showed no effect on pod yield, percent extra-large kernels, percent total sound mature kernels, and crude protein levels of seed under a range of environmental and edaphic conditions with four cultivars.

PGR-IV

Another example of a combination of compounds in a PGR includes PGR-IV, which is a combination of gibberellic acid, indolebutyric acid, and a propriety fermentation broth. Various responses were observed for yield enhancement in studies by Oosterhuis and Zhao (1994). Foliar application gave yield enhancement that was associated with increases in leaf growth, nutrient uptake, and boll number, whereas yield enhancement from a soil application was associated with enhanced root growth and nutrient uptake. Subsequent work indicated that PGR-IV application made to plants grown in growth chambers with water stress imposed had the ability to partially alleviate the detrimental effects of water stress on photosynthesis and dry matter accumulation (Zhao and Oosterhuis, 1997). Additional studies were conducted to determine if PGR-IV was beneficial for increasing fruit retention of shaded cotton (Zhao and Oosterhuis, 1998). Shade shelters provided a 63% sunlight reduction. Shading during early squaring did not affect yield; however, shading after the first flower stage significantly increased leaf chlorophyll concentration and fruit abscission and also decreased leaf photosynthetic rate, nonstructural carbohydrate concentrations, and lint yield. An application of PGR-IV to the foliage before shading gave a numeric increase of 6 to 18% in lint yield compared with shaded plants not treated with PGR-IV. Guinn (1976a, 1982) had previously reported that ethylene and abscisic acid contents increased dramatically under low-light conditions resulting in boll abscission. A study by Biles and Cothren (2001) in Texas examined the use of PGR-IV and mepiquat chloride on cotton flowering when applied alone or used in sequential applications. The mepiquat chloride and PGR-IV + mepiquat chloride treatments caused plants to have a season-long average of 0.55 and 0.48 more flowers m-1 of row day-1, respectively, than the untreated plants. Earlier work (Oosterhuis and Zhao, 1993; Robertson and Cothren, 1995) suggested that yield increases resulted from increased boll numbers and boll weight (Faircloth, 2007).

Mepiquat-Based PGRs

One of the most widely used PGRs in cotton production is mepiquat chloride (MC) and similar products (Table 1).

Table 1. Mepiquat and Mepiquat-like growth regulators.

Common Name	Trade Name	Company
Mepiquat chloride	Mepex	DuPont™
Mepiquat chloride + kinetin	Mepex Ginout	DuPont™
Mepiquat chloride + cyclanilide	Stance	Bayer
Mepiquat pentaborate	Pentia	BASF

The original intent of this product was to suppress vegetative growth and reduce plant height. In situations where excess moisture and nitrogen were problems, this compound effectively reduced plant height in most instances (Nuti et al., 2006; Zhao and Oosterhuis, 1999), but was not necessarily associated with yield increases (Heilman, 1981; Boman and Westerman, 1994). Mepiquat chloride acts as an anti-gibberellic acid compound, thus decreasing cell elongation and usually reducing number of main-stem nodes (Kerby et al., 1986; Pettigrew and Johnson, 2005), although this is not always the case (Zhao and Oosterhuis, 1999). Earlier maturity has also been reported from mepiquat chloride use (Gwathmey and Craig, 2003; Oosterhuis et al., 1991).

Dodds et al. (2010) reported that a Beltwide evaluation of numerous mepiquat-based products showed reduced end of season plant height with application of all MC and MC-type PGRs examined.

However, the PGR applications did not impact lint yield, micronaire, or uniformity in any region of the study (Dodds et al., 2010). Of the four products evaluated, no single product provided superior performance with regard to growth regulation, yield, or fiber quality. Several have reported acceleration of maturity whereas others have indicated that mepiquat chloride had no effect on earliness (Stewart et al., 2000). Gwathmey and Craig (2003) found that mepiquat chloride significantly hastened time to cutout, defined as NAWF=5 (Bourland et al., 2001), but the cultivars examined differed in this response as well as in the treatment regime. Comparisons across cultivars indicated that cutout occurred four to six days earlier with MC than in the untreated control. However, a single application of MC did not hasten flowering progress in STV 132, the earliest cultivar, relative to the untreated control. Low-rate multiple applications suggest that the growth habit of later cultivars which are more indeterminate may be shifted more by MC than earlier, more determinate types. Bader and Niles (1986) reported similar responses for cotton cultivars. Mepiquat chloride has also been used in efforts to improve carbohydrate source-sink relations to enhance efficiency of yield formation in cotton (Gwathmey and Clement, 2010). The understanding of carbon partitioning in cotton is not straight forward, as each boll can receive photosynthate from multiple sources (Pace et al., 1999). Autoradiography work by Brown (1968) showed photosynthate to a boll was provided by its bract, boll wall, subtending leaf, main-stem leaf subtending the sympodium and depended mainly on the subtending and other nearby leaves. Others found that the subtending leaf was the primary source for the boll (Ashley, 1972; Benedict et al., 1973), but Wullschleger and Oosterhuis (1990) contend that the total carbon needs of most bolls could not be supplied by the subtending leaf.

The premise of Gwathmey and Clement (2010) was that by increasing plant population density (PPD) through planting in narrower rows, boll retention would be reduced more than leaf area. Thus, leaf-to-boll ratio would be increased and the concentration of residual starch in stem tissue would be increased during boll filling. However, higher PPD tended to reduce bolls per plant more than leaf area per plant in narrower rows. They also hypothesized that application of MC would effectively decrease leaf-to-boll ratio and stem starch reserves, thus promoting yield formation at higher PPD. Their finding supported the hypothesis that boll set and yield formation in narrow-row systems benefit from a reduction in LAI. In this situation MC increased boll set percentage.

1-Methylcyclopropene (1-MCP)

The compound 1-methylcyclopropene (1-MCP) is a gaseous ethylene antagonist that blocks ethylene receptors, consequently inhibiting its perception and preventing ethylene effects in the plant tissues (Blankenship and Dole, 2003; Sisler and Serek, 1997). The affinity of 1-MCP to ethylene receptors is 10x greater than the affinity of ethylene to its receptors (Blankenship and Dole, 2003). 1-MCP is widely used in horticultural production (Fan and Mattheis, 2000). Studies in horticulture mainly focused on post-harvest physiology of climacteric fruit to counter the detrimental effects of ethylene. Its beneficial impact has been conclusively documented in fruit production and processing (Vilas-Boas and Kader, 2006; Zhou et al., 2006) as well as in flower quality (Porat et al., 1995; Reid and Celikel, 2008). These studies showed that the compound impacts a variety of physiological processes, such as decreasing ethylene synthesis (Blankenship and Dole, 2003; Dong et al., 2001; Jeong et al., 2002), respiration (Blankenship and Dole, 2003; Dong et al., 2001; Fan and Mattheis, 2000), and chlorophyll degradation (Blankenship and Dole, 2003; Fan and Mattheis, 2000; Jiang et al., 2002), thus extending shelf-life (Fan and Mattheis, 2000).

Ethylene, a plant stress hormone, is known to increase under environmental stresses such as high temperature (Davis et al., 1990) and water deficits (Pettigrew 2004a and 2004b). Morgan et al. (1992)

observed a burst in ethylene levels that lasted four days before abscission and concluded that this peak in ethylene may have been the necessary signal to initiate cell wall hydrolysis in the abscission zone followed by abscission. Since ethylene plays an important role in abscission (Guinn, 1976a) and young cotton fruit are more vulnerable to abscission than older fruiting forms, it is desirable to protect yield by preventing fruit loss induced by a peak in ethylene synthesis before abscission. Although squares can abscise at any age, most do so during the first seven days after appearance (Crozat et al., 1999). However, opinions vary as to the most susceptible stage for vulnerability to shedding. The most susceptible boll stage has been cited as occurring during the week following anthesis (open-flower stage) (Crozat et al., 1999), whereas for modeling purposes Hearn and da Roza (1985) assumed bolls were not susceptible to shedding 10 days after anthesis (flowering). Moreover, according to Guinn (1998), bolls are almost immune to shedding only after three weeks following anthesis. Since yield in cotton is generally associated with the number of bolls produced per unit area (Boquet et al., 2004; Wu et al., 2005), regardless of genotype and environment (Wells and Meredith, 1984), any means of reducing boll loss is important relative to increases in yield. Thus, if ethylene is a causal factor in boll abscission or in leaf senescence (Grbic and Bleecker, 1995), or other physiological processes, reducing the impact of ethylene on these processes provides a potential for increasing yield, and 1-MCP provides a mechanism for obtaining this goal. However, knowing the appropriate time to apply 1-MCP is critical for optimizing responses for reducing stress responses.

With the aforementioned information, da Costa and Cothren (2011a) established studies to investigate how drought affects plant growth/development and yield components of 1-MCP-treated cotton plants during the peak of reproductive phase under greenhouse conditions. A secondary objective was to determine if gas exchange, plant growth/development and yield component responses to drought could be altered by the presence of 1-MCP treatment. The compound 1-MCP was delivered as a gas one day before water-deficit stress was imposed as a protection agent to the fruiting sites already present. Utilizing plant mapping, dry matter partitioning and chlorophyll content data analyses, da Costa and Cothren (2011a) observed that water-deficit stress reduced plant height, internode length, nodes above white flower, total leaf area and weight, vegetative weight, number of squares, reproductive growth, number and retention of bolls. On the other hand, drought increased specific leaf weight, chlorophyll content, and harvest index. 1-MCP treatments had little or no positive effect on plant mapping, dry matter partitioning and chlorophyll content. The application of 1-MCP decreased the number of vegetative nodes, and increased the number of squares and reproductive nodes by 9% when plants were well-watered and by 17%, when under stress. The 1-MCP treatment showed a potential to improve lint yield in cotton, as it increased reproductive nodes per plant basis mainly for cotton under water stress during its reproductive phase. However, this greater number of reproductive nodes did not lead to a better harvest index, since 1-MCP caused high fruit abscission. In unpublished data (da Costa and Cothren, 2008 and 2009), it was observed that 1-MCP temporarily increased ethylene emission in cotton leaves above the untreated control one day after its application. Because ethylene is one of the main stimuli in abscission, it was speculated that this increase of ethylene early in the reproductive stage was one of the major factors for the high fruit shed that was observed 22 days after 1-MCP application. Loka and Oosterhuis (2011) reported that 1-MCP application to water-stressed cotton had no alleviating effect on stomatal conductance, leaf photosynthesis, and respiration. Similarly, leaf and pistil carbohydrate content remained unaffected by 1-MCP application with the exception of pistil sucrose content where 1-MCP decreased sucrose accumulation due to water-deficit stress.

Additional work has been completed with timing of application of 1-MCP (Kawakami et al., 2010) temperature conditions where the maximum temperatures during the period of cotton fruit development were well above the optimum 30° C temperature for cotton. Plants receiving 1-MCP at first

flower and first flower plus 2 weeks had significantly higher seed cotton and lint yields than the untreated control. Since no effect on cotton fruit abscission was observed, one possible reason offered for the yield increase was that the 1-MCP treated bolls in the middle of the plant canopy had significant increases in boll weight. Stress levels were also decreased by 1-MCP treatment; a higher maximum quantum efficiency of Photosystem II and lower activity of the leaf antioxidant glutathione reductase were noted as well. Loka and Oosterhuis (2011) were unable to show that 1-MCP alleviated the effects of water-deficit stress on leaf photosynthesis, respiration, and stomatal conductance. Work continues to refine the use of 1-MCP in cotton production systems.

1-MCP and Synthetic Ethylene

Field studies were conducted by da Costa and Cothren (2011b) to evaluate 1-MCP capabilities to ameliorate the negative effects (if any) of ethephon, an ethylene-releasing chemical, as a source of abiotc stress on growth and yield components of cotton plants. Cotton plants are known to have the ability to compensate for early season fruit loss (Stewart *et al*., 2001), however, nothing is known regarding such a loss later in the season. Thus, the authors also investigated to what extent cotton plants can compensate for fruit loss during the late season as a secondary objective. Following manufacturer's recommendations at that time, 1-MCP was applied prior to the stress event in combination with a surfactant. One day later, ethephon was delivered as the source of stress.

Nodes above white flower (NAWF) value refers to the number of mainstem nodes that are above a sympodial (reproductive) branch. In order to be counted, such branch has to have a white flower in its 1st fruiting position. NAWF assessment provides researchers the progression of the reproductive stages, and where the plant is relative to its maturity development (Pettigrew, 2004a). Both rates of 1-MCP detrimentally effected NAWF in the absence of the surfactant resulting in an acceleration of crop maturity, meaning that the reproductive phase was shortened (Table 2; da Costa and Cothren, 2011 b). Such shortening was also supported by a low number of square (flower buds) counts. On the other hand, when both rates of 1-MCP were applied together with the surfactant (as recommended by the manufacturer) and compared against untreated-control, such combination ameliorated the negative effects of 1-MCP rates on NAWF and preserved the normal rhythm of the crop maturity.

It is important to highlight that vegetative nodes have minimum contribution in the overall lint yield. Reproductive nodes, which originate on sympodial branches, account for the vast majority of the cotton lint yield (da Costa and Cothren, 2011b). Ethephon alone reduced the number of reproductive nodes while all treatments with 1-MCP were not different than the untreated-control, demonstrating that 1-MCP overcame the unfavorable effect of ethephon on the number of reproductive nodes (da Costa and Cothren, 2011 b). This ability of 1-MCP to improve the number of reproductive nodes on stressed plants was also observed when cotton plants were under water deficit. Our associated studies showed that 1-MCP increased the number of reproductive nodes per plant basis by 17% when compared to untreated plants also under water deficit (da Costa and Cothren, 2011b).

Such an improvement in the number of reproductive nodes caused by 1-MCP treatments, however, did not generate greater lint yields. When applied alone, 1-MCP had the lowest lint yields. While in combination with the surfactant, 1-MCP lint yield was not different than the untreated-control (da Costa and Cothren, 2011 b). Therefore, 1-MCP alone showed a negative effect on lint yield. Numerically, the treatment with the highest lint yield, however, was ethephon alone (da Costa and Cothren, 2011 b). Ethephon alone also caused the greatest fruit abscission (Table 2). Such abscission consequently favored the formation of more bolls as it was demonstrated by the linear relationship (r^2 = 0.89) between fruit shed and green bolls over the 2 yr experiments (Fig. 1).

Table 2. Effects of 1-MCP, surfactant and ethephon on plant height, internode length, counts of vegetative, reproductive, and mainstem nodes, and nodes above white flower (NAWF) per plant 50 days after treatments were initiated at the Texas AgriLife Field Laboratory in Burleson County, TX, 2007-2008 (adapted from da Costa and Cothren, 2011 b).

1-MCP	Surfactant 0.37% v v^{-1}	Ethephon 292 mL ha^{-1}	Lint yield	Abscised fruit number	Square number	Reproductive nodes	NAWF
g a.i. ha^{-1}			Kg ha^{-1}				
0.0	-	-	1348ab	21.6ab	0.34ab	15.5a	1.1a
0.0	-	+	1440a	26.2a	0.41ab	13.9b	1.1a
0.0	+	+	1359ab	19.6b	0.60ab	14.9ab	0.8a
25.0	-	+	1170c	21.8ab	0.09b	14.6ab	0.0b
25.0	+	+	1208bc	23.4ab	0.84a	15.8a	1.3a
50.0	-	+	1083c	21.3ab	0.19b	15.2a	0.0b
50.0	+	+	1207bc	20.9b	0.54ab	15.1a	1.3a

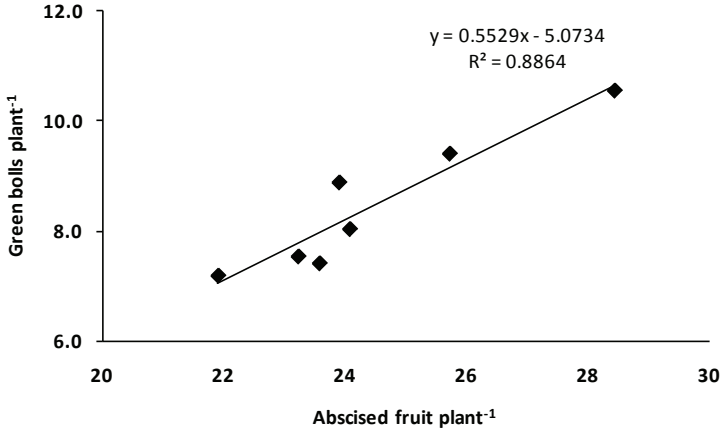

Figure 1. Relationship between the number of green bolls and abscised fruit per plant at 50 days after treatments were initiated at the Texas AgriLife Field Laboratory in Burleson County, TX, 2007-2008 (adapted from da Costa and Cothren, 2011 b).

Even though 1-MCP treatments favored the formation of reproductive nodes on stressed cotton plants, lint yields were not improved. The most logical question is to investigate why such yields were not improved as well. In order to address such a logical question, yield components were investigated at harvest. It was observed that both rates of 1-MCP favored the formation of fruit set in the upper canopy. Nevertheless, this increase in the total fruit number in the upper canopy did not lead to increased lint yield because the majority of this fruit increase was due mainly to a 76% improvement (data not shown) in the number of what appeared to be full size but still yet immature bolls (not cracked). Thus, both rates of 1-MCP showed potential to increase lint yield, but this potential was not converted into lint yield because the extra bolls set did not open in time for the mechanical harvest. Ethephon alone, on the other hand, had greater total number of fruit located in the lower portion of the canopy. Most of these bolls were already opened during harvest, granting ethephon treatment with the highest lint yield. Thus, these findings demonstrated that cotton plants treated with ethephon were still able to compensate for the fruit loss occurred later in the season (mid-bloom; da Costa and Cothren, 2011 b).

SUMMARY

From the previous discussion, it is obvious that cotton yield is affected by a number of factors. Although we can partially control some of these factors through cultural inputs, our ability to control the environment is often beyond our control. Two of the major environmental constraints limiting yield are temperature and water supply. When moisture is available through irrigation, we can effectively remove the water limitation. However, a large portion of cotton is grown under dry land production, and these areas are often prone to temperature stress as well. Plant growth regulators are used during the fruiting cycle of cotton in an effort to overcome the constraints of water stress and high temperature. The mepiquat chloride products can effectively reduce overall plant growth through reductions in plant height and leaf area and have been shown to benefit the crop by changes that lead to more efficient water uses. Other PGRs have also shown potential for increasing flower production, lint yield, and tolerance to water and temperature stresses. Means of more effectively monitoring the stress level of the crop also show utility for better timing and use rate in the crop.

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Chapter 8

POLYAMINES AND COTTON FLOWERING

Derrick M. Oosterhuis and Dimitra A. Loka
Department of Crop, Soil, and Environmental Sciences
University of Arkansas, Fayetteville, AR 72701

INTRODUCTION

Cotton production is plagued by various biotic and abiotic stresses. Even though cotton originates from areas that are characterized by adverse environmental conditions, the cotton crop is still vulnerable to stresses particularly during the reproductive stage. Environmental stress during floral development is a major reason for the disparity between actual and potential yields in crops with valuable reproductive structures (Boyer, 1982; Oosterhuis, 2002; Castiglioni, *et al.*, 2008). In cotton, even moderate stresses can result in reproductive failure in a multitude of ways, such as by disabling pollen vectors, or by causing sterility, asynchrony in pollen and ovule production, or even abortion of reproductive structures (Chiariello and Gulmon, 1991). The physiological functions that occur during flowering have been well documented (Mauney and Stewart, 1986; Stewart *et al.*, 2010). However, information is limited about the metabolic functions in the cotton flower and how these are affected by adverse conditions. Despite some recent reports on the effects of high temperature stress on metabolism in the cotton flower (Burke and Wanjura, 2010; Snider *et al.*, 2010, 2011a, 2011b, 2011c) and a number of older publications on hormone affects and interactions in cotton fruiting forms (Guinn, 1981, Guinn and Brummett, 1988), the overall picture is still not clear. Polyamines were first accepted as plant growth regulators at the International Conference of Plant Growth Substances in 1982 (Davies, 1987). However, controversial opinions exists as to whether PAs qualify to be classified as hormones, due to their abundance in plants, and the much higher concentrations that are required to induce biological responses. Most of the published work on hormones in cotton deals with ethylene, abscisic acid and auxin, with little known about the role and importance of the "new" phytohormones such as polyamines. This review covers the role of polyamines in reproductive development and what is known in cotton.

POLYAMINES

Polyamines (PAs) are low-molecular-weight organic polycations with two or more primary amino groups (NH_2), and are ubiquitous in bacteria, plants and animals. They were discovered as early as 1678, when three-sided crystals from human semen were first described (van Leeuwenhoek, 1678), but their correct chemical composition and structure were only determined in the 1920's (Galston and Kaur-Sawhney, 1990).

Diamine putrescine (PUT) and its derivatives, triamine spermidine (SPD), and tetramine spermine (SPM) are the most common polyamines in plants. Spermine is found specifically in flowering plants, while its isomer thermospermine is present in all plants (Minguet *et al.*, 2008). PUT derives

either directly from ornithine or indirectly from arginine through ornithine decarboxylase (ODC) or arginine decarboxylase (ADC), respectively (Adiga and Prasad, 1985; Kao, 1997). Exceptions exist in the biosynthetic pathways with arabidopsis (*Arabidopsis thaliana*) using only the arginine-ADC pathway. In general, the activities of ADC and ODC enzymes appear to regulate overall PUT biosynthesis in plants (Bagni and Tassoni, 2001) with ADC being associated with tissues undergoing cell expansion and ODC with tissues growing by cell division. The triamine SPD and tetramine SPM are synthesized from PUT and SPD, respectively, with addition of one aminopropyl moiety (Palavan-Ünsal, 1995). The aminopropyl group, decarboxylated S-adenosylmethionine (dcSAM), is produced from S-adenosylmethionine (SAM) mediated by S-adenosylmethionine decarboxylase (SAMDC) (Bagni and Tassoni, 2001). Polyamines are degraded by diamine oxidases (DAO) or polyamine oxidases (PAO) (Bagni and Tassoni, 2001). DAOs occur in high levels in dicots and catalyze the oxidation of PUT to 4-aminobutanal, hydrogen peroxide (H_2O_2) and ammonia (Cona *et al.*, 2006). PAOs on the other hand, are found at high levels in monocots (Sebela *et al.*, 2001) and they are either involved in terminal catabolism of SPD and SPM to 4-aminobutanal, H_2O_2 and 1,3-diaminopropane (Cona *et al.*, 2006) or participate in back-conversion of SPM to SPD with simultaneous production of H_2O_2 (Moschou *et al.*, 2008).

Polyamines occur in free forms, conjugated with phenolic acids or bound to other low molecular weight compounds or macromolecules (Galston and Kaur-Sawhney, 1990). Their high concentrations that range from micromolar to millimolar levels in the plant cells allow them to function as minerals without being toxic (Galston and Kaur-Sawhney, 1990). Free polyamines have been quantified in a variety of plants such as olives (*Olea europea* L.) (Pritsa and Vogiatzis, 2004), tomatoes (*Lycopersicum esculendum* L.) (Fos *et al.* 2003), peas (*Pisum sativum* L.) (Carbonell and Navarro, 1989), and Arabidopsis (Todorova *et al.* 2007). Only free PAs have been reported to be translocated throughout the plant in the phloem and the xylem sap mainly as PUT (Antognoni *et al.*, 1998). Short distance PA translocation is mediated by specific transporters (Antognoni *et al.*, 1993; Tachihara *et al.*, 2005), while long distance transport is both basipetal and acropetal despite their polycation nature (Friedman *et al.*, 1986; Rabiti *et al.*, 1989; Caffaro *et al.*, 1993). Polyamine uptake has been reported to be either active or passive (Kerschbaum *et al.*, 2003). Additionally, different rates of PA transport have been reported between leaves of different ages, with the younger leaves exporting PAs substantially slower than the older leaves (Antognoni *et al.*, 1998).

The importance of PAs in plants is due to their participation in a multitude of plant metabolism functions, including photosynthesis, enzyme activation and maintenance, cell proliferation, division and differentiation, morphogenesis and embryogenesis as well as organogenesis (Evans and Malmberg, 1989; Galston *et al.*, 1997; Kakkar *et al.*, 2000). In addition, PAs are involved in the correct conformation of nucleic acids, gene expression and translation, mediating hormone action, modulation of cell signaling, membrane stabilization and ion channel regulation, as well as heat shock protein and macromolecular synthesis. Besides all the aforementioned functions, PAs also act as second messengers in leaf senescence and apoptosis and more importantly for biotic and abiotic stresses (Kumar *et al.*, 1997; Walden *et al.*, 1997; Malmberg *et al.*, 1998; Bouchereau *et al.*, 1999; Liu *et al.*, 2000; Konigshofer and Lechner, 2002; Alcazar *et al.*, 2006b; Groppa and Benavides, 2007; Ioannidis and Kotzabasis, 2007; Kusano *et al.*, 2008).

Despite the extensive research on other crops, limited information for polyamine exists for cotton (*Gossypium hirsutum* L.) with the only reports being on the distribution of polyamines in the cotton plant (Bibi *et al.*, 2011), polyamine content just prior to rapid fiber elongation (Davidonis, 1995), the effect of heat stress on PAs (Bibi *et al.*, 2010), and the occurrences of uncommon polyamines (norspermidine, norspermine, pentamine, and hexamine) (Kuehn, *et al.*, 1990).

POLYAMINE DISTRIBUTION IN THE PLANT

Both the type and quantity of polyamines have been reported to vary within the plant. Kakkar and Rai (1993) reported that PAs and their titers were different in meristems and vegetative parts. Urano *et al.* (2003) reported that PA biosynthesis showed different profiles during plant development in arabidopsis as well as in response to environmental conditions. Increased concentrations of free and conjugated PAs have been reported to occur in the shoot apex and the flowering parts of many plants. The response appears to be time specific, with arabidopsis rosettes and bolts containing small quantities of SPD that increase dramatically in flowers. However, root and leaf PA concentrations appear to be unaffected from flower initiation or flower development (Li and Wang, 2004). Roots in general accumulate lower concentrations of PAs compared to the flowering parts of the plant, with leguminous plants being the only exception since the nodules accumulate 5 to10 times higher concentrations compared to other organs (Fujihara *et al.*, 1994). Differences in PA concentrations have been noticed between photosynthetic and non-photosynthetic tissues (Lefevre *et al.*, 2001) as well as within floral organs with flowers of Nicotiana species containing higher concentrations of PAs in their stamens compared to the pistils (Pedrizet and Prevost, 1981; Martin-Tanguy, 1997).

In cotton, PA concentrations of the leaf have been observed to be significantly lower compared to the ovary, while the ovary and the style have been reported to contain similar levels of Pas with the exception of PUT that occurs in considerably higher concentrations in the ovary than the style (Loka *et al.*, in press). Additionally, Bibi *et al.* (2011) showed that the concentration of PUT, SPD and SPM in the ovules of first-position white flowers varied up the main-stem of the cotton plant. PUT concentration decreased acropetally along the main-stem of the cotton plant with the highest concentration observed at the 7th node and the lowest at the 13th node. SPD concentration decreased below and above the 9th node, with the 9th node showing the highest amount of SPD and the 13th node the lowest in both years of the study. Similarly, the SPM concentration decreased below and above the 9th node. The highest amounts of PAs were observed at the 7th and the 9th node of cotton, and this may be associated with the majority of the yield distribution coming from these nodal positions. Genotypic differences were also observed in ovarian polyamine content (Bibi *et al.*, 2011).

INTERACTION OF POLYAMINES WITH OTHER METABOLIC FACTORS

Polyamines participate in a number of metabolic and hormonal pathways that regulate plant growth and development as well as plant responses under conditions of environmental stress.

Polyamines and Ethylene

Ethylene and polyamine (SPD and SPM) biosynthesis pathways share the common precursor S-adenosylmethionine. However, their plant responses appear to be different if not occasionally antagonistic, i.e., PAs are considered to be plant growth promoters whereas ethylene is mostly associated with plant growth inhibition and senescence. Opposite opinions exist on the interdependence of the two biosynthetic pathways. However, utilization of the precursor SAM by the plants for either production of ethylene or polyamines, appears to be dependent on the type of tissue, the developmental stage as well as the growing conditions. Specifically, polyamine levels and synthesis rates are high in tissues that undergo cell division compared to the non-dividing or senescing tissues (Kushad and Dumbroff, 1991). Ethylene synthesis, on the other hand, increases significantly in older tissues whereas it is very limited in meristematic tissues. Research has indicated that preventing the conversion of SAM into ethylene can dramatically increase polyamine synthesis; however, in young tissues, where ethylene enhances growth, polyamine levels have been reported to increase. Conversely, in cases where ethylene acts as a growth inhibitor, for example under adverse conditions or senescence, polyamine responses vary. For example, PUT has been shown to increase in accordance with ethylene, while SPD and SPM remain unaffected (McDonald and Kushad, 1986; Corey and Barker, 1989; Gomez-Jimenez *et al.*, 2010). In experiments with exogenous application of polyamines, ethylene biosynthesis is inhibited (Suttle, 1981; Apelbaum *et al.*, 1981) however, the opposite has also been observed. In general, ethylene and polyamine functions may appear to be opposite (Kumar *et al.*, 1996; Pang *et al.*, 2006), however their responses are dependent on the species, the type of tissue as well as the experimental conditions (Gomez-Jimenez *et al.*, 2010; Quinet *et al.*, 2010). There does not appear to be any published research on the interaction of PAs and ethylene in cotton.

Polyamines and Abscisic Acid

Abscisic acid (ABA) has been reported to control PA levels in plants by affecting their synthesis, degradation, transfer and conjugation with other molecules under conditions of stress (Liu *et al.*, 2005; Nieves *et al.*, 2001). In maize (*Zea mays* L.), low concentrations of ABA due to chemical inhibition resulted in low PA levels (Liu *et al.*, 2005). Inhibition of ABA synthesis in wheat (*Triticum aestivum* L.) resulted in lower PUT concentrations due to decreased ADC activity (Lee *et al.*, 1997). Similar results were observed in arabidopsis (Cuevas *et al.*, 2008), however, Kim *et al.* (2002) reported that ABA and PAs had no interdependent action in leaves of cold-stressed tomato. Bueno and Matilla (1992) observed that exogenous application of ABA increased PA levels in isolated embryonic axes of chickpea (*Cicer ariennum* L.) seeds. Nieves *et al.* (2001) reported similar results in sugarcane (*Beta vulgaris*). In contrast, ABA application to cucumber cotyledons (Suresh *et al.*, 1978) resulted in decreased PA levels and a similar pattern was observed in rice (*Oryza sativa* L.) embryos (Choudhuri and Ghosh, 1982). As with ethylene, there have been no published results of PAs and ABA interactions in cotton.

Polyamines and Gibberellins

Gibberellins appear to regulate PA biosynthesis. Dai *et al.* (1982) observed that exogenous application of GA_3 resulted in significant increases in PUT and SPM concentrations in internodes of dwarf peas, while Kaur-Sawnhey *et al.* (1986) reported that only ADC activity increased, whereas application of PA inhibitors resulted in reduced length of internodes. Enhancement of GA_3 induced elongation has also been observed in lettuce hypocotyls (Cho, 1983). Increases in PUT levels, but not in SPD and SPM, were reported in seedless berries as well after application of GA_3 (Shinozaki *et al.*, 1998). Increases in PUT, SPD and SPM were reported by Smith *et al.* (1985) in dwarf peas. In addition, Kyriakidis *et al.* (1983) reported that application of GA_3 caused ODC activity to increase in barley seeds (*Hordeum vulgare*) and indicated that PAs and especially SPD were regulating GA_3 action and α-amylase formation. To the contrary, Lin (1984) reported that GA_3 application had no effect on polyamine levels in aleurone layer cells in barley seeds. As with ethylene and abscisic acid, there does not appear to be any reported research on interactions between GAs and PAs in cotton.

Polyamines and Cytokinins

Polyamine biosynthesis has been reported to be regulated by cytokinins (Galston, 1983; Bouchereau *et al.*, 1999). Additionally, PAs have been observed to block cytokinin-action (Romanov *et al.*, 2002). Romanov *et al.* (2004) reported that exogenous application of PAs to Arabidopsis plants resulted in blocking cytokinin activity and the efficiency of the PAs depended on their concentrations, with SPM being the most efficient at all concentrations. Similar interactions between cytokinins and PAs have been reported in Amaranthus seedlings (Feray *et al.*, 1992), but the opposite was reported in beet root cells (Naik *et al.*, 1980). Levels of PUT and cytokinins were observed to increase in embryogenic cell cultures of celery (*Apium graveolous* L.) at embryogenesis, while application of PA biosynthesis inhibitors resulted in restriction of cell division (Danin *et al.*, 1993). No apparent reports exist of cytokinins and PAs in cotton.

Polyamines and Nitric Oxide

Nitric oxide (NO), a reactive gaseous molecule, has been shown to act as a signal in plant responses to biotic and abiotic stresses (Delledonne *et al.*, 2001), as well in regulation of a variety of plant growth and developmental processes such as germination and flowering, fruit ripening, and senescence (Hung and Kao 2003; Pagnussat *et al.*, 2004). The amino acid arginine is the common precursor for both polyamine and nitric oxide pathways (Yamasaki and Cohen, 2006). Recent evidence has indicated that PAs are involved in NO production in plants (Tun *et al.*, 2006; Groppa *et al.*, 2008; Arasimowicz-Jelonek *et al.*, 2009), and exogenous application of PAs to arabidopsis seedlings was observed to enhance NO production (Tun *et al.*, 2006). Similar results were reported by Manjunantha *et al.* (2010) in banana fruits (*Musa acuminate* L.) and by Silveira *et al.* (2006) in Brazilian pine (*Araucaria angustifolia*) after application of PUT, while application of either SPD or SPM had the opposite results (Silveira *et al.*, 2006). In conclusion, PAs have an effect on NO production although the exact mechanism involved is yet to be elucidated. The relationship between PA and NO has not been quantified for cotton.

Polyamines and Hydrogen Peroxide

Polyamines are involved in antioxidant metabolism through their function as free radical scavengers, and they also participate in generation of hydrogen peroxide (H_2O_2) through their catabolism by DAOs or PAOs. Hydrogen peroxide, a reactive oxygen species at high concentrations leads to plant cell death, and in lower concentrations serves as a signaling molecule inducing tolerance to biotic and abiotic stresses due to its high permeability across membranes and relatively long life (Quan *et al.*, 2008). Additionally, H_2O_2 is involved in vascular development of plants through lignin polymerization (De Marco and Roubelakis-Angelakis, 1996). Previous research has indicated that increased levels of PAs resulted in decreased production of H_2O_2 and lipid peroxidation (Nayyar and Chander; 2004; Verma and Mishra, 2005).The relationship between PA and H_2O_2 has not been documented for cotton.

POLYAMINES AND REPRODUCTIVE DEVELOPMENT

A relationship between PAs and reproductive development has long been established due to the significant increase in their concentrations as plants transition from their vegetative to reproductive stage of growth (Kakkar and Rai, 1993). Kloareg *et al.* (1986) indicated that PAs are indispensable to plants at the time of flowering and early fruit development. In support of this observation, experiments with PA-deficient mutants or mutants with unbalanced PA metabolism resulted in abnormal growth and flowering patterns as well as delayed flowering (Galston *et al.*, 1997; Kakkar and Sawhney, 2002; Hanzawa *et al.*, 2002; Alcazar *et al.*, 2005). Polyamines have been implicated in flower induction (Evans and Malmberg, 1989; Faust and Wang, 1992; Bagni *et al.*, 1993;Bouchereau *et al.*, 1999; Kakkar and Sawhney, 2002), flower initiation (Kaur-Sawhney *et al.* 1988), pollination (Falasca *et al,.* 2010), fruit growth and ripening (Kakkar and Rai, 1993). Sexual differentiation of tissues appears to be dependent on PA biosynthesis and catabolism, as well as their free or conjugated forms (Martin-Tanguy, 1997).

Ornithine decarboxylase is reported to be the main enzyme controlling PA concentrations and may be required for flower development (Burtin *et al.*, 1989; Aribaud *et al.*, 1994; Tarenghi and Martin-Tanguy, 1995). In support of that theory, application of ODC inhibitors during early fruit growth resulted in complete inhibition of flowering in tomato and potato (*Solanum tuberosum* L.) (Cohen *et al.*, 1982). However, in tobacco (*Nicotiana tabacum* L.), ODC inhibitors had no effect on flowering, while the opposite was observed with ADC inhibitors (Tiburcio *et al.*, 1988). In general, however, the ODC pathway is associated with meristematic tissues, while ADC is common in mature tissues (Flores, 1991). Additionally, use of SPD biosynthesis inhibitors resulted in flower abortion and abscission in tobacco (Burtin *et al.*, 1989). Inhibition of SPD biosynthesis has been reported to result in anther malformation and pollen sterility in a variety of crops (Martin-Tanguy, 1996). Similar results were reported in kiwi fruit (*Actinidia deliciosa* L.) (Falasca *et al.*, 2010), tobacco (Chibi *et al.*, 1994), apple (*Malus domestica* L.) (Bagni *et al.*, 1981) and tomato (Song *et al.*, 2002). Kakkar and Rai (1993) suggested that SPD concentrations could be used as potential markers for floral induction. In maize, PAs have been observed to be involved in normal and aborting kernels, through their involvement in early endosperm devel-

opment (Liang and Lur, 2002). In addition, the PUT:SPD ratio appears to play an important role in plant regeneration capacity (Shoeb *et al.*, 2001). The levels of PUT against the levels of SPD +SPM have been associated with totipotency as well as with embryo development and germination (Papadakis *et al.*, 2005; Silveira *et al.*, 2006).

In addition to flowering, PAs have also been reported to participate in regulation of post-fertilization development (Galston, 1983; Slocum and Galston, 1985; Lin, 1984; Evans and Malmberg, 1989). Numerous reports on PAs and their role in stimulating fruit set and fruit development exist, i.e., in apple (Costa and Bagni, 1983; Biasi *et al.*, 1991), pear (*Pyrus communis* L.) (Crisosto *et al.*, 1986; Crisosto *et al.*, 1988), pepper (*Capsicum annuum* L.) (Serrano *et al.*, 1995), olive (Rugini and Mencuccini 1985), mango (*Mangifera indica* L.) (Singh and Singh, 1995), tomato (Antognoni *et al.*, 2002), and strawberry (*Fragaria amanassa* Dutch.) (Tarenghi and Martin-Tanguy, 1995). In cotton, there has only been limited research on PAs and reproductive development. Bibi *et al.* (2010) measured PA content in cotton ovaries as affected by heat stress, showing that SPD and SPM, but not PUT, were decreased with elevated temperatures, and successful seed fertilization was also significantly decreased by the high temperature (Bibi *et al.*, 2007). Similar results were reported by Loka and Oosterhuis (2011) for the effect of water deficit on PAs in cotton flowers. PUT concentrations of water-stressed ovaries remained at the same levels as the control, whereas both SPD and SPM concentrations significantly decreased under conditions of water-deficit stress.

POLYAMINES AND PLANT RESPONSE TO STRESS

The first observation of PA involvement in plant response under adverse conditions was reported in potassium deficient barley plants that over-accumulated PUT (Richards and Coleman, 1952). Further research revealed that the ADC pathway is more active compared to the ODC pathway under stress conditions (Smith and Richards, 1964; Flores and Galston, 1984). Since then, extended investigation in a number of plant species has shown that changes in PA production is a common plant response to a variety of abiotic stresses, including salinity, chilling, heat and drought as well as biotic stresses (Bouchereau *et al.*, 1999; Alcazar *et al.*, 2006b; Groppa and Benavides, 2007). Additionally, recent advances indicate that the ratio of PA catabolism to PA anabolism is an important factor in PA mediated stress tolerance (Moschou *et al.*, 2009).

Salinity

Polyamine responses to salinity have been reported to vary in different plant species as well as within a plant species (Russak *et al.*, 2010), but there are no reports for cotton. Significant increases in PUT and SPM have been reported in arabidopsis under conditions of salt stress (Urano *et al.*, 2003) and similar results were found in rice (Basu and Ghosh, 1991) and mung beans (*Vigna radiata*L.) (Friedman *et al.*, 1986). Krisnamurthy and Bhagwat (1984) reported that SPD and SPM levels of salt-tolerant rice cultivars were increased under conditions of high salinity while PUT concentrations decreased. Similar findings were reported in maize (Jimenez-Bremont *et al.*, 2007), in soybean (*Glycine max* L.) (Campestre *et al.*, 2011), and in a variety of vegetable crops (Zapata *et al.*, 2004). However, Prakasch *et al.* (1988) observed that PA

concentrations in rice plants under water stress significantly decreased compared to the control, and similar results were also observed in tomato (Santa-Cruz *et al.*, 1997). Furthermore, in experiments with transgenic arabidopsis plants, Yamaguchi *et al.* (2007) observed that SPM-deficient mutants were significantly more salt-sensitive compared to control plants. Similarly, salt-resistant plant varieties were found to have increased concentrations of PAs under conditions of salt stress (Erdei *et al.*, 1996; Basu and Ghosh, 1991), whereas over-production of PUT was also reported to increase salt tolerance in rice (Roy and Wu, 2001; Quinet *et al.*, 2010) and tobacco (Kumria and Rajam, 2002; Waie and Rajam, 2003).

Cold Stress

Chilling treatment was reported to increase PUT accumulation in Arabidopsis (Urano *et al.*, 2003; Cuevas *et al.*, 2009) while SPD and SPM concentrations remained unaffected or slightly decreased. PUT accumulation under conditions of cold stress was also observed in alfalfa (*Medicago sativa*) and wheat (Nadeau *et al.*, 1987; Kovacs *et al.*, 2010), in citrus and lemon (*Citrus* sp.) (McDonald and Kushad, 1986; Kushad and Yelenosky, 1987), in cucumber (*Cucumis sativus*) (Shen *et al.*, 2000),as well as in beans (*Phaseolus* sp.) (Guye *et al.*, 1986), and in rice (Lee *et al.*, 1997). However, differential responses to cold stress were reported within wheat species with a winter wheat variety showing increased PUT and SPD concentrations under stress while in a spring variety only the SPD and SPM concentrations were increased (Szalai *et al.*, 2009). Further research with exogenous application of PUT on tomato resulted in decreased electrolyte leakage due to the cold treatment whereas the use of PUT synthesis inhibitors had the opposite effect (Kim *et al.*, 2002). Freezing tolerance due to increased accumulation of PAs was also reported in Arabidopsis (Kasukabe *et al.*, 2004; Altabella *et al.*, 2009). Low temperatures are not usually a problem in cotton production except in some areas during germination; however, no work has been published on PAs during cotton early season growth.

Heat Stress

The most frequent response of polyamines to heat stress is an increase in one or more PAs (Narçin-Ünsal, 1995). In experiments with rice callus Roy and Ghosh (1996) reported that levels of free and bound PAs were significantly higher in heat tolerant varieties compared to the heat sensitive. Additionally, over-production of SPD and SPM in transgenic tomato plants resulted in significantly more heat tolerant plants (Cheng *et al.*, 2009). In support of these observations, Song *et al.* (2002) reported that extreme temperatures significantly decreased SPD and SPM concentrations in tomato pollen, while PUT concentrations increased. In cotton the negative effect of high temperatures during reproductive development is known (Oosterhuis and Snider, 2011), but research on free PAs under high temperature stress is limited. Kuehn *et al.* (1990) reported on the occurrences of uncommon polyamines in cotton under heat stress. Bibi *et al.* (2007) documented a negative correlation of temperature and PAs, with polyamine content in cotton ovaries decreasing with increased canopy temperature. Subjecting the plants to high temperatures (38°C) compared to the optimum (30°C) significantly decreased SPD and SPM levels but not PUT (Bibi *et al.*, 2010). Successful seed fertilization was significantly decreased by the high temperature,

and significantly increased by exogenous application of PUT (Bibi *et al.*, 2010). Information is lacking about the specific role of PAs in the leaf and pistil in the response of the cotton plant to high temperature, and the possible protective role of PAs in thermostability in the leaf and pistil.

Water-deficit Stress

Polyamines have been reported to be involved in plant responses under limited water conditions either as signaling molecules due to their connection to ABA metabolism (Alcazar *et al.*, 2006a) or as protective agents against water stress (Capell *et al.*, 1998). In support of these observations, Alcazar *et al.* (2010) reported that transgenic Arabidopsis plants that over-accumulated PUT had increased drought tolerance compared to the control as well as reduced stomatal aperture. Similar results were observed in rice plants over-producing SPD and SPM by Capell *et al.* (1998) while after imposition of water stress on cell cultures of drought tolerant alfalfa, SPD and SPM concentrations increased while PUT levels decreased (Yamaguchi *et al.*, 2007). The opposite was observed in detached oat leaves under conditions of water stress with PUT significantly increasing while SPD and SPM showed a dramatic decrease (Flores and Galston, 1984). Furthermore, SPM-deficient arabidopsis plants were observed to be significantly more drought sensitive compared to the control (Yamaguchi *et al.*, 2007). Regarding cotton, experiments with cultivars differing in drought tolerance revealed that water-deficit stress resulted in significant increases in PUT, SPD, and SPM levels in both ovary and leaf of the drought-sensitive cultivar, whereas no significant effect was observed on PA concentrations of the drought-tolerant cultivar (Loka *et al.*, in press).

Biotic Stress

PAs have been reported to interact with fungal (Greenland and Lewis, 1984) and viral (Torrigiani *et al.*, 1997) pathogens as well as mycorrhizae (Walters, 2000). Apart from their role as signaling molecules that induce defensive mechanisms against the pathogen (Takahashi *et al.*, 2004), they also participate in resistance mechanisms against infections (Yamakawa *et al.*, 1998). Additionally, PAs provide the possibility of controlling fungal plant diseases by specific inhibition of their biosynthesis (Rajam and Galston, 1985; Walters, 2003) since experiments with certain PA biosynthesis inhibitors resulted in complete disruption of microcycleconidiation (Khurana *et al.*, 1996). Furthermore, polyamine catabolism pathways are also potentially able to regulate plant-pathogen interactions due to the concentrations of H_2O_2 and NO that are ultimately produced (Romero-Puertas *et al.*, 2004; Tun *et al.*, 2006; Walters, 2003). We are not aware of any published work on the involvement of PAs in biotic stress in cotton.

EXOGENOUS APPLICATION OF POLYAMINES

The documented importance of polyamines in reproductive development has directed many researchers to exogenously applying polyamines in an effort to enhance fruit development. Reports for mango (Singh and Janes, 2000) and apricot (*Prunus armeniaca* L.) (Alburquerque *et al.*, 2006) showed improved fruit retention and yield with exogenous PAs, while application of SPD and SPM alleviated high temperature inhibition of pollen germination and pollen tube growth in tomato (Song

et al., 2002). In apricot the exogenous application of PUT on flowers increased the percentage of functional ovules about 16 % (Alburquerque *et al.* 2006). Also, exogenous application of PUT has been shown to improve yield in litchi (*Litchi chinensis* L.) (Stern and Gazit, 2000), apple (Costa *et al.*, 1983), and olive (Rugini and Menciccini, 1985). Earlier, Galston and Kaur-Sawhney (1990) reported that the application of exogenous PAs to plants produced visible effects such as the prevention of senescence and the formation of embryos or floral primordial in certain otherwise vegetative tissue. According to Cohen *et al.* (1982) the development of pollinated tomato ovaries was prevented by inhibitors of ODC and could be counteracted by exogenous PUT application. Besford *et al.* (1993) showed that exogenously applied SPD and SPM prevented the destruction of chlorophyll, Rubisco and molecular complexes of thylakoids from occurring in osmotically stressed oat (*Avena sativa* L.) leaves, and Roberts *et al.* (1986) showed that exogenous PAs altered membrane fluidity in bean leaves. Various reports of exogenous polyamine application suggest a possible involvement of PAs in plant adaptation to several environmental stresses by preserving membrane functions and reversing growth inhibition as well as by decreasing ROS concentrations and increasing antioxidant enzyme activities (Ali, 2000; Iqbal and Ashraf, 2005a; Tang and Newton, 2005; Ndayiragiji and Lutts, 2006; Afzal *et al.*, 2009; Yiu et al, 2009; Zhang *et al.*, 2009). Farooq *et al.* (2008) reported that exogenous application of PAs increased drought tolerance in rice by improving leaf water status and decreasing membrane leakage. Similar results were reported by Rajasekaran and Blake (1999) who observed that application of SPD to drought stressed Jack pine (*Pinus banksana Lamb*.) seedlings reduced ethylene production and membrane leakage. Application of SPD was also reported to ameliorate chilling injury of rice seedlings (Tajima and Kabaki, 1981), while Shi *et al.* (2008) observed that PUT application significantly ameliorated salt stress in cucumber.

In cotton, Bibi *et al.* (2007) reported that exogenous application of PUT to floral buds in controlled environment studies, 24 hours prior anthesis, increased the level of PUT in ovaries, but there was no effect on SPD and SPM concentrations. The increase in PA was associated with increased seed set. These authors also showed that increased temperatures decreased SPD concentrations in the pistil and seed set in cotton flowers, and exogenous application of PUT ameliorated the negative effect of high temperature on seed set.

The use of exogenous application of polyamines in row crops may not be a practical management practice due to the high cost of the material (and because it is still not in mass production (SPM=$180/5g, SPD=$118/5g, PUT= $55/25g). Nevertheless, an affordable alternative is to apply synthetic plant growth regulators. The plant growth regulator BM86 (active ingredient GA_{14}; Goëmar Laboratories, Saint-Malo, France) has been reported to regulate the synthesis of endogenic PAs. Broquedis *et al.* (1995) investigated the effect of GA_{14} on the composition of PAs in grapes (*Vitis vinifera* L.), and showed an increase in the accumulation of PAs particularly at the end of the first stage of fruit development. Treatment with GA_{14} increased the numbers, weight and growth of the fruit, and this was related to a significant increase in polyamine content in the flowers and in the fruit. It appears, therefore, that the BM86 stimulated the metabolism of PAs in the plant. Numerous anecdotal reports with horticultural plants have shown that BM86 acts to stimulate reproductive development of the plant. According to these reports (Anonymous, 2008), BM86 increased yield and oil production of olives, promoted uniform fertilization and fruit set in grapes and resulted in increased fruit size and total yield in citrus.

In cotton, (Rethwich *et al.*, 2006) associated BM86 application with increased yield, while Bibi *et al.* (2007) subsequently showed that application of BM86 at the first flower stage and two weeks later at 2.34 mL/ha had a positive effect on ovarian polyamine content of cotton. PUT and SPD concentrations one week after the first BM86 application and PUT concentration two week after the first BM86 application, were significantly increased compared to the untreated control. Additionally, higher seed set efficiency with the BM86 application was observed. Only small differences in ovarian polyamine content were detected among the genotypes tested, possibly due to the narrow genetic pool of the commercial cotton cultivars used. They concluded that application of BM86 could significantly increase cotton seed number by enhancing PAs biosynthesis, but cautioned that further research was needed.

SUMMARY

Polyamines are growth regulating compounds that occur widely in bacteria, plants and animals. They are involved in numerous plant metabolic activities and in plant response to stress. Polyamines are of particular importance in reproductive development and are considered indispensable to plants at the time of flowering and early fruit development, since they have been implicated in flower induction, flower initiation, pollination, fruit growth and ripening. Both the type and quantity of polyamines have been reported to vary within the plant, and during plant development, as well as in response to environmental conditions. In cotton, PA concentrations are generally higher in the ovary, and genotypic differences exist in ovarian polyamine content. Polyamines participate in a number of metabolic and hormonal pathways that regulate plant growth and development as well as plant responses under conditions of environmental stress, including salinity, chilling, heat, drought and biotic stresses. Despite the extensive research on other crops, limited information exists for cotton with the only reports being on the distribution of polyamines in the cotton plant, polyamine content just prior to rapid fiber elongation, the effect of heat stress on PAs, and the occurrences of uncommon polyamines. Exogenous application of PAs have been reported to enhance fruit retention, development, and yield in a number of horticultural crops, and improve plant adaptation to several environmental stresses. In cotton, exogenously applied putrescine increased the level of putrescine in ovaries, which was associated with increased seed set, and amelioration of the negative effect of high temperature on seed set. Understanding of the role of polyamines in cotton is limited, but given what we know in horticultural plants, it is obvious that as our understanding of the involvement of PAs in metabolic functions and in stress response increases, it would appear that research with cotton in this field will be a fruitful and rewarding endeavor.

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Chapter 9

COTTON SEED DEVELOPMENT: OPPORTUNITIES TO ADD VALUE TO A BYPRODUCT OF FIBER PRODUCTION

Qing Liu, Danny J. Llewellyn, Surinder P. Singh and Allan G. Green
CSIRO Plant Industry, PO Box 1600, ACT 2601, Australia

INTRODUCTION

Unlike other major crops in which seeds provide most of the economic value, cotton seed is largely a by-product of more valuable fiber production and represents only approximately 15% of the farm gate value of the cotton crop. A cotton plant normally produces about 1.6 kg of seed for every kg of lint. Following ginning to remove the lint fiber, fuzzy cotton seed is either used directly as animal feed, or processed into four major products including oil, meal, hull and short linter fibers (Cherry and Leffler, 1984; O'Brien *et al.*, 2005). With about 21% oil and 23% protein by weight in the seed, however, this makes cotton the fifth largest oil crop in the world and the second most important potential source of plant proteins. As a by-product of fiber production, the continued large scale production of cotton seeds is assured because of the on-going demand for the fiber by the textile industry. Until recently, the quality of cotton seed has been relatively neglected as the main research priority has been devoted to enhancing fiber quality and fiber yield. This continual human selection for high yield and better quality of fiber has narrowed the genetic variation available for cotton seed quality improvement through breeding. An in-depth understanding of metabolic events that determine the overall components of the storage reserves in cotton seeds is therefore of vital importance for improving the yield, quality and ultimately the value of seed products and opens the possibility for significant value-adding by engineering novel attributes into the seed. While in this review we have focused primarily on the development of the cotton seed, other more studied species such as *Arabidopsis thaliana* can serve as a model for cotton and for completeness those other species are mentioned where data for cotton are limited.

EMBRYO AND SEED DEVELOPMENT ARE HIGHLY COORDINATED WITH FIBER PRODUCTION

As in other angiosperm species, seed development in cotton is triggered by the double-fertilization event that gives rise to the zygote and the triploid cell that gives rise to the endosperm tissue. At the same time that the zygote and endosperm are generated, the maternally derived seed coat begins to differentiate from the ovule outer integuments that surround the embryo sac. The seed coat plays a major role in protecting the embryo and transferring nutrients from the maternal plant to all parts of the seed. It also generates the seed fibers that provide a dispersal mecha-

nism for seeds in the wild. Individual epidermal cells within the expanding seed coat elongate rapidly at anthesis to produce the long lint fibers that thicken and desiccate into the fibers that are harvested from the seed at maturity. The development of the fiber is described in detail in Chapter 10. A schematic drawing of a typical cotton seed at maturity is shown in Figure 1.

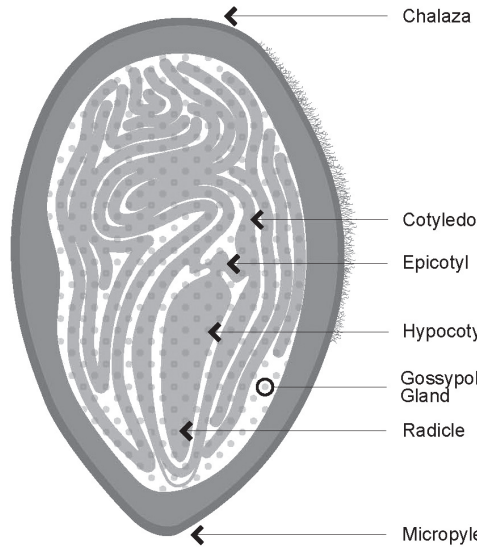

Figure 1. A schematic drawing of a typical cotton seed at maturity.

Embryogenesis

The development of the embryo can be generally classified into three overlapping stages, namely embryogenesis, maturation and desiccation (West and Harada, 1993; Turley and Chapman, 2010). Following fertilization, the endosperm proliferates to occupy most of the post-fertilization embryo sac and nourishes the embryo during early development (Schulz and Jensen, 1977). The zygote divides asymmetrically, giving rise to the apical cell that develops into the embryo proper and the suspensor structure (funiculus) that supports and feeds the embryo until it degenerates later in development (Goldberg *et al.,* 1994). The zygote undergoes its first cell division at 3-5 days post anthesis (DPA). The formation of the heart stage embryo occurs at 6-17 DPA. All embryonic organs and vascular tissues are differentiated and developed by 18 DPA at the torpedo stage. Embryos then continue to expand until approximately 25 DPA. The fiber and embryo share synchronous phases of development (Stewart, 1986). Rapid cell growth in both fiber and embryos occurs in the period of the first 25 DPA. Zygotic differentiation and growth through the globular, heart, and torpedo stages are well documented, but the chronology of the major events occurring during each phase is variable, likely due to different varieties and environmental conditions (Reeves and Beasley, 1935; Forman and Jensen, 1965; Dure, 1975). Cotton embryos have two large cotyledons that accumulate lipid, storage protein, and phytin, but no starch. In contrast to some other dicotyle-

donous plant species, such as legumes, cotton cotyledons remain thin, become highly convoluted, and wrap themselves around the embryo axis (Fig. 1).

The embryo maturation stage over the next 20 days (25-45 DPA) is characterized by an increase in the size and weight of the cotyledons and rapid accumulation of oil and storage proteins that are the major reserves of carbon and nitrogenous compounds needed for seed germination and early seedling growth, respectively (Reeves and Beasley, 1935; Forman and Jensen, 1965). During this embryo maturation period the fiber on the seed coat is depositing its thick secondary cell wall of almost pure cellulose (Stewart, 1986). The strengthening and lignification of the non-fiber seed coat epidermal cells also occurs at this time. Approximately 80% of the dry weight of the mature cotton seed kernel consists of storage lipids and proteins. Different from some other oilseed plants such as rapeseed (Murphy, 1993), the temporal accumulation of storage lipids in cotton seeds coincides with the accumulation of storage proteins (Chapman unpublished and referred to in review by Turley and Chapman, 2010).

At the final stage of cotton seed development after approximately 45 DPA, the funiculus begins to disintegrate and both seeds and fiber desiccate through lack of a vascular connection (Benedict *et al.*, 1976). The embryo reaches its maximum dry weight while its water content declines (Hughes and Galau, 1989; Turley and Chapman, 2010). Developmental arrest and the ability to withstand desiccation are the characteristics of cotton embryos at this phase and enable the embryo to remain in a quiescent state without undergoing precocious germination.

Endosperm Development

The endosperm nucleus divides freely within hours after fertilization forming a multinucleate mass surrounding the embryo. Cellular endosperm forms at about 10 DPA. The increase in its dry weight peaks about 24 DPA, then gradually decreases as the embryo expands and stabilizes by 35 DPA (Stewart, 1986). Little is known about the co-ordination between the developing embryo and surrounding endosperm and seed coat. There is emerging evidence in arabidopsis, at least, that the endosperm is not only a source of nutrients, but also acts as an integrator of seed growth and development through intercellular signal transduction pathways involving small secreted peptide ligands (Berger *et al.,* 2006; Fiume and Fletcher, 2012).

Molecular Regulation of Seed Development

Despite the obvious genetic control of cotton seed development and physiology, global gene expression programs involved in cotton embryogenesis, especially the early events following fertilization, are still largely unknown. In recent years arabidopsis and legumes have been used as model plants for genome-wide expression profiling and have provided some insights into the processes that may also occur in cotton (McElver *et al.,* 2001; Tzafrir *et al.,* 2004).

The developmental events that culminate in the production of a mature seed from a single-cell zygote are precisely coordinated and relatively conserved in the majority of angiosperm species (Goldberg *et al.,* 1994). The recent characterization of regulatory genes identified through chemical and insertional mutagenesis has provided the first glimpses of the developmental pathways involved in zygotic embryogenesis. By searching the Seed Genes database (http://www.

seedgenes.org) and recent literature, Hsu *et al.* (2010) found that 339 non-redundant genes are required for proper embryo formation. Of these, 108 likely encode plastid-targeted proteins, and 19 are necessary for development of globular stage embryos.

A large number of genes with complex expression patterns are also involved in somatic embryogenesis (Zeng *et al.,* 2007). Various signal transduction pathways are involved in activating/repressing numerous gene sets, many of which are yet to be identified and characterized. Molecular and physiological events leading to seed formation are therefore still far from being completely understood. A large number of genes required for seed development were revealed via T-DNA insertional mutagenesis in arabidopsis (McElver *et al.,* 2001) and surprisingly, not all were embryo-specific in their pattern of expression. This is consistent with a requirement for their basal functions throughout the life cycle of the plant (Tzafrir *et al.,* 2004).

While little has yet been done in cotton to understand the gene networks and signaling pathways necessary for embryogenesis it is unlikely that they will dramatically depart from that seen in model plants. Early embryogenesis in arabidopsis is regulated by transcription factors, signal transduction pathways mediated by kinases, and proteins that establish and maintain auxin hormone gradients (Willemsen and Scheres, 2004). For example, WUSCHEL-related homeobox (WOX) transcription factor genes mark cell fate decisions during early embryogenesis (Jenik and Barton, 2005). This gene is necessary for cell divisions that form the apical embryo domain (Haecker *et al.,* 2004). PIN-formed (PIN) genes encode transporter-like membrane proteins that are important for regulating auxin transport and mutations in *PIN1* and *PIN7* disrupt the establishment of the embryogenic apical-basal axis (Steinmann *et al.,* 1999). Seed development is also controlled epigenetically and DNA methylation, for example, is critical for plant embryogenesis and for seed viability. Arabidopsis plants with loss-of-function mutations in *MET1* (METHYLTRANSFERASE1) and *CMT3* (CHROMOMETHYLASE3) produce embryos that develop improperly and have reduced viability (Xiao *et al.,* 2006). Genes that specify embryo cell identity are mis-expressed and auxin hormone gradients are not properly formed in abnormal *met1* embryos. A fundamental aspect of the transition phase of embryogenesis, when apical and basal organ fates are established, is the repression of the root-promoting gene cascade in the apical embryo domain. This is provided by the activity of the TOPLESS and TOPLESS-RELATED proteins, co-repressors that function together with AUX/IAA proteins in auxin-mediated transcriptional repression (Szemenyei *et al.,* 2008).

Several transcription factors such as LEAFY COTYLEDON1 (LEC1), LEAFY COTYLEDON2 (LEC2), FUSCA3 (FUS3) and abscisic acid insensitive3 (ABI3) have been identified as master regulators of seed development and maturation, activating genes encoding seed proteins that define each phase of embryo development (Abid *et al.,* 2010). LEC proteins stimulate abscisic acid (ABA) levels and activate genes that repress gibberellic acids (GA) levels, contributing to the high ABA to GA ratio characteristic of the maturation phase. Ectopic expression of all three *LEC* genes, *LEC1*, *LEC2* and *FUS3*, caused cells in vegetative and reproductive tissues to adopt characteristics of maturation phase embryos (Lotan *et al.,* 1998; Santos-Mendoza *et al.,* 2005; Braybrook and Harada, 2008). Ectopic expression of *LEC1* has demonstrated that this gene is required for the maintenance of the suspensor cell fate, the specification of cotyledon identity, the initiation and maintenance of the maturation phase, the promotion of embryogenic

cell identity and division and the suppression of germination (Casson and Lindsey, 2006). Seed storage protein gene expression is controlled by LEC1 through the regulation of *ABI3* and *FUS3* expression (Kagaya *et al.,* 2005). Yamagishi *et al.* (2005) isolated *TANMEI* (*TAN*) that controls various aspects of both early and late phases of embryo development. Analysis of *tan* mutants indicates that this gene plays a role in both the morphogenesis and maturation phases of embryogenesis. Moreover, *tan* mutants share many characteristics with *lec* mutants suggesting that *TAN* has overlapping functions with the *LEC* genes. For example, both *tan-1* and *lec* mutations cause desiccation intolerance and defects in storage protein and lipid accumulation.

MicroRNAs (miRNAs) are 21-nucleotide single stranded RNA molecules that act by binding complementary target mRNAs to promote their cleavage or interfere with their translation (for review, see Voinnet, 2009). During early embryogenesis of arabidopsis, specific miRNAs down-regulate one or more miRNA targets to both promote the repression of maturation and, in the advanced embryogenic and developmental stages, a reduction in those miRNAs leads to the induction of maturation (Willmann *et al.,* 2011). DICER-LIKE1 (DCL1) that is required for miRNA biogenesis was found to be critical for multiple embryonic cell differentiation events as early as the eight cell stage. Genome wide transcript profiling revealed that DCL1 was required for the early embryonic repression of nearly 50 miRNA targets among which two redundant SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) transcription factors, SPL10/SPL11, regulated by miR156, were most depressed in eight-cell *dcl1* embryos (Nodine and Bartel, 2010). Molecular analyses of miRNAs in cotton (Zhang *et al.*, 2007; Barozai *et al.*, 2008; Wang *et al.*, 2012) indicate that most miRNA families and their specific gene targets are highly conserved between cotton and arabidopsis so will likely carry out the same roles in seed development.

Somatic Embryogenesis as a Model for Embryo Development

In a parallel with the developmental pathway of zygotic embryos in seeds, somatic embryos can be induced in cotton *in vitro* from a group of meristematic cells that go through globular, heart shaped, torpedo shaped and cotyledonary stages (Price and Smith, 1979; Shoemaker *et al.,* 1986). The zygote is intrinsically embryogenic, while somatic embryogenesis requires the induction of embryogenic competence. Despite these differences, some common features between zygotic and somatic embryogenesis have been reported (Trolinder *et al.,* 1987, 1988, 1989). One of the major obstacles to understanding in detail the events that govern early embryo formation in cotton is the relative inaccessibility of the young embryo to experimental manipulation, particularly at the early stages of embryogenesis involving only one or a few cells. Somatic embryogenesis offers an alternative approach that circumvents this problem in some respects as large quantities of early embryos can be simultaneously induced and studied at the molecular or biochemical level.

Gene expression associated with somatic embryogenesis in somatic embryogenic responsive cotton cultivars including Coker 201 and CCR124 was analyzed using a suppression subtractive hybridization (SSH) approach (Zeng *et al.*, 2006; Wu *et al.,* 2009). Genes encoding transporters such as lipid transfer protein (LTP) were found to be the major group of genes responsive to

somatic embryogenesis. Expression of LTP genes is strongly associated with the first differentiated tissue of somatic embryos and exerts a regulatory role in controlling cell expansion during embryo development (Dodeman *et al.,* 1997). This induction of different transporters was followed by induction of genes encoding proteins involved in regulating transcription and post-transcriptional processing, including Zinc Finger-HD homeobox genes, ethylene-responsive transcriptional co-activators, a putative RNA helicase, a SCARECROW (SCR)-like gene, and genes encoding nonsense-mediated mRNA decay proteins and high mobility group proteins. Homeodomain genes play a key regulatory role in pattern formation and differentiation in all multi-cellular organisms. SCR is critical in regulating the asymmetric division of the cortex/endodermis initial and radial patterning of tissues in both the root and shoot during embryogenesis (Di Laurenzio *et al.,* 1996; Wysocka-Diller *et al.,* 2000).

METABOLISM IN THE DEVELOPING SEED AND ITS MANIPULATION THROUGH BIOTECHNOLOGY

A hallmark of plant embryos is the accumulation of storage reserves and secondary metabolites. Cotton seed is a rich source of oil and proteins that have been the subject of many investigations and evaluated as a wholesome, nutritious and versatile ingredient in animal feed as well as human food products (review by Cherry, 1983; Alford *et al.,* 1996; Liu *et al.,* 2009). Cotton seed is also characterized by the accumulation of lysigenous gossypol glands containing terpenoid aldehydes, principally gossypol that is toxic to non-ruminant animals and humans (Fryxell, 1968; Stipanovic *et al.,* 1977). Recent advances in the understanding of the biochemical, cellular and molecular mechanisms underlying biogenesis of seed storage compounds and secondary metabolites, coupled with the cloning of many of the genes involved in these processes, have facilitated the production of designer cotton seeds with improved nutritional benefits and enhanced functional properties that will add value to this by-product of fiber production.

Carbohydrate Metabolism

Developing cotton fruits (bolls) are very strong carbohydrate sinks. The fruit can increase in weight by as much as 15% per day (Schubert *et al.,* 1986). During the early stage of seed development, sucrose derived from the photosynthetic leaves is delivered to the seed coat and endosperm, via the funiculus, and it is either cleaved by sucrose synthase (Sus) producing UDP-glucose and fructose or hydrolysed by invertase into glucose and fructose (Hendrix, 1990; Ruan *et al.,* 2008). The comparison of relative activities of sucrose synthase and invertase in the seed coats at this stage suggested that the Sus seems to predominate over invertase (Hendrix, 1990; Ruan *et al*., 2008). UDP-glucose could be transported to fiber cells and directly used in cellulose biosynthesis (Benedict *et al.,* 1976; Haigler *et al.,* 2001). However, much of the hexoses are used to synthesize starch in the seed coat and endosperm, at least transiently (Hendrix, 1990; Ruan *et al*., 2008).

Cotton embryos accumulate starch beginning at 20 DPA when starch in the seed coat begins to decline. At about 40 DPA, the starch in the embryo starts to disappear and approximately the

same quantity of complex sugars appears as galactosides including raffinose and stachyose. By about 45 DPA, carbon importation into the seed has ceased and re-arrangement of non-structural carbohydrates becomes a major metabolic activity driving seed maturation (Benedict *et al.*, 1976). Raffinose is synthesized by the transfer of galactose to sucrose from galactinol, while a second galactose addition produces stachyose. Mature cotton seeds contain up to 10% by weight of these soluble storage carbohydrates (Muller and Jacks, 1983; Mellon and Cotty, 1999). Raffinose and stachyose disappear rapidly early after germination and they serve as readily mobilizable carbon sources before the lipid utilizing enzymes become fully active (Bortman *et al.,* 1981; Doman *et al.,* 1982; Miernyk and Trelease, 1981; Trelease *et al.,* 1986).

It has been well established that Sus plays an important role in young cotton ovule carbohydrate partitioning and that sucrose phosphate synthase and the galactoside synthesizing enzymes are important in carbohydrate partitioning in maturing cotton seeds (Hendrix, 1990; Ruan, 2005). In early seed development, Sus was localized in the cellularising endosperm, but not in the heart shaped embryo at 10 DPA (Ruan *et al.,* 2008). In developing cotton embryos, Sus activity was found to be about five-fold that of vacuolar invertase and more than 10-fold that of alkaline invertase (Ruan *et al.,* 2003), indicating a key role for Sus in degrading incoming sucrose during early cotton seed development. Suppressing the expression of Sus in the cotton seed coat led to a fiberless phenotype (Ruan *et al.,* 2003), whereas silencing its expression in the filial tissue resulted in stunted and unviable seeds and loss of transfer cells (Ruan *et al.,* 2003; 2008). On the other hand, elevation of Sus activity by over-expressing a potato *Sus* gene in transgenic cotton resulted in an increased seed number (Xu *et al.,* 2011). The higher Sus activity in the filial tissues might significantly enhance seed sink strength (Pugh *et al.,* 2010) and consequently reduce seed abortion to increase the number of seeds set.

Oil Biosynthesis and Accumulation in Cotton Seeds

Oil is one of the major compounds synthesized and deposited during cotton seed development. It is composed almost entirely of triacylglycerol (TAG) molecules that consist of three fatty acids (FAs) bound to a glycerol backbone. FA biosynthesis and TAG assembly are highly compartmentalized processes that are located in several different sub-cellular organelles. *De novo* biosynthesis of FAs occurs in the stroma of plastids in the developing seeds. FAs are then exported to the cytoplasm in the form of acyl-CoA thioesters, and some of these FAs may also be modified while attached to phospholipids on the surface of endoplasmic reticulum (ER) prior to TAG assembly and storage in the oil bodies or oleosomes (see review by Browse and Ohlrogge, 1995).

In most plant species, including cotton, the sugars providing the carbon skeleton for lipids synthesized in the seed are first transported into the endosperm from the source tissues via the seed coat and are then absorbed by the embryo. The sugars include sucrose, glucose and fructose. The cleavage products of sucrose generated by Sus and invertase are utilized through both the cytosolic and the plastidic glycolytic pathways. Multiple transporters, including the glucose-6-phosphate (Glc-6-P) translocator, the triose phosphate translocator, and the phosphoenolpyruvate (PEP) translocator, which are localized in the plastid membrane, exchange intermediates

that are generated during glycolysis from the cytosol to the plastid (Fischer and Weber, 2002). The PEP and pyruvate generated are utilized in plastids as the carbon source for FA biosynthesis. Malonyl-CoA, produced from pyruvate, serves as a two-carbon unit donor in incremental FA synthesis catalysed by a series of fatty acid synthases (Browse and Ohlrogge, 1995).

While little detailed molecular analyses have been done in cotton, Bourgis *et al.* (2011) recently compared the transcriptome and metabolomes of oil accumulating developing mesocarp of oil palm (*Elaeis guineensis*) and sugar accumulating developing mesocarp of date palm (*Phoenix dactylifera*) to gain an understanding of the reasons for the large difference in carbon partitioning between these two closely related palm species. Among all the genes involved in fatty acid biosynthesis and TAG assembly, only transcripts encoding the 18 plastidial fatty acid biosynthetic enzymes that are involved in the conversion of pyruvate to fatty acids were found to be significantly up-regulated in oil palm by, on average, 13 times. Plastidial transporters for hexose, pentose, triose phosphate, and phosphoenolpyruvate were all strongly up-regulated in oil palm, pointing to increased capacity for carbon flow into the plastid. Surprisingly, most enzymes of TAG assembly and cytosolic glycolysis were not very different between the two species, indicating that it is FA biosynthesis and transfer rather than TAG assembly that exert the most control over oil accumulation in palm and possibly other plants.

Sucrose and glucose are the major source of carbon provided by maternal tissues to developing seeds. In embryos and endosperm of four oilseeds, including *B. napus*, *Ricinus communis*, *Euonymus alatus* and *Tropaeolum majus*, Sus ESTs were 20-40 fold higher than neutral invertases (Tronocoso-Ponce *et al.,* 2011). While there are many different levels of regulation of metabolic flux through pathways other than at the transcriptional level, these EST data suggest Sus may play a key role in generating hexoses during oil accumulation in embryonic cotyledons. Such a result appears to be consistent with the biochemical analysis in developing cotton seeds (Ruan *et al.*, 2003; 2008).

In the plastids of all plants, acetyl-CoA carboxylase (ACCase) catalyses the first committed step in the pathway of oil synthesis by addition of a carboxyl group to acetyl-CoA to form malonyl-CoA. The malonyl group is then transferred from CoA to an acyl-carrier protein (ACP), which serves as the carrier for the growing FA chain. The acetyl-CoA condensing enzyme, ketoacyl-ACP synthase III (KASIII) catalyses the elongation step of acetyl-CoA and malonyl-ACP to yield acetoacetyl-ACP, the first step in the fatty acid elongation pathway (Clough *et al.,* 1992). The repeated process of adding two-carbon units onto the elongated fatty acid chain is catalysed by KASI leading to the formation of palmitoyl-ACP (C16:0-ACP). KASII catalyses the elongation of palmitoyl-ACP to stearoyl-ACP (C18:0-ACP). A soluble stearoyl-ACP Δ9-desaturase (SAD) introduces the first double bond into stearoyl-ACP to convert it to oleoyl-ACP in the plastid. Cotton seed oil accumulates only about 2% stearic acid as much of stearate is converted to oleate *via* SAD. SAD enzymes are encoded by a multigene family consisting of at least five genes per diploid genome in cotton (Liu *et al.,* 1996; 2009). The growing saturated fatty acyl chain and the monounsaturated oleate are cleaved off the ACP by a substrate specific thioesterase enzyme, FatB or FatA, enabling them to exit the plastid into the cytoplasm. On ER membranes, oleic acid becomes associated with Phosphatidylcholine (PC) and can be further modified by a membrane-bound omega-6 (or Δ12) fatty acid desaturase, FAD2. FAD2 intro-

duces a double bond into oleic acid to form the linoleic acid that accounts for more than 50% of total fatty acids in cotton seed oil. There are at least four different genes encoding cotton FAD2, with *ghFAD2-1* playing a major role in the production of linoleic acid in cotton seed oil (Liu *et al.,* 1999a,b; Pirtle *et al.,* 2001; Zhang *et al.,* 2010). The expression of *ghFAD2-1* is seed-specific and reaches its highest level at the middle maturity stage, between 25-35 DPA, before drastically declining when seeds approach maturity (Liu *et al.*, 1999a). In cotton, oleic acid is also the precursor for a group of carbocyclic fatty acids, i.e. dihydrosterculic acid, sterculic acid and malvalic acid, which account for less than 1% of total FAs. In a developing cotton seed, these carbocyclic fatty acids are exclusively synthesized in the embryo axis, but not in cotyledons (Wood, 1986). A fatty acid methyltransferase, known as cyclopropane FA synthase (CPA-FAS), was identified as the first enzyme converting oleic acid to dihydrosterculic acid using *S*-adenosylmethionine as the methyl donor (Bao *et al.,* 2003; Liu *et al.*, 2004; Yu *et al.,* 2011).

The FAs formed may then be incorporated into membrane and storage lipids *via* the Kennedy pathway by the sequential esterification of glycerol-3-phosphate through the action of glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid, followed by 1-acyl-sn-glycerol-3-phosphate acyltransferase (LPAAT) to form phosphatidic acid (PA). Dephosphorylation of PA by phosphatidic acid phosphatase results in the formation of diacylglycerol (DAG), which is then acylated to form TAG by a DAG acyltransferase (DGAT) (Browse and Ohlrogge, 1995). The recently discovered enzyme, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) is required for the efficient biosynthesis of polyunsaturated fatty acid during TAG accumulation in seeds (Lu *et al.,* 2009). PDCT catalyses the inter-conversion of PC and DAG by transferring the phosphocholine headgroup between these two molecules, thus enabling more oleic acid be desaturated by the FAD2 enzyme. TAG can also be formed in plants *via* an acyl-CoA independent pathway, catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist *et al.,* 2000). Finally TAGs are stored in seeds in specialized oil body structures. Chapman studied the enzyme activities of some of these TAG assembly enzymes in developing cotton seed and found LPAAT and DGAT are highest in cotton embryos between 25 to 35 DPA (Chapman unpublished data, referred by Turley and Chapman, 2010).

Metabolite analysis (Perry *et al.,* 1999) and other studies (Zheng *et al.,* 2008) suggest that DGAT may be one of the rate-limiting steps in plant seed lipid accumulation. Over-expression of the arabidopsis *DGAT1* in wild type plants led to an increase in seed oil content and seed weight (Jako *et al.,* 2001). Subsequently, *DGAT* expression has been genetically manipulated to produce crops with increased oil content (Lardizabal *et al.,* 2008; Weslake *et al.,* 2008; Zheng *et al.,* 2008; Taylor *et al.,* 2009). The major reduction in oil content in seeds of arabidopsis with RNAi-mediated silencing of *DGAT1* and *PDAT1* indicates that these enzymes have overlapping functions in seed TAG synthesis, and also led to the discovery that TAG synthesis or other functions of DGAT1 and PDAT1 are essential for normal embryo and pollen development (Zhang *et al.,* 2009). TAG synthesis clearly plays an important role not only as a storage reserve, but also in normal growth and development.

In arabidopsis LEC1 and WRI1 have been identified as two key transcription factors involved in the regulation of oil accumulation (Baumlein *et al.,* 1994; Cernac and Benning, 2004) and over-expression of either or both of *LEC1* and *WRI1* resulted in oil increases without negative

impacts on grain yield (Shen *et al.,* 2010). In the developing seeds, carbon derived from photosynthesis is partitioned into different storage compounds. The carbon flux into oil may be in competition with other metabolic pathways, such as starch. It has been recently demonstrated that redirecting carbon partitioning by genetic down-regulation of *APS1*, a major catalytic isoform of the small subunit of ADP-glucose pyrophosphorylase involved in starch biosynthesis, in combination with up-regulation of oil biosynthesis through over-expressing *WRI1*, could substantially increase TAG accumulation and therefore lead to increased energy density of the seed biomass (Sanjaya *et al.,* 2011). However, this may not be applicable to cotton as it does not accumulate much starch in mature seeds.

Nutritional Enhancement of Cotton Seed Oil

Other than the fiber, oil is the most valuable product of a cotton seed, and is widely used as a cooking oil and an ingredient in marinades, dressings, pastries, margarines, and shortenings. Furthermore, oil is by far the most efficient form for energy storage, since it contains more than twice the energy on a dry weight basis than can be stored in starch or proteins. Consequently whole cotton seed has been regarded by the dairy industry as a special feed ingredient with advantageous energy and dietary fiber properties required by the high-producing dairy cow. There is increasing emphasis on greater energy density in cotton seed through increasing seed oil content at the expense of carbohydrate concentrations (Coppock *et al.,* 1987; O'Brien *et al.*, 2005).

In cotton seed, the ratio of oleic and linoleic acids is largely determined by the seed-specific enzyme, ghFAD2-1, which converts about 80% of oleic acid to linoleic acid in developing cotton seed. High levels of linoleic acid have made cotton seed oil prone to auto-oxidation. Partial hydrogenation is often used to extend the oil's stability and shelf life, producing various levels of *trans* fatty acids that are recognized to have cholesterol-raising properties and associated increased risk of cardiovascular heart disease (Mozaffarian *et al.*, 2006). Seed-specific down-regulation of *ghFAD2-1* using an RNAi approach was able to raise oleic acid levels up to 78%, at the expense of linoleic acid that was reduced to 4% (Liu *et al.,* 2002). Cotton seed oil also contains the highest level of palmitic acid among commodity vegetable oils produced in the temperate climates. Palmitic acid is widely reported to raise total plasma cholesterol and low-density lipoprotein cholesterol levels (Kris-Etherton *et al.,* 1993). The small amount of carbocyclic fatty acids that exist in cotton seed oil are potent inhibitors of mammalian fatty acid desaturases and are therefore believed to be nutritionally undesirable (Shenstone and Vickery, 1961; Cherry, 1983). Significant reduction of these nutritionally undesirable fatty acids would therefore increase the health appeal of cotton seed oil and competitiveness relative to other vegetable oils. In the subsequent experiments of Liu and co-workers (Liu *et al.*, 2008b), two other genes, *ghFatB* and *ghCPA-FAS,* were simultaneously down-regulated together with *ghFAD2-1*. The transgenic cotton plants had reduced palmitic acid and carbocyclic fatty acids and a further increase of oleic acid, representing potentially enhanced nutritional value.

Seed-specific RNAi mediated gene silencing of *ghSAD-1* led to a raised stearic acid level up to 40% on the single seed basis, accompanied by a reduction of all other fatty acids in cotton seed oil: i.e., linoleic acid was reduced to 38% from the normal 56%; palmitic acid to 17% from 26%; and oleic acid to 10% from 15% (Liu *et al.,* 2002). Such high-stearic oil was found to have a markedly increased melting point compared to the conventional cotton seed oil control

(Qing Liu, unpublished data). Different from palmitic acid, its shorter chain saturate counterpart, stearic acid does not raise cholesterol and therefore does not impact heart health negatively (Mensink and Katan, 1992). Because of its high oxidative stability and high melting point, cotton seed oil with raised stearic acid level could be used to manufacture *trans*-free margarine and as a substitute for cocoa butter in confectionery.

Albeit lacking omega-3 fatty acids, traditional cotton seed oil does contain high levels of linoleic acid that could provide a significant endogenous substrate pool for the Δ6 desaturase that produces γ-linolenic acid (C18:$3^{\Delta6,9,12}$, GLA) and the Δ15 desaturase that produces α-linolenic acid (C18:$3^{\Delta9,12,15}$, ALA) that are found in other plants. The Δ6 desaturase could also catalyse further desaturation of ALA to produce stearidonic acid (C18:$4^{\Delta6,9,12,15}$, SDA), a precursor for the production of omega-3 long chain polyunsaturated fatty acids (LC-PUFA). Transgenic expression of a Δ6 desaturase from *Echium plantagineum* and a Δ15 desaturase from *Brassica napus* has led to the accumulation of 25% GLA and 35% ALA in cotton seed oil, respectively (Liu *et al.*, 2008a). Up to 6% SDA, in addition to both ALA and GLA, were observed in homozygous F_1 seeds following the hybridization of these two transgenic genotypes. Such genetically modified cotton seed oil may provide an alternative source for these C18 polyunsaturated fatty acids that are currently obtained from herbal oils such as those from evening primrose, borage and black current. The transgenic cotton seeds expressing the entire omega-3 LC-PUFA pathway may also be able to replace the current marine sources for omega-3 LC-PUFA, such as eicosapentaenoic acid (20:$5^{\Delta5,8,11,14,17}$, EPA) and docosahexaenoic acid (22:$6^{\Delta4,7,10,13,16,19}$, DHA).

Improving cotton seed oil quality may not be limited to just increasing its nutritional value. The accumulation of vernolic acid, an epoxygenated FA with valuable industrial applications, increased to up to 17% of total fatty acids in the seed oil of transgenic cotton plants expressing a Δ12 fatty acid epoxygenase derived from *Crepis palaestina* (Zhou *et al.*, 2006). However this level of vernolic acid in cotton seed oil is still considerably less than the 70% found in *C. palaestin*a, the source of the epoxygenase transgene. It is apparent that metabolic bottlenecks are limiting the full potential for cotton seed in accumulating this unusual fatty acid at an economically viable level, and further research would be required to overcome this bottleneck. The production of unusual fatty acids in oilseed crops in general has been a great challenge because of the apparent complexity of the biosynthesis pathways for these fatty acids (Napier, 2007).

Synthesis and Accumulation of Seed Storage Proteins in Cotton Seeds

In higher plants, seed storage proteins are synthesized on the rough ER, using amino acids directly taken up by the embryo, or obtained after transamination reactions. Subsequently, they are transported into protein storage vacuoles by a vesicle-mediated pathway (Jolliffe *et al.*, 2005). In cotton seed, two major classes of storage proteins are globulins and albumins, which differ in their solubility properties. Both globulins and albumins are synthesized and compartmentalized in storage protein vacuoles during cotton seed maturation (Dure and Chlan, 1981). Globulins can be further classified based on the sedimentation rate of their aggregated forms into the 7S vicilins (or α-globulin) and 11/12S legumins (or β-globulin; Youle and Huang, 1981). In a recent

survey of the most abundant cotton seed storage proteins, nearly all of the proteins identified belong to the vicilin and legumin families, comprising 60-70% of the total seed proteins (Hu *et al.,* 2011).

Vicilin A and Vicilin B, which share 72% amino acid similarity, represent the first discovered cotton seed storage proteins (Chlan *et al.,* 1987). Each vicilin is encoded by a single-copy genes in the diploid *Gossypium* genomes, and their corresponding homeologous copies were identified in allotetraploid cotton. Similarly there are two legumin isomers, Legumin A and Legumin B, which are more diverged compared to the vicilin gene family, sharing only 58.5% amino acid similarity. As with the vicilins, each legumin is encoded by a single-copy gene in the diploid cotton genomes.

The expression of seed storage proteins genes is regulated temporally and spatially in cotton seeds (Galau *et al.,* 1983; Chlan *et al.,* 1987; Galau *et al.,* 1992). Despite the existence of mainly single genes encoding each protein in the diploid genome, the mRNA pool in developing cotton seeds was composed of 30% legumin mRNAs, 15% vicilin mRNAs, and 2% 2S albumin mRNAs (Hughes and Galau, 1989; Galau *et al.,* 1992). Therefore, regulatory sequences from genes encoding seed storage proteins represent a valuable source of promoters that could be utilized to drive the expression of transgenes in a seed-specific manner. The promoter region of α-globulin gene B was isolated and identified as highly seed-specific (Sunilkumar *et al.,* 2002), and it has been successfully used for seed-specific reduction of toxic gossypol levels in cotton seed (Sunilkumar *et al.,* 2006).

Cotton seed protein contains large amounts of arginine, especially compared to some legume species such as soybean (Capdevila and Dure, 1977). Arginine has been shown to slow down cancer progression (Lowell *et al.,* 1990), to act as a principal regulator of blood pressure, and to cause a relaxation of cardiovascular smooth muscle cells following conversion to nitric oxide (Moncada and Higgs, 1993). However, cotton seed is severely deficient in lysine and slightly deficient in isoleucine and the sulphur amino acids, including methionine and cysteine, compared to other major oilseeds, such as soybean and canola (Capdevila and Dure, 1977). Additionally, when pigment glands in the cotton seed are disrupted during processing, the free gossypol can bind to the epsilon amino group of lysine and reduce its availability to below acceptable levels for animal nutrition (Zarins and Cherry, 1981; Calhoun *et al.,* 1995).

Genetic improvement of cotton seed storage protein and amino acid profiles are clearly necessary if it is to be used as a source of protein for non-ruminant animals or humans, but little if any progress has been documented in the literature. The feasibility of raising lysine content in a plant seed has been demonstrated in maize in recent years (Huang *et al.,* 2006; Frizzi *et al.,* 2008), and this could also be applicable to cotton. To imitate the natural high lysine maize *opaque* mutant with its reduced level of the seed storage prolamine α-zein, RNAi was used to specifically suppress α-zein production in transgenic maize kernels, resulting in a doubling of the lysine content from 2400 to 4800 ppm (Huang *et al.,* 2006). To further enhance the accumulation of free lysine, the bifunctional enzyme lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), which is responsible for lysine catabolism, was also expressed in transgenic maize and increased lysine content about 30 fold. An additional bottleneck for lysine accumulation was identified as the lysine feedback effect, which was by-passed by express-

ing a lysine feedback-insensitive enzyme from *Corynebacterium glutamicum*, CordapA. This resulted in further enhancement of lysine levels up to 100 fold in maize endosperm relative to non-transgenic controls (Frizzi *et al.,* 2008).

Cotton seed is also deficient in sulphur-containing amino acids because the sulphur-rich proteins, such as albumin, constitute a low fraction of the total seed proteins (Galau *et al.,* 1992; Hu *et al.,* 2011). The sulphur amino acids, such as methionine, in cotton seeds could be genetically enhanced by either over-expressing a sulphur-rich protein or reducing the relative abundance of endogenous sulphur-poor proteins, such as globulins. It has been demonstrated in rapeseed and lupin seeds that methionine content can be increased substantially by the introduction of foreign genes encoding naturally sulphur-rich proteins, such as the 2S albumin from Brazil nut that contains 18% methionine and 8% cysteine (Altenbach *et al.,* 1992; Molvig *et al.,* 1997). These approaches take advantage of the plant's homeostasis mechanisms such that seeds generally appear to compensate for a shortfall of a major protein by accumulating other seed proteins to maintain a relatively constant protein content. For example, the genetic reduction of β-conglycinin in soybean resulted in a concomitant increase in the accumulation of glycinin, which contains higher levels of sulphur amino acids (Kinney *et al.,* 2001). However, despite the observed increase of methionine in transgenic seeds, total seed sulphur amino acids remained virtually unchanged relative to the control plants. Molvig *et al.* (1997) expressed the sunflower seed albumin (SSA) protein that is rich in methionine (16%) and cysteine (8%) in transgenic lupin, resulting in a 94% increase in methionine accompanied by a 12% reduction of cysteine, another sulphur-rich amino acid. The unexpected decrease in cysteine suggested that synthesis of the transgenic protein simply caused a redistribution of limited sulphur resources. A substantial increase in the bioavailable sulphur pool would be necessary for any real gain in these nutritionally desirable sulphur-rich amino acids.

The sulphur-rich 2S albumins, unfortunately, have been recognized as potent allergens and correlated with potentially lethal anaphylaxia in numerous plant seeds such as Brazil nuts, peanuts and sunflower seeds (see review by Herman and Burks, 2011). Despite some early efforts in biochemical characterization of cotton 2S albumin, much remains poorly understood about the potential allergenic effects of cotton seed storage proteins (Youle and Huang, 1979). Such an issue needs to be addressed before genetic enhancement of 2S albumin in cotton seeds could be undertaken.

A class of 18 proteins termed as late embryogenesis abundant (LEA) proteins was found to be highly up regulated in the seed desiccation period in cotton (Hughes and Galau, 1989). It is hypothesized that some of these LEA proteins are functionally involved in eliciting desiccation tolerance in the seed, and their synthesis is correlated with abscission of the funiculus that terminates nutrition and water transport to the seed from the mother plant (Hughes and Galau, 1989; Turley and Chapman, 2010).

Oil Body Proteins: Oleosins

Oleosins are a class of small proteins associated with the oil body membrane in plant seeds (Huang, 1992; Hughes *et al.,* 1993; Chapman *et al.,* 2012). They play dual physiological roles,

acting as protectors for stabilizing the oil bodies in developing and mature seeds and as the recognition signal for lipase binding in germinating seeds. Oleosins are alkaline and hydrophobic proteins having three domains including amphipathic N and C termini and a central hydrophobic domain that is highly conserved and could penetrate through the phospholipid monolayer into the oil body matrix. The N and C termini of the oleosin polypeptide are located on the oil body surface and interact with the phospholipid membrane, forming a highly stable structure that surrounds the oil body in an amphipathic shell (Huang, 1992). Two distinct genes encoding oleosin, *MatP6* and *MatP7* that are 77% identical, were expressed during the maturation and post-abscission stages of cotton embryogenesis (Hughes and Galau, 1989; Hughes *et al.,* 1993).

Taking advantage of the oleosin's capability for anchoring onto the surface of oil bodies, artificial oil bodies (AOBs) have been successfully constituted and used for purification, refolding, and immobilization of recombinant proteins in transgenic oilseed plants. The heterologous protein fused to the N/C-terminus does not alter the functional domains of the oleosin, and the fusions can still anchor normally onto the surface of oil bodies. Since oil bodies are of low density and free of contaminating proteins, they can be separated from other cellular components simply by flotation centrifugation. The desired protein along with the oil body fraction can be readily removed without the need for costly and time consuming chromatographic steps for protein purification. The desired protein can then be released from oil bodies by using a site-specific protease to cleave at a sequence engineered into the recombinant protein. This property has been exploited for numerous "molecular farming" applications, as reviewed recently by Boothe *et al.* (2010). For example, fusion proteins of oleosin with either a human precursor insulin (Des-B30) (Nykiforuk *et al.,* 2005) or a human insulin-like growth factor 1 (hIGF-1) have been successfully expressed in arabidopsis seeds (Li *et al.,* 2011). Similar strategies could be used to generate valuable recombinant proteins in cotton.

Gossypol Synthesis and Accumulation in Cotton Seeds

Lysigenous glands in cotton plants, including cotton seed tissues, contain terpenoid aldehydes. These are sesquiterpenes (C15) derived from a cytosolic branch of terpenoid metabolism via the mevalonate pathway (Stipanovic *et al.,* 1999; Cai *et al.,* 2010), and they provide a defense against herbivory. Farnesyl diphosphate (FPP) is generated as the linear carbon skeleton of the sesquiterpenes, and its cyclisation (catalysed by a terpene cyclase enzyme, (+)-δ-cadinene synthase, to form (+)-δ-cadinene) is the first committed step in gossypol biosynthesis (Chen *et al.,* 1995). (+)-δ-cadinene is then hydroxylated at the C-8 position (leading to 8-hydroxy-(+)-δ-cadinene) through the action of a cytochrome P450 enzyme, (+)-δ-cadinene-8-hydroxylase (CYP706B1). Subsequently, 8-hydroxy-(+)-δ-cadinene is converted to desoxyhemigossypol (dHG) by a yet uncharacterized process and further oxidized by one electron into hemigossypol. Finally, gossypol is formed by a phenolic oxidative coupling of two molecules of hemigossypol, catalysed by a hydrogen peroxide-dependent peroxidase enzyme (Dewick, 2009).

Gossypol is the predominant sesquiterpenoid formed in cotton seed, with only traces existing of dHG and hemigossypol (Cai *et al.,* 2010). Gossypol occurs in either free or bound to proteins, and the former is toxic. Gossypol in its unbound form causes anorexia, slow growth, and in-

creased fat deposition in liver tissue when fed to fish in excess (Wood and Yasutake, 1956). Gossypol is particularly toxic to non-ruminant animals and has inhibitory effects on male fertility when cotton seed is used for feeding either directly or as a meal following oil extraction. During the oil extraction process, gossypol is deactivated in the meal through moist heating, which causes the formation of a double bond between the ε-amino group of lysine and the aldehyde group. Although effective in reducing the toxicity of gossypol, the binding of gossypol reduces the amount of soluble protein and bioactive lysine in the meal (Zarins and Cherry, 1981).

Development of edible protein products from cotton seed for non-ruminants or humans has been impeded by the presence of the gossypol glands and has been a serious impediment for widespread cotton seed processing and use (Hopper, 1959). Mutant glandless cotton that is free of gossypol throughout the whole plant was first reported by McMichael (1954), and glandless cotton seed kernels have been used to produce snack foods, peanut butter, and baked and confectionary products. Despite initial optimism, these varieties have proven to be commercially unviable because the systemic absence of the protective terpenoids have made the aerial part of cotton plant much more susceptible to insect pests and pathogens than normal glanded cotton varieties.

Because (+)-δ-cadinene synthase catalyses the first committed step in gossypol biosynthesis, considerable efforts have also been made in characterizing and genetically manipulating this enzyme. A cDNA encoding (+)-δ-cadinene synthase was first cloned and functionally characterized from the A-genome diploid cotton (*G. arboreum*) (Chen *et al.,* 1995). The enzyme is encoded by a multigene family with different temporal and spatial regulation, and the isoforms may be responsible for different branches of the cotton sesquiterpene pathway. For example, (+)-δ-cadinene synthase mRNA and enzyme are highly up-regulated in response to infection by *Xanthomonas campestris* pv. *malvacearum* strains (now called *Xanthomonas axonopodis*) and *Verticillium dahliae* (Townsend *et al.,* 2005). RNAi down regulation of this gene led to a drastic seed-specific reduction of gossypol levels without reducing this compound and other related terpenoids in somatic tissues (Sunilkumar *et al.,* 2006). An average gossypol value of 0.2 µg/mg has been observed in the F_2 transgenic seeds, compared to 10 µg/mg in wild type, a value that falls well within the safety limit (0.6 µg/mg) set by World Health Organization (Lusas and Jividen, 1987).

Gene introgression from wild cotton species

An ideal cotton plant should have glandless seeds with low or no gossypol for optimal food and feed uses while retaining glanded foliage with a higher level of gossypol to limit pest attacks. Such a trait only exists naturally in some Australian wild diploid cotton species, such as *G. sturtianum* (Brubaker *et al.,* 1996). In cotton breeding programs inter-specific hybrids have been developed attempting to transfer the glandless-seed and glanded-plant trait from wild cotton to cultivated cotton (Vroh Bi *et al.,* 1999). A trispecies bridge hybrid strategy involving [(*G. hirsutum* x *G. raimondii*)^2 x *G. sturtianum*] was able to generate progeny plants that produced nearly or completely glandless seeds while still having normal gossypol gland density on their aerial parts. However, this combination of traits has yet to be introgressed into commercial cot-

ton cultivars because of the lack of recombination between the C genome of *G. sturtianum* and A and D genomes of tetraploid cotton. Attempts were also made to characterize the introgression of chromosomal segments from an Australian wild diploid cotton using RFLP markers of known chromosomal locations (Vroh Bi *et al.,* 1999). However, the mechanism for the repression of gossypol biosynthesis in the seeds of *G. sturtianum* remains unclear. It appears that multiple genes are involved as indicated by the high level of heterozygosity in the segregation of gossypol levels in the progeny testing following up to five generations of backcrossing and selfing (Benbouza *et al.,* 2010).

Germplasm selection for high (+)/(-) gossypol ratio

Gossypol is synthesized by a free radical dimerization of hemigossypol that yields two optically active enantiomers, (+)-gossypol and (-)-gossypol due to restricted rotation around the binaphthyl bond. Only (-)-gossypol is toxic to animals, while toxicity to insects and pathogens is independent of the (+)- to (-)- ratio. Therefore, a high (+)- to (-)- gossypol ratio in the seed would retain their natural defense capability while producing seeds that could be fed to non-ruminant animals. For example, broiler chickens fed a diet containing cotton seed with a higher ratio of (+)- to (-)- gossypol gained more weight compared to those fed on seeds with a low (+)- to (-)- gossypol ratio (Bailey *et al.,* 2000). On the other hand, a low ratio of (+)- to (-)-gossypol was found to be more effective in inhibiting the growth of various cancer cells, in anti-HIV activity, and in reducing male fertility (Lin *et al.,* 1993; Matlin *et al.,* 1985). The (+)- to (-)- gossypol ratio is genetically determined. In most US commercial cotton, the ratio is about 3:2. Several new sources of wild cottons that produce high levels of (+)-gossypol have been identified, including (+)- to (-)- gossypol ratio as high as 98:2 in some Moco cotton (*G. hirsutum* var. marie galante) accessions. Attempts have been made to breed cotton plants that incorporate such a trait from Moco cotton in order to maximise (+)-gossypol in the seed (Stipanovic *et al.,* 2000; Cai *et al.,* 2010).

Gossypol, especially in its (-)-enantiomer form, has great pharmacological interest due to its potential as an anti-cancer agent and for its male contraceptive effects (Dodou, 2005). Gossypol could effectively inhibit the enzyme activities of glycolysis and the TCA cycle, severely crippling energy metabolism and ATP production and lowering the mobility of human sperm cells in a dose-dependent manner (Medrano and Andreu, 1986).

CONCLUDING REMARKS

Currently, more than 1 billion people are undernourished with food intake below the recommended minimum daily energy requirement (OECD/FAO, 2010). Cotton seed, being a readily available by-product of more valuable cotton fiber production, is increasingly being recognized to have excellent potential as a source of additional food, feed and even biofuels for both developed and under-developed countries. In this review, we have attempted to emphasize the role of cotton seed as a renewable platform for the large-scale production and storage of many diverse biological molecules for agriculture and even for pharmaceutical and industrial enterprises. Value added cotton seed with broader applications through genetic improvement of both

seed production and quality without compromising fiber production is clearly in accord with the strategy for sustainable intensification of agricultural production by producing more food without increasing land use (Baulcombe, 2010).

Although histological, morphological, molecular and biochemical studies have provided descriptive information on embryogenesis and seed metabolism in cotton, the molecular and physiological events leading to the seed formation and storage compound accumulation are still far from being completely understood. Mutagenic silencing of genes and characterization of gene function through T-DNA tagging (techniques that are commonly used to generate loss-of-function alleles in model plants such as arabidopsis) might never be appropriate in cotton, primarily because of its relatively poor transformation efficiency and the inability to generate large numbers of transgenic lines. Furthermore, cotton is an allotetraploid with a moderately sized genome, and the presence of homeologous genes may mask knockout phenotypes because of the effects of redundant genes in the different genomes. However, the remarkable progress in the area of high throughput transcriptomics and proteomics over the last few years has now made large-scale investigations of genes and proteins achievable in cotton. Comprehensive web-based databases integrating updated EST collections, comparative transcriptome analyses between cotton and other plant species, expressed miRNAs and their putative targets, and simple sequence repeats (SSR) analyses, have become available (www.leonxi.com/, Xie *et al.,* 2011; cottonevolution.info/, Udall *et al*., 2006; and www.cotton marker.org/, Blenda *et al.*, 2006). In addition, because of its smaller genome size and low genome complexity, the worldwide cotton community has prioritized the D-genome progenitor *Gossypium raimondii* for complete sequencing by the Joint Genome Institute (Lin *et al.,* 2010) and a draft (as yet) unannotated assembly is now available for searching on-line (http://phytozome.net/cotton.php). Sequences of the A-genome progenitor *G. arboreum*, as well as a number of tetraploid (A+D) cottons are likely to follow in quick succession. These new genomic resources should now make it possible to study gene expression during cotton embryogenesis and seed development at a global level using whole genome microarrays or next generation sequencing technologies (RNAseq). They will no doubt provide an invaluable resource for identifying and characterizing genes that play critical roles during cotton embryogenesis and seed development. Insight into the complex process of seed development, and the identification and dynamic expression profiling during seed development of genes and proteins at the genome scale in many different plant species is beginning to provide a general framework for more in depth comparative studies that will aid our understanding of seed development and help identify targets for manipulation or transfer into crop plants to achieve specific desired outcomes.

Implementation of the tools of molecular biology and biotechnology has opened the door to the development of improved end-uses for cotton seed products for food, feed, as well as industrial applications. The biosynthetic pathways responsible for the synthesis and accumulation of many of the main storage compounds of seeds have largely been elucidated, and significant progress have already been made in the genetic improvement of a number of seed traits in cotton, including carbohydrate (Ruan *et al.,* 2003; Xu *et al.,* 2011), fatty acid composition (Chapman *et al.,* 2001; Liu *et al.,* 2002; 2008a,b) and gossypol (Sunilkumar *et al.,* 2006). The amenability of cotton seed to genetic modification has been clearly demonstrated in these ex-

periments, although none of the traits developed has yet to be commercialized. Despite this success, it is still unclear what factors determine the overall partitioning of seed reserves to the main storage components, and this will be critical if fiber development and quality is not to be impacted. More studies in cotton metabolic regulation will be necessary to understand the complex flux-control in the various biosynthetic pathways, especially in response to varying physiological and environmental conditions. Future advances in making a better cotton seed will be greatly facilitated by the wealth of genomics tools becoming available, and combined with efforts targeting metabolomics and flux map analysis, will allow for more rational design of genetic manipulation of the key metabolic control points to create substantial benefits for the global environment as well as the world-wide-economy and industry.

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Chapter 10

RECENT ADVANCES IN COTTON FIBER DEVELOPMENT

Michael R. Stiff[1] and Candace H. Haigler[2]
Department of Crop Science[1] and Department of Plant Biology[2]
North Carolina State University
Raleigh, NC 27695

INTRODUCTION

Cotton fiber is the world's most important natural textile fiber. In the U.S.A. in 2010, cotton fiber had a 61% share of the market for apparel and home textiles (Bearden, 2010), with synthetic fibers having most of the remaining market share. A similar demand for renewable cotton fiber occurs worldwide. A highly regulated cellular differentiation process governs the morphogenesis of the fiber. Each long cotton "lint" fiber originates from a single epidermal cell on the ovule surface that transforms into the highly elongated and reinforced dead fiber through dramatic polar expansion and cell wall thickening. Fiber morphogenesis proceeds through several stages that will be described further in this chapter: initiation, elongation, transitional primary wall remodeling, secondary wall synthesis, and maturation. These differentiation processes, which typically last at least 50 days, directly determine cotton fiber quality characteristics. The number of fibers initiated in the outer integument of the ovule is a major factor in fiber yield. Fiber fineness (weight per unit length) and micronaire are determined by the fiber perimeter and the extent of secondary cell wall thickening. Fiber length and length uniformity are both valued in modern spinning mills, and cell elongation is strongly affected by developing and maintaining high turgor pressure within the central vacuole as well as carbon supply in different regions of the seed. Fiber strength is affected by properties of the transitional "winding" cell wall layer as well as secondary wall cellulose (*e.g.*, degree of polymerization and microfibril angle). High fiber tensile properties (including strength and mechanical elongation, or elongation-to-break) help to preserve fiber length during processing, and they are also required to produce strong yarns and fabrics. The potential of cotton fibers to pickup dye molecules and to absorb water are determined by the amount and the degree of crystallinity of the secondary wall cellulose. The final collapse of the fiber into the typical kidney bean shape that facilitates spinning (through improved friction properties) relies on adequate filling, but not over-filling, with secondary wall cellulose (Wakelyn *et al.*, 2007). Since a cotton fiber is a cell wall composite, many of its important developmental processes relate to plant cell wall deposition (Haigler *et al.*, 2012).

At first, it was surprising that the single-celled cotton fiber expresses a large percentage of the genes present in a complex allotetraploid genome (Hovav *et al.*, 2008b), but the morphogenetic processes leading to commercial cotton fiber encompass many aspects of whole plant growth. Many years will be required to understand how most of the genes and regulatory networks ex-

ert their effects on the fiber differentiation process. Cotton fiber has many advantages for such experiments. It is a single cell that undergoes semi-synchronous differentiation in a series of overlapping developmental stages. Cotton fibers are easily separated from the developing seed. Therefore, researchers can sample a large population of one cell type and have clear knowledge of the predominant cellular activities at that time. Given that cotton fibers are expendable for plant growth, there is unrestricted ability to manipulate fiber development experimentally. The genus *Gossypium* includes living, non-domesticated, diploid and allotetraploid progenitor species that provide valuable comparisons and contrasts with commercial fiber (Kim and Triplett, 2001; Hovav *et al.*, 2008b; Rapp *et al.*, 2010).

Below we provide a review of emerging experimental evidence on the cellular and physiological processes underlying the morphogenesis of domesticated *G. hirsutum* fiber with emphasis on research published since 2005. Useful related information can be found in other reviews discussing: cotton fiber-ovule culture as a unique experimental tool (Kim and Triplett, 2001); the evolutionary and domestication history of cotton (Wendel *et al.*, 2009); genomic scale research on the secondary wall thickening phase (Haigler *et al.*, 2005; Haigler *et al.*, 2009); transcriptional changes during fiber development (Wilkins and Arpat, 2005; Shangguan *et al.*, 2010); and transcriptional and hormonal regulation of fiber development (Lee *et al.*, 2007).

COTTON FIBER DEVELOPMENT

Cotton Fiber Initiation

The first step in cotton fiber morphogenesis is the differentiation of selected ovule epidermal cells into fiber initials, or rounded protrusions above the ovule surface. Fiber initiation typically begins on the day of anthesis and continues to at least 5 days post anthesis (DPA) when each ovule has about 16,000 fiber initials (inclusive of young elongating fibers) in modern cultivars (Fig. 1; Stewart, 1975; Seagull and Giavalis, 2004). As initials expand, the central vacuole forms and the nucleus migrates from the base toward the middle of the developing fiber. The density of fiber initials at 0 and 1 DPA was positively correlated with lint percentage and lint index in five *G. hirsutum* cultivars (Li *et al.*, 2009). Humans selected for higher density and more synchronous fiber initiation during cotton domestication (Butterworth *et al.*, 2009). Generation of turgor pressure within the central vacuole drives the expansion of fiber initials. Ruan and coworkers (Ruan *et al.*, 2003) showed that both fiber initiation and fiber elongation were inhibited by suppressed expression of the *SS3* isoform of sucrose synthase (Sus) in transgenic cotton (Ruan *et al.*, 2003). Plants with lower Sus protein in the seed coat compared to wild-type cotton produced fewer fibers that appeared shrunken and collapsed when viewed by scanning electron microscopy. The transgenic fibers had lower Sus activity, which correlated with decreased levels of hexoses and starch, but not sucrose. Possibly decreased hexoses caused lower osmotic potential and reduced turgor, but the fiber defects might also have been at least partly explained by factors such as less Sus-generated UDP-glucose to provide the substrate for cell wall synthesis (see further discussion below) and/or disruption of signaling pathways that might depend on hexose.

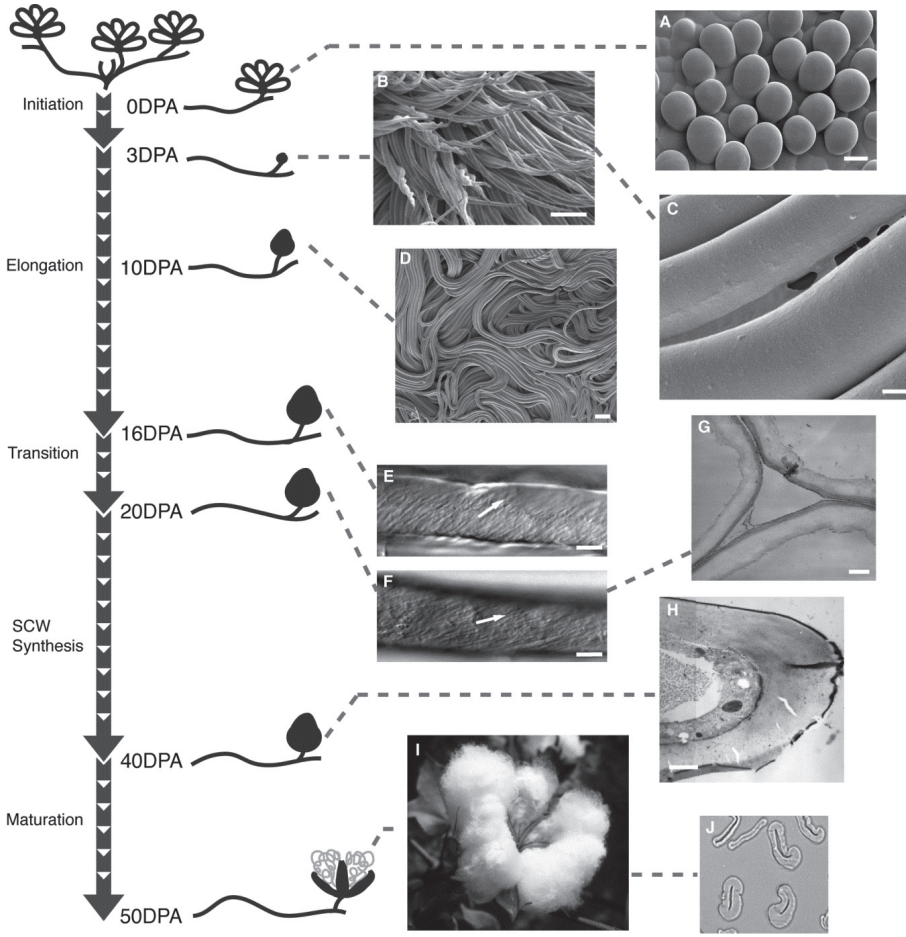

Figure 1. Representation of a mature cotton plant containing bolls and fibers at all stages of development. The stem indicates the fastest timeline for cultivated fiber development when plants are grown in an optimal (30°C) environment. Branches indicate days post-anthesis (DPA) and the images show many of the key features of fiber development. Cryo-field-emission scanning electron microscopy of (A) fiber initials on the ovule surface (bar = 10 μm); (B) twisting and elongating 3 DPA fibers (bar = 100 μm); (C) cotton fiber middle lamella (CFML) stretched between two 3 DPA fibers (bar = 4 μm); (D) ordered bundles of fibers inside the boll (bar = 100 μm). Differential interference contrast micrographs indicating microfibril angle of (E) 16 DPA and (F) 20 DPA fiber, with a steeper angle at 20 DPA (bars = 10 μm). TEM fiber cross-section showing (G) an early stage of secondary wall thickening (bar = 300 nm); and (H) a more advanced stage of secondary wall (bar = 1 μm). (I) Mature cotton boll and (J) cross-section of mature fiber viewed in the light microscope. SCW, secondary cell wall.

Transcriptional Regulation of Initiation

Transcription is regulated by various small RNAs, such as small interfering RNAs (siRNAs), as well as the activity of transcription factors. Small RNAs occur in size classes that regulate gene expression differently. siRNAs are predominantly 24 nucleotides long and direct DNA methylation and chromatin remodeling. The typically 18-21 nucleotide microRNAs (miRNAs) are derived from an endogenous hairpin structure and participate in the RNA-induced silencing complex (RISC) to target homologous mRNA for degradation or inhibit translation (Voinnet 2009). Some of the miRNAs expressed in cotton fiber have been identified by bioinformatics (Zhang *et al.*, 2007) and, along with siRNAs, through sequencing approaches (Pang *et al.*, 2009; Kwak *et al.*, 2009). Ovules engaged in early fiber development (0 and 3 DPA) showed a marked increase in the expression of 24 nucleotide siRNAs compared to -3 DPA ovules and leaves, which led to the proposal that siRNA-mediated chromatin remodeling may contribute to fiber initiation. In general, miRNAs were expressed more highly in -3 DPA ovules compared to 3 DPA fiber-bearing ovules and 10 DPA fibers, suggesting that miRNAs may maintain low expression of target genes prior to initiation (Pang *et al.*, 2009). Consistent with a decline in miRNAs by 3 DPA, a large percentage of the cotton genome is expressed in 2 to 25 DPA fiber (Hovav *et al.*, 2008b). In the *fuzzless-lintless* (*fl*) mutant that does not initiate fiber, more miRNA families were expressed compared to wild-type, leading to the hypothesis that targeted down-regulation of genes controlling fiber initiation could underpin the mutant phenotype (Kwak *et al.*, 2009).

Transcription factors, often working together in complexes, bind with specific genomic sequences to promote, enhance, or block transcription. Given the importance of fiber initiation for fiber yield, several studies have focused on identifying transcription factors that control initiation. In the regulatory cascade controlling fiber initiation, GhMYB25-like (Walford *et al.*, 2011) acts up-stream of GhMYB25 (Machado *et al.*, 2009). The promoter of *GhMYB25-like* drove GUS expression from -3 DPA to 3 DPA in the ovule surface and elongating fibers, and RNAi suppression of *GhMyb25-like* caused fiberless seeds. However, over-expression of *GhMYB25-like* did not increase the number of fiber initials. Consistent with these data, -1 to 4 DPA ovules of *fl* mutant had reduced *GhMYB25-like* expression, and only a mutated A genome homolog with a single amino acid substitution in the DNA binding domain was expressed in contrast to expression of wild-type A and D homologs in the parental line (Walford *et al.*, 2011). *GhMYB25* was identified in Southern blots of diploid species *G. arboreum* (A_2 genome), *G. raimondii* (D_5 genome), and the allotetraploid *G. hirsutum* cv. Coker 315 (AD genome) (Machado *et al.*, 2009). *GhMYB25* was highly expressed in 0 DPA ovules, and its promoter activated the *GUS* reporter in epidermal cells of 0 DPA ovules and 10 DPA fibers. RNAi suppression of *GhMYB25* resulted in 10-20% fewer fiber initials, and the initials that formed had delayed expansion and elongation compared to wild-type. In contrast, over-expression of *GhMYB25* resulted in 15-35% more fiber initials with no alteration of final fiber length, supporting the participation of the GhMYB25 transcription factor in controlling fiber initiation. Notably, as discussed by Walford and coworkers (Walford *et al.*, 2011), GhMYB25-like and GhMYB25 are more similar to petal-expressed MYBs in several species than they are to the MYBs that regulate arabidopsis leaf trichome formation, which correlates with known differences between the pathways regulating formation of cotton fibers and arabidopsis leaf trichomes (Serna and Martin, 2006).

Other initiation-associated transcription factor genes have recently been characterized. The RAD-Like *GbRL1* from *G. barbadense*, a SANT/MYB-type transcription factor, was expressed most highly in cotton ovules at -3 and 0 DPA. Over-expression in arabidopsis caused dwarfing and delayed flowering, similar to the effects of other *RAD* genes (Zhang *et al.*, 2011a). *GaHOX1* was isolated from *G. arboreum* (Guan *et al.*, 2008) as a homolog of arabidopsis *GLABRA2* (*GL2*), a gene required for cell expansion, branching, and cell wall maturation in leaf trichomes. *GaHOX1*, a member of the class IV homeodomain-leucine zipper (HD-ZIP) family of transcription factors, was expressed in many *G. arboreum* tissues but most strongly in 0 DPA ovule epidermal cells and 1 DPA fiber. In *G. hirsutum* the strongest expression was during early fiber elongation. Supporting a possible role in fiber initiation, *GaHOX1* complemented the trichome-less phenotype of the arabidopsis *gl2-2* mutant (Guan *et al.*, 2008). Evidence for a temporal cascade of transcription factors in early fiber development was obtained by comparing gene expression profiles in TM-1 and its derivative lintless mutant, *N1N1*, over the period of -3 to 10 DPA (Lee *et al.*, 2006). Five distinct gene expression profiles were identified, and certain genes were up-regulated in a logical progression (as often inferred from the function of their arabidopsis homolog). For example, *GhPDF1* (protodermal factor 1 that is possibly involved in cell fate determination) was highly expressed in -3 DPA ovules, then *GhMYB25* was expressed at 0 DPA, followed by the up-regulation of other genes such as *E6*, *EF-1*, and *RDL1* by 3 DPA and afterwards as fiber elongation progressed. Guan and coworkers (Guan *et al.*, 2011) suggested a specific pairing of a transcription factor and its downstream target based on co-expressing *GhMYB2* and *GhRDL1* in arabidopsis, which induced ectopic seed and silique trichomes. GhMYB2 functioned similarly to arabidopsis GL1 (involved in leaf trichome development), and GhMYB2 targeted the *GhRDL1* promoter. Expressing either gene alone increased the number of arabidopsis seeds producing trichomes (~6%) compared to wild-type (~0.4%). Co-expression led to ~8% or ~10% seed trichomes in wild-type or the *try* mutant background, respectively. The induced seed trichomes were at least two times longer than in wild-type. The results led to a regulatory model involving GhMYB2, GhMYB25, GhMYB109, and GhTTG1 as controllers of the downstream transcription of *GhHOX1* and *GhRDL1*. The data also predicted the existence of a cotton protein that functions as a negative regulator of fiber initiation, similar to TRIPTYCHON (TRY) in arabidopsis trichomes.

Action and interaction of phytohormones on fiber initiation

Phytohormones participate in signaling processes to regulate almost every aspect of plant growth and adaptation. Many years ago, gibberellic acid (GA) and auxin (IAA) were shown to be required for fiber growth on cultured cotton ovules (Beasley and Ting, 1973), and recent large scale gene expression data on -3 to 3 DPA ovules (or ovules with fibers) compared to other cotton tissues implicated extensive networks related to the biosynthesis and signaling of IAA, GA, and brassinosteroids (BR) as important for early fiber development (Yang *et al.*, 2006).

There is experimental evidence for a positive effect of IAA on fiber initiation. Zhang and coworkers (Zhang *et al.,* 2011b) observed IAA accumulation in the outer integument of the ovule at 0 DPA, but not in ovules treated with an IAA transport inhibitor (1-napthalamic acid, NPA) or

in the fiberless, *fl*, mutant. Cotton was transformed with an IAA biosynthesis gene, *iaaM*, under the control of the fiber-specific *FBP7* promoter, which resulted in 128% higher IAA concentration at 0 to 3 DPA, 13.5-28.6% more fiber initials, and 19.9-39.8% increase in the number of lint fibers compared to wild-type. The *FBP7* promoter was strongly effective between -2 to 0 DPA, which might reflect the period that IAA acts in the epidermis to stimulate cotton fiber initiation. This hypothesis was supported by unchanged fiber initiation when the *iaaM* coding sequence was over-expressed under the control of the promoter of the *E6* gene, which has maximal expression at 5 to 24 DPA (Zhang *et al.*, 2011b). Ovules from -3 to 3 DPA also expressed homologs of genes from other species that are related to IAA biosynthesis (*YUCCAs*, *CYP83B1s, NIT2*) as well as IAA signaling and transport (*ARFs*, *AUX1*, *TIR1*, *PIN1*) (Yang *et al.*, 2006).

GA has a positive effect on both initiation and elongation of the cotton fiber. Addition of GA to the medium of cultured ovules stimulated fiber elongation, whereas addition of the GA biosynthesis inhibitor paclobutrazol resulted in fewer, shorter fibers compared to no hormone controls (Liao *et al.*, 2009). Ovules from -3 to 3 DPA expressed homologs of genes from other species that are involved in GA biosynthesis (*GA20OX*, *GA2OX*, *POTH1*, *KO*) and GA signaling (*GAI*, *RGL2*, *RGL1*, *DDF1*, *PHOR1*, *RSG*, *PKL*, *GL1*, *GAMYB*, *AGAMOUS*, *LUE1*) (Yang *et al.*, 2006). Constitutive over-expression of *GhGA20ox1* in cotton resulted in more fiber initials and elongating fibers at 0 to 3 DPA and significantly higher level of bioactive GA in 0 DPA ovules and 10 DPA fibers (Xiao *et al.*, 2010). The role of GA in fiber elongation will be discussed further below.

Experimental evidence also supports a role for BR in fiber development. Treating cotton flower buds with the BR biosynthesis inhibitor, brassinazole2001, arrested fiber initiation, possibly because inhibition of BR biosynthesis altered epidermal cell differentiation (Sun *et al.*, 2005). The expression of genes known from other species to be involved in BR biosynthesis (*SMT1*, *SMT2*, and *BR60X*) and signaling (*BRI1s*, *BAK1*, *BES1*, *CPD*) was enriched in -3 to 3 DPA ovules compared to elongating fibers (Yang *et al.*, 2006). The cotton gene *GhDET2* is homologous to arabidopsis *AtDET2,* which is responsible for the rate-limiting step in BR biosynthesis. *GhDET2* had highest expression in ovules and elongating fibers, and heterologous expression showed that the encoded protein functioned as expected as a progesterone reductase. When *GhDET2* expression was suppressed in transformed cotton, fewer fiber initials formed and fiber elongation was inhibited. In contrast, increasing *GhDET2* expression in the seed coat led to more, longer fibers (Luo *et al.*, 2007).

Effect of ROS and Ca^{2+} on fiber initiation

Other signaling molecules that have been studied recently for their role in cotton fiber development include calcium (Ca^{2+}) and reactive oxygen species (ROS), specifically superoxide ($O_2^{\bullet-}$) and H_2O_2. ROS are produced in response to various biotic and abiotic stressors, and high levels cause oxidative stress and often cell damage. ROS are also important signaling molecules during plant development (Mittler *et al.*, 2004; Swanson and Gilroy, 2010). In cotton, ROS were detected in fiber initials at 0 DPA through fluorescence of the ROS indicator 2′, 7′-dichlorodihydrofluoroscein diacetate (2, 7-DCH_2FDA) (Mei *et al.*, 2009). When bolls from *G. hirsutum* fiber initiation mutants, naked seed (*N1*) and fuzzless Xinxianxiaoji (*XinFLM*), were treated

with H_2O_2, fiber initials were induced in both mutants by 0 DPA (Zhang *et al.*, 2010). These experimental data support a role for ROS in fiber initiation.

Ca^{2+} is key to many cellular processes (Kudla *et al.*, 2010), and research has begun to describe how Ca^{2+} participates in cotton fiber initiation and elongation. Ca^{2+} accumulation was correlated with fiber initiation and ER development in 0 DPA ovules compared to -1 DPA ovules. Microarray analysis showed that genes encoding components of Ca^{2+} signaling, such as calmodulin binding protein, were up-regulated at 1 DPA (Taliercio and Boykin, 2007).

Cotton Fiber Elongation

After initiation, polar expansion and rapid fiber elongation proceed from ~2 to 20 DPA until the fiber is 2 to 3 cm long. Beginning as early as 2 to 3 DPA, elongating fibers twist together to form bundles of fiber (Fig. 1B, C; Singh *et al.*, 2009a; see further discussion below on the cotton fiber middle lamella). The recently characterized cotton vacuolar invertase (GhVIN1) appears to play a role during early elongation by increasing osmotica for the control of turgor. *GhVIN1* expression and VIN activity peaked at 0 to 5 DPA in *G. hirsutum* fiber, but VIN activity was higher and persisted at a high level until 10 DPA in *G. barbadense* fiber that was also longer than in *G. hirsutum* (Wang *et al.*, 2010b). Other specific cellular mechanisms facilitate the most rapid phase of cotton fiber elongation. For example, increased synthesis of callose was correlated with closing of plasmodesmata at the fiber foot from 10 to 16 DPA. The isolation of the fiber from the ovule was proposed to result in higher turgor pressure, which in turn facilitated the period of most rapid fiber elongation (Ruan, 2007). Aquaporins, membrane proteins that facilitate the movement of water across biological membranes, may also have a role in cotton fiber elongation. Cotton aquaporins include GhγTIP1 located in the tonoplast and GhPIP1-2 located in the plasma membrane (Liu *et al.*, 2008). Both genes were expressed at 5 to 15 DPA during fiber elongation, and further work is needed to clarify a potential role of aquaporins in regulating turgor-driven elongation. Starch exists in elongating cotton fiber, and at 10 DPA there was peak expression of the genes encoding the subunits of ADP-glucose pyrophosphorylase that are required for starch biosynthesis (Taliercio, 2011). However, starch composed only ~0.3% of fiber dry weight, which correlates with only rare small starch grains observed at the level of transmission electron microscopy (TEM) in cotton fiber (Haigler and coworkers, unpublished).

Transcriptional and proteome-level regulation of elongation

The R2R3 MYB family transcription factor, GhMYB109, is structurally similar to AtGL1 and AtWER, which help to regulate arabidopsis leaf trichome development (Pu *et al.*, 2008). Logically, *GhMYB109* acts downstream of initiation-related *GhMYB25-like* and *GhMYB25* (Walford *et al.*, 2011). The *GhMYB109* promoter drove fiber-specific gene transcription, and antisense suppression of *GhMYB109* led to decreased fiber length. In addition, the decreased expression of *GhMYB109* was also associated with the suppression of genes known to be involved in fiber elongation, *e.g.*, *GhACO1* and *GhACO2* that support ethylene (ET) biosynthesis and *GhTUB1* and *GhACT1* encoding cytoskeletal proteins. Therefore, GhMYB109 likely acts upstream of phytohormone and cytoskeletal changes during fiber elongation (Pu *et al.*, 2008).

Moving beyond the transcriptome to analyze proteins showed that the cotton fiber proteome is dynamic and temporally regulated. Total protein (% of extracted fiber frozen weight) decreased from 5 to 20 DPA, then again from 30 to 35 DPA during fiber development in *G. hirsutum* cv. CRI 35. A total of 235 proteins showed changing abundance between 5 to 25 DPA in two dimensional gel electrophoresis, and these clustered into four distinct abundance patterns during fiber development (Yang *et al.*, 2008). Possibly helping to control the dynamic fiber proteome through regulating protein degradation, a cotton RING-type ubiquitin ligase (E3), GhRING1, was identified. Transcription of *GhRING1* increased from 5 to 15 DPA then decreased from 15 to 23 DPA, and recombinant GhRING1 had ubiquitin ligase activity *in vitro* (Ho *et al.*, 2010).

Action and interaction of phytohormones in fiber elongation

In recent years, the exogenous effect, endogenous amount, and genes involved in the signaling and biosynthesis of phytohormones have been studied in the elongating cotton fiber. Complementing the work of Liao and coworkers already described (Liao *et al.*, 2009), Aleman and coworkers (Aleman *et al.*, 2008) determined the effect of exogenous GA on fiber development while describing components of GA perception in cotton. Adding GA_3 to cultured ovules for 10 days stimulated ovule growth, fiber elongation, and expression of elongation-related genes such as *EXP*, *XTH1*, and *XTH2*. In addition, cotton homologs of the rice GA receptor gene *OsGID1*, *GhGID1a* and *GhGID1b*, and cotton DELLA-type genes, *GhSLR1a* and *GhSLR1b*, were identified. DELLA proteins repress GA-mediated gene expression and cell growth and enable fine control of hormone responses. The function of *GhGID1a* as a GA receptor was supported by its ability to rescue rice *gid1-3* mutants. The role of *GhSLR1b* as a DELLA protein was supported by its over-expression in arabidopsis causing a dwarfed phenotype (Aleman *et al.*, 2008). In further analysis of *GhSLR1*-type genes, the expression of a similar gene, called *GhRGL,* increased in ovules/fiber until 10 DPA (Liao *et al.*, 2009). (Note: *GhRGL* was amplified using primers designed from the same full-length cotton nucleotide sequence as one of the two used by Aleman *et al.* (2008) to identify *GhSLR1a* and *GhSLR1b*. *GhRGL* may be an allele of or the same sequence as one of these two genes.) Similar to results for *GhSLR1b* and consistent with function as a DELLA protein, arabidopsis constitutively expressing *GhRGL* was dwarfed, and plant height was negatively correlated with gene expression (Aleman *et al.*, 2008). Study of GA biosynthesis in the fiber has added more to this story. *In vivo* levels of GA in *G. hirsutum* were found by mass spectrometry (LC-ESI-MS) to be highest in fibers at 10 DPA, which was also the time of highest expression of the GA biosynthesis gene, *GhGA20ox1* (Xiao *et al.*, 2010). Over-expression of *GhGA20ox1* in *G. hirsutum* led to significantly longer fibers at 5 DPA and at maturity. The effects of altering GA *in vitro* and *in vivo* on fiber length and the dynamic nature of GA concentration during fiber development demonstrated a role for GA in regulating elongation. Consistently, genes relevant to the biosynthesis, perception, and control of GA demonstrate the phytohormone's relevance within cotton fiber.

The effect of BR on fiber elongation has been explored by manipulating ovule cultures and characterizing the expression of BR biosynthesis and signaling genes. The most biologically active BR, brassinolide, and the BR biosynthesis inhibitor brassinazole2001 have been used

to examine the effect of BR on fiber length and gene expression (Sun *et al.*, 2005). The addition of brassinolide increased fiber length by 12.7%, whereas inhibition of BR biosynthesis decreased fiber length 38% after 14 days in ovule culture. Further, brassinolide stimulated the expression of the elongation-related genes *EXP*, *XTH*, *AGP*, and *GhTUB1*, whereas brassinazole2001 inhibited their expression. Fiber elongation was also decreased when finasteride, another BR biosynthesis inhibitor, was added to cultured ovules for 5 or 15 days, but the inhibition was reversed by adding BR to the medium (Luo *et al.*, 2007). The cotton homologs of *AtBIN2*, encoding a negative regulator of BR signaling that makes plants insensitive to BR have also been investigated (Sun and Allen, 2005). The expression of *GhBIN2-C* and *GhBIN2-E* were highest in cotton tissues undergoing rapid cell expansion and/or vascularization, including ovules at 5 to 8 DPA bearing elongating fiber. This work further showed that, as predicted, over-expression of *GhBIN2* in arabidopsis led to dwarfism that correlated with the level of transgene expression, *i.e.*, transgenic arabidopsis expressing more *GhBIN2* resulted in shorter plants. BR biosynthesis is also active and required for fiber elongation, as already described for *GhDET2* (Luo *et al.*, 2007).

Abscisic acid (ABA) is a phytohormone associated with the inhibition of elongation in some plant cells (Lee *et al.*, 1994). The effect of ABA on cotton fiber elongation was examined for three cotton genotypes representing long, medium, and short fibers: *G. hirsutum* Hybrid-4 and Hybrid-8 as well as *G. arboreum* Gujarat Cotton-15 (Dasani and Thaker, 2006). In ovule culture experiments, addition of ABA decreased fiber elongation compared to no hormones, and fiber elongation was negatively correlated with increasing ABA concentration when NAA and GA_3 were also in the medium. In field-grown fiber, the ABA concentration measured by ELISA increased after fiber elongation ended even though the maximum fiber length occurred at 33 DPA in both *G. hirsutum* cultivars or 19 DPA in the *G. arboreum* cultivar. Together the *in vitro* and *in vivo* results suggest that ABA may actively inhibit elongation and/or positively signal the beginning of fiber wall thickening.

Ethylene is involved in signaling mechanisms related to fruit ripening, flower development, and stress responses (Bleecker and Kende, 2000). ET has been detected in fibers, it affects fiber elongation, and genes for ET biosynthesis are transcribed during elongation. The level of ET in cultured ovules was significantly decreased by treatment with the ET biosynthesis inhibitor, L-(2-aminoethoxyvinyl)-glycine (AVG). ET promoted fiber length, and AVG inhibited fiber elongation in a concentration dependent manner. Microarray analysis and metabolic mapping of gene expression in developing fiber identified ET biosynthesis as the most up-regulated pathway. PCR confirmed that the ET biosynthesis genes, *ACO1* and *ACO2,* were up-regulated in 5 to 15 DPA fiber, whereas *ACO3* peaked earlier at 10 DPA. Yeast expressing each of the three ET biosynthesis genes produced ET, verifying their function in ET biosynthesis (Shi *et al.*, 2006).

ET may work in conjunction with other signaling pathways such as BR and ROS. Treating cultured ovules with ET overcame the hindrance of fiber elongation resulting from inhibition of BR biosynthesis, and ET or BR treatment stimulated the expression of biosynthesis genes of the other hormone. Action within an integrated pathway is supported by no additive effect on fiber length after dual ET and BR treatment (Shi *et al.*, 2006). With regard to ROS, transcription of the cotton ascorbate peroxidase gene, *GhAPX1,* APX activity, and fiber length were increased

after young fibers in culture were treated with H_2O_2. ET treatment also increased the level of H_2O_2 in fibers (Li *et al.*, 2007). Later work showed that treatment of cultured ovules with H_2O_2 promoted ET production, which led to the proposal that ET stimulates both H_2O_2 production and fiber elongation while H_2O_2 stimulates additional ET synthesis (Qin *et al.*, 2008).

There is evidence that ET responds to an extracellular ATP/ADP signal, with a low dose of extracellular ATP/ADP stimulating fiber elongation whereas a higher concentration is inhibitory. The authors argued that the effects were due to perception of the molecule within a signaling process rather than phosphate transfer (Clark *et al.*, 2009). For example, 30 μM of the ATP-analog, ATPγS, stimulated fiber elongation, whereas 150 μM ATPγS inhibited fiber elongation. Neither effect occurred when ET biosynthesis was inhibited by AVG or $AgNO_3$. Addition of the ET precursor, ACC, also reduced the concentration of ATPγS required to stimulate elongation from 30 μM to 10 μM. The effects were also observed using poorly hydrolysable ATP- and ADP-analogs. A similar biphasic effect of high and low concentrations of the extracellular nucleotide on arabidopsis root hair showed that ROS and nitric oxide (NO) were required to propagate the effect of the nucleotides (Clark *et al.*, 2010), and similar interactions may take place in elongating fiber. ET in cotton fiber is also influenced by very-long-chain fatty acids (VLCFA) (Qin *et al.*, 2007a), which will be discussed below. Further work to elucidate the connection among extracellular VLCFA, ATP, ET, BR, and ROS could reveal a complex chemical communications network coordinating the elongation stage of fiber development.

ROS occurrence throughout fiber elongation

ROS are important regulators of cotton fiber elongation as shown by fiber transcriptomics and proteomics, manipulation of intracellular ROS in fibers of cultured ovules, and characterization of fiber-specific genes involved in ROS management. The most stable ROS as a transmissible signaling molecule is H_2O_2, which accumulates at low levels during early elongation until it peaks at 20 DPA (Potikha *et al.*, 1999; Yang *et al.*, 2008). Microarray comparison of global gene expression between the long fibered *G. herbaceum* and the short fibered *G. longicalyx* revealed genes involved in stress-responses, including oxidative stress, were over-represented during early fiber development in the short fibered species. This led to the hypothesis that the longer fiber in more recently evolved or selected cotton had been supported by more robust systems for ROS management (Hovav *et al.*, 2008a). The expression of three genes involved in ROS management (*GAST1-like*, *Cop1/BONZAI*, and *Pex1*) was higher in long fibered species (*G. herbaceum*, *G. arboreum*, *G. hirsutum* cv. TM1, and even wild *G. hirsutum* var. *yucatanense*) compared to short fibered species (*G. longicalyx* and *G. raimondii*). Microarray analyses of fiber gene expression in domesticated *G. hirsutum* and *G. barbadense* and their wild progenitors also showed that both domesticated species displayed increased expression of antioxidant genes during early elongation (Chaudhary *et al.*, 2009). Changes in the fiber proteome during elongation support these observations. For example, dehydroascorbate reductase involved in redox homeostasis was most abundant at 5 to 15 DPA. The activity of ascorbate peroxidase, which uses ascorbate to reduce two H_2O_2 to two H_2O and O_2, peaked at 10 DPA and subsequently dropped while H_2O_2 concentration then increased (Yang *et al.*, 2008). Another study showed that *GhAPX1* transcript levels and APX activity were highest at 5 to 10 DPA, and APX

protein was more abundant in wild-type cotton fiber compared to the *fuzzless-lintless fl* mutant (Li *et al.*, 2007). Cu/Zn-superoxide dismutase (SOD) genes have also been identified in cotton. Cytosolic *GhCSD1* and plastidic *GhCSD2* were expressed at the highest during early fiber elongation, which may support the maintenance of low H_2O_2 levels (Kim *et al.*, 2008).

The maintenance of low levels of ROS during early elongation may occur during normal development, *e.g.*, by dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 by SOD and the oxidation of H_2O_2 to H_2O by APX. However, the role of ROS throughout elongation may be more complicated. First, ROS is increased throughout the fiber cell during late elongation as discussed above. Second, addition of H_2O_2 to cultured ovules increased fiber length (Li *et al.*, 2007) and ROS appears to be required for fiber elongation (Mei *et al.*, 2009). In addition to *GhCSD1* and *GhCSD2*, other Cu/Zn-SOD genes, *GhCSD3* and *GhCSD3s*, were expressed two-fold higher only in 16 DPA fiber and SOD proteins were localized to the primary and secondary wall (Kim *et al.*, 2008; Kim and Triplett, 2008). *GhCSD3* may perform a particular role in the transition from elongation to secondary wall synthesis and will be discussed below. In contrast with the low levels of H_2O_2 maintained during early elongation, $O_2^{\bullet-}$ increased in fiber-bearing ovules during elongation from 0 to 10 DPA compared to fiberless *fl* ovules (Mei *et al.*, 2009). By the use of the ROS indicator 2, 7-DCH$_2$FDA, fluorescence was detected at 0 to 2 DPA while initiation and polar elongation occurred in wild-type, but no fluorescence was detected at -1 DPA in wild-type or at any DPA in ovules of the *fl*. When ovules in culture were treated with diphenyleneiodium (DPI), an inhibitor of NADPH oxidase activity, or a peroxidase inhibitor, salicylichydroxamic acid (SHAM), $O_2^{\bullet-}$ production and fiber length decreased in a concentration dependent manner in fiber-bearing ovules. The expression of another Class III plant peroxidase, *GhPOX1*, peaked at 10 to 15 DPA, which correlated with the peak in total peroxidase activity. *GhPOX1* is homologous to *AtPOX13*, which was determined by mutant analysis to be required for lateral root initiation and elongation in arabidopsis. Therefore, GhPOX1 could have a similar role in cotton fiber. Although many peroxidases, such as APX, remove H_2O_2 from the cell, others produce ROS. GhPOX1 was proposed to produce ROS to promote fiber elongation (Mei *et al.*, 2009).

The exact mode of action for ROS is unclear at this time, but the study of ROS effects on the development of other plant systems may suggest avenues of research. For instance, ROS promote cell wall loosening and elongation in maize roots (Liszkay *et al.*, 2004) and the tip-growth of arabidopsis root hairs and tobacco pollen tubes (Monshausen *et al.*, 2007 and Potocký *et al.*, 2007, respectively). The dynamic nature of ROS during fiber elongation may support multiple modes of action for ROS or specific modes for different forms of ROS, *i.e.*, $O_2^{\bullet-}$ and H_2O_2.

Ca^{2+} signaling during fiber elongation

Ca^{2+} signaling is a vital part of plant growth and development participating in responses to abiotic stress and phytohormones. Ca^{2+} was a central player in the polar growth of root hairs and pollen tubes (Monshausen et al, 2007; Cheung and Wu, 2008). Ca^{2+} signaling also functions in cotton fiber polar growth and elongation. In ovule culture experiments, fiber elongation did not occur without Ca^{2+} in the medium (Huang *et al.*, 2008). However, consistent with the fine control of cytosolic Ca^{2+} levels required for Ca^{2+} signaling, lower (0.1 mM) Ca^{2+} promoted fiber initiation, early elongation, and the expression of expansin, compared to higher (1 mM) Ca^{2+}

in ovule culture medium. The window of sensitivity to high Ca^{2+} was the 0 to 3 DPA, but fiber elongation did not recover from early inhibition up to 10 DPA. The effects on fiber elongation of antagonists of Ca^{2+} signaling in the presence of low or high calcium were consistent with Ca^{2+} in the medium acting through signaling pathways, versus effects on other processes such as cell wall rigidity through pectin cross-linking (Taliercio and Haigler, 2011). Calmodulin is often a key component in Ca^{2+} signaling pathways, and the calmodulin inhibitor, trifluoperazine, inhibited fiber elongation *in vitro* in a concentration dependent manner (Huang *et al.*, 2008). Trifluoperazine inhibited fiber elongation more strongly in low calcium medium, which likely allowed more normal internal Ca^{2+} concentration within fibers (Taliercio and Haigler, 2011).

Other components of Ca^{2+} signaling have recently been characterized and shown to communicate with one another. Comparing gene expression in elongating fibers to leaves using a suppression subtraction cDNA library strategy identified fiber-specific/preferred genes involved in Ca^{2+} signaling: calcineurin B-like (CBL) -interacting protein kinase (*GhCIPK1*), calmodulin (*GhCaM*), and glutamate decarboxylase (*GhGAD*). Each of these genes was expressed most strongly in 9 to 15 DPA wild-type fiber, but high expression ended at 9 DPA in fibers of the *ligon lintless* mutant that terminate elongation at <6 mm (Gao *et al.*, 2007). GhCIPK1 functioned similarly to other CBL-interacting proteins during *in vitro* autophosphorylation kinase activity assays. Two CBLs, *GhCBL2* and *GhCBL3*, that may interact with GhCIPK1 were also expressed in an elongation-specific manner, and both GhCBLs interacted with the C-terminus of GhCIPK1 in yeast two-hybrid and affinity pull-down assays (Gao *et al.*, 2008). Calcium dependent protein kinases (CDPK) are another component of Ca^{2+} signaling present in the elongating fiber. *GhCDPK1* was expressed most strongly in 9 to 15 DPA wild-type fiber. Furthermore, GhCDPK1 protein fused to GFP localized in the plasma membrane of onion epidermal cells during transient transformation assays. GhCDPK1 performed autophosphorylation and phosphorylation of histone III-S only in the presence of Ca^{2+}, as expected for a CDPK (Huang *et al.*, 2008). Calcium is also often involved in the cellular activity of annexins, which comprise a multifunctional protein family associated with Ca^{2+}-dependent membrane transport, GTPase activity, binding to filamentous actin (F-actin), and ROS reduction. *GhFAnnx* expression was ovule/fiber specific, and, in transient transformation assays, GhFAnnx-GFP fusions localized to the plasma membrane of onion epidermal cells in a Ca^{2+}-dependent manner and to the plasma membranes of cotton fibers after 14 days in culture (Wang *et al.*, 2010c). In summary, fiber elongation was inhibited by interfering with Ca^{2+} sensing, and the expression of proteins required for Ca^{2+} signaling was correlated with the time of high-rate polar growth of fibers. Further work can be beneficially directed toward understanding connections within the Ca^{2+} signaling pathway as well as connections with other signaling pathways.

Emerging importance of fatty acids in fiber elongation

Fatty acid metabolism is responsible for the biosynthesis of many cellular lipids especially membrane components, so rapid fatty acid synthesis in an elongating cotton fiber is expected. Indeed, genes required for fatty acid synthesis were highly expressed in elongating fiber (Shi *et al.*, 2006). The total fatty acid content as determined by gas chromatography was also highest in elongating 7 to 21 DPA fibers compared to 28 DPA fibers. In the same study, cotton fiber EST libraries were

analyzed to implicate many genes that may be involved in *de novo* fatty acid biosynthesis in elongating fiber (Wanjie *et al.*, 2005). Fatty acids also provide building blocks of signaling molecules such as phosphoinositol and sphingolipids, and very-long-chain fatty acids (VLCFA) that often exist within sphingolipids have a particular role in fiber elongation. VLCFA accumulate preferentially in elongating fibers compared to ovules, and the cotton homologs of several genes supporting VLCFA biosynthesis (*KCS12*, *KCS6*, *KCS13*, and *KCS2*) were up-regulated in elongating fiber. The cotton genes were able to complement the yeast *elo2Δ elo3Δ* double mutant with defects in fatty acid elongase activity, confirming their function. When two types of VLCFA, C24:0 and C26:0, were added to cotton ovule culture, fiber elongation strongly increased compared to controls. In contrast, the inhibition of VLCFA biosynthesis by 2-chloro-*N*-[ethoxymethyl]-*N*-[2-ethyl-6-methyl-phenyl]-acetamide (ACE) led to a concentration dependent decrease in fiber length and ovule size (Qin *et al.*, 2007a). *GhCER6* also complemented yeast *elo2Δ elo3Δ* double deletion mutants for fatty acid elongase activity (Qin *et al.*, 2007b). Other genes involved in VLCFA biosynthesis in cotton fiber include *GhECR1* and *GhECR2*, which perform the reduction of *trans*-2-enoyl-CoA (ECR activity). These genes have expression peaks in elongating cotton fiber, although *GhECR2* was more fiber-specific, and both could complement the yeast *tsc13Δ* mutant lacking ECR activity (Song *et al.*, 2009). Three genes encoding 2-ketoacyl-CoA reductase (with KCR activity) were also expressed in cotton fiber. *GhKCR1* and *GhKCR2* have peak expression at 5 to 10 DPA and functionally complemented yeast *ybr159wΔ* mutants lacking KCR activity (Qin *et al.*, 2005). *GhKCR3* was expressed in 10 DPA fiber and also functionally complemented the yeast *ybr159wΔ* mutant. GhKCR3 had a higher affinity for long chain fatty acids (Pang *et al.*, 2010b).

Experiments in cotton fiber suggest that VLCFA signal the expression of ET biosynthesis genes and ET accumulation with subsequent stimulation of fiber elongation (Qin *et al.*, 2007a). Fibers on cultured ovules treated with combinations of VLCFA and ET and/or ET biosynthesis inhibitors showed that VLCFA-enhanced elongation occurred only if ET was provided exogenously or through biosynthesis. VLCFA addition to ovule cultures also led to higher ET and sphingolipid accumulation, and ET and sphingolipids levels decreased when VLCFA biosynthesis was inhibited. Analysis of the arabidopsis *cut1* mutant in VLCFA biosynthesis using exogenous VLCFA or ectopic expression of *GhKSC13* supported a role for VLCFA in elongation of roots and root hairs. VLCFA has been proposed to signal the expression of ET biosynthesis genes and ET accumulation with subsequent stimulation of fiber cell elongation (Qin and Zhu, 2010).

Significance of the cytoskeleton for fiber elongation

Cotton fiber elongation requires organized and efficient transport of new membranes and cell wall materials, processes that are aided by a dynamic cytoskeleton, including F-actin and microtubules. Genes associated with the cytoskeleton and intracellular transport had higher expression in microarray analysis in the chromosomal substitution line *G. hirsutum* cv. CS-B22sh compared to its progenitor (Wu *et al.*, 2008). CS-B22sh contains a substitution of the chromosome 22 short arm from *G. barbadense* cv. 3-79 in the TM-1 genetic background, and it has improved lint percent, micronaire, and fiber length (Saha *et al.*, 2006). This supports the impact of the cytoskeleton and intracellular transport on fiber quality.

During high-rate fiber elongation, microtubules are oriented transversely to the fiber axis and have a role in controlling the orientation of cellulose fibrils in cotton fiber (Seagull, 1993), as commonly occurs in plants (Paradez *et al.*, 2006). At least nine b-tubulin genes are preferentially or differentially expressed in elongating fiber, and many of these, including *GhTUB1*, complemented a tubulin-deficient yeast mutant. Different combinations of these b-tubulin genes were stimulated in fibers of cultured ovules treated with ET, BR, GA, or VLCFA (He *et al.*, 2008). The antisense suppression of the transcription factor *GhMYB109* led to decreased fiber length and suppressed expression of *GhACO1*/*GhACO2* that support ET biosynthesis, *GhTUB1*, and *GhACT1* (Pu *et al.*, 2008). Therefore, key regulatory processes in cotton fiber ultimately exert their effects through the modulation of microtubules and downstream cellular effects related to the cytoskeleton.

Actin exists in thick microfilaments oriented axially and in a fine network in the cortical cytoplasm that sometimes parallels the microtubules during high-rate elongation (Seagull, 1993). The *in vitro* disruption of F-actin with cytochalasin D resulted in shorter fibers with a less rigid morphology (Wang *et al.*, 2005; Wang *et al.*, 2010d). Other recent work focused on the earliest phase of fiber elongation at 1 to 5 DPA when thick actin filaments (stained with rhodamine phalloidin) were arrayed parallel to the long axis of wild-type fiber (Li *et al.*, 2005). Five of sixteen actin genes identified in cotton were expressed preferentially in fibers, *GhACT1*, *GhACT2*, *GhACT4*, *GhACT5*, and *GhACT11*. When *GhACT1* expression was reduced ~10-fold by RNAi in transgenic cotton, fiber length at 0 to 3 DPA was reduced 1.5- to 3-fold. The shorter 1 to 5 DPA fibers had fewer, more randomly arranged actin filaments compared to wild-type (Li *et al.*, 2005).

The dynamic activity of the actin cytoskeleton is enabled by actin-modifying proteins. These include profilins (PFN), such as GhPFN1 and GhPFN2 that promote polymerization into F-actin, and actin depolymerization factors (ADF) such as GhADF1. Wang and coworkers worked extensively on fiber profilins, and there is evidence that GhPFN1 may help to control fiber elongation (Wang *et al.*, 2005; Wang *et al.*, 2010d). *GhPFN1* was strongly expressed in 3 to 18 DPA fibers, and cultured tobacco cells constitutively over-expressing *GhPFN1* elongated more and had thicker, more abundant F-actin bundles compared to controls. *GhADF1*, expressed in fiber at 6 to 27 DPA, is likely to support actin depolymerization at multiple stages of fiber development. Down-regulation of *GhADF1* expression in transgenic cotton using RNAi resulted in less GhADF1 protein and a heritable increase in fiber length (+5.6%) along with thicker, longer actin cables within fiber, presumably due to increased stability of actin in the presence of less actin depolymerizing protein (Wang *et al.*, 2009).

Primary wall structure during cotton fiber elongation

Cotton fiber initiation and elongation depend on the rapid synthesis of a primary wall outside the plasma membrane. At a gross level, the cotton fiber primary wall is similar to those in other dicotyledonous plants (Doblin *et al.*, 2010). It contains ~22% semi-crystalline (ß-1, 4-glucan) cellulose fibrils, which are surrounded by a matrix composed mainly of other polysaccharides including xyloglucan and pectin (Meinert and Delmer, 1977; Singh *et al.*, 2009a). The

high-strength cellulose fibrils are oriented transversely to the long fiber axis during elongation, which laterally constrains turgor pressure so that fiber elongation occurs. The elongating fiber wall must compromise strength and flexibility. For example, genes encoding wall-loosening expansin proteins (Sampedro and Cosgrove, 2005; Cosgrove, 2005) are expressed in rapidly-elongating fiber (Harmer *et al.*, 2002) and are associated with QTLs related to fiber length (An *et al.*, 2007).

The presence and modification of the matrix components also contribute to cell wall properties and growth potential (Cosgrove, 2005) and may regulate fiber elongation, as will be discussed further below. Pectins are a complex family of galacturonic acid-rich polysaccharides including: (a) homogalacturonan, with/or without side-groups or side-chains; and (b) rhamnogalacturonan I with a repeating disaccharide (galacturonic acid-rhamnose) in its backbone and a wide variety of side-groups or side-chains (Mohnen, 2008). Xyloglucan has a ß-1, 4-glucan backbone with a variable pattern of side-chains composed of α-1, 6- xylose and sometimes galactose and fucose (O'Neill and York, 2003). Research is continuing on how cellulose and the matrix components work together through largely non-covalent interactions to generate strength, flexibility, and developmental plasticity within the composite primary wall (*e.g.*, Abasolo *et al.*, 2009; Boyer, 2009).

Specialization of cell walls is often a key feature of cellular differentiation (Cosgrove, 2005), and changes in cell wall structure and chemistry are a signature of the progression of fiber development. Experimental or breeding lines with differences in fiber quality frequently show differential gene expression related to cell walls and/or related processes such as cytoskeletal organization (*e.g.*, Wu *et al.*, 2008; Hinchliffe *et al.*, 2010). A montage of micrographs in Seagull (1993) shows the changes in the orientation of cellulose fibrils in successive cell wall layers as fiber development proceeds, and equally dramatic changes occur in cell wall matrix components. Recently, immunolabeling of cotton fiber from wild-type and fiberless lines with antibodies raised against isolated rhamnogalacturonan I showed that cotton initiation and early elongation at 0 to 2 DPA were characterized by: (a) loss of an epitope characteristic of (1–6)-ß-D-galactan carrying arabinose (possibly contained *in situ* within arabinogalactan protein); and (b) appearance of an epitope characteristic of (1–4)-ß-D-galactan, one of the possible side chains of rhamnogalacturonan I (Bowling *et al.*, 2011). This study also confirmed the existence of an outer pectin-rich sheath surrounding an inner primary wall that was enriched in cellulose and xyloglucan, as described previously (Vaughn and Turley, 1999). This outer pectin sheath is not known in other plant cells, demonstrating that aspects of cotton fiber cell wall structure are likely to help to control the differentiation program and unique features of cotton fiber.

Pectin synthesis and modification as regulators of cotton fiber elongation

The importance of pectin in modulating cotton fiber elongation has been shown in two recent studies. Pang and coworkers (Pang *et al.*, 2010a) compared 10 DPA whole ovules of wild-type and the fuzzless-lintless cotton mutant by proteomics, which implicated proteins associated with nucleotide sugar metabolism and pectin biosynthesis as supporting fiber elongation. In

ovule culture experiments, transcripts of the pectin biosynthesis genes responded positively to the addition of ET or a VLCFA (lignoceric acid, C24:0, $C_{23}H_{47}COOH$), which are positive regulators of fiber elongation (Qin *et al.*, 2007a) as described previously. Fiber elongation in culture also responded positively to the addition of UDP-activated rhamnose, galacturonic acid, or glucuronic acid, and UDP-rhamnose or UDP-galacturonic acid allowed fiber elongation in the presence of an ET perception inhibitor. Together with experiments on arabidopsis mutants with defects related to pectin biosynthesis, C24:0 biosynthesis, or ET signaling, these results supported the model that pectin biosynthesis was a target for signaling pathways that promote fiber elongation (Pang *et al.*, 2010a). A positive correlation between cell expansion and pectin supply occurs frequently in plants (Boyer, 2009).

In addition, the molecular structure of pectin is critically important for cotton fiber elongation. Wang and coworkers (Wang *et al.*, 2010a) studied the effect of increased levels of de-esterified homogalacturonan in elongating cotton fiber. They showed that a gene encoding an authentic pectate lyase, *GhPEL,* was preferentially expressed in fibers with peak expression at 10 DPA. The recombinant GhPEL protein was able to degrade polygalacturonic acid (de-esterified homogalacturonan) *in vitro*. Peak pectate lyase activity occurred in wild-type fiber at 10 DPA, and the amount of de-esterified pectin (in a crude extract that could contain other cell wall polymers) declined continuously from 5 to 20 DPA in wild-type fiber. The 10 to 15 DPA fiber of transgenic cotton with reduced *GhPEL* expression had less pectate lyase activity and increased content of (putative) de-esterified pectin. Mature transgenic fiber was proposed to be about 3 – 16% shorter across several homozygous lines, and reduced fiber elongation beginning at 10 DPA occurred in one line compared to wild-type. Potentially shorter cotton fibers when the amount of de-esterified homogalacturonan increased could be explained by more pectin gel formation leading to cell wall rigidification, a phenomenon that occurs when calcium acts as a cross-linker for de-esterified homogalacturonan (Cosgrove, 2005; Boyer, 2009).

Xyloglucan modification as a regulator of cotton fiber elongation

Many researchers have explored the potential of xyloglucan to help regulate plant cell growth. Surprisingly, xyloglucan-depleted arabidopsis plants grew almost normally in the laboratory, although cell wall mechanical properties were changed and root hairs were short with bulging bases (Cavalier *et al.*, 2008). For cotton researchers, the main targets of exploration have been a family of xyloglucan endo transglycosylase/hydrolase (XTH) genes encoding proteins that have one or both abilities to degrade xyloglucan irreversibly (xyloglucan endo-transglycosylase, XET, activity) or to cleave and transfer chain ends between molecules (xyloglucan endo-hydrolase, XEH, activity). In species characterized so far, this is a large gene family and each encoded enzyme must be analyzed individually in biochemical and cell biological experiments to determine its actual function (Eklöf and Brumer, 2010). Modulations in one or both of the XET/XEH enzyme activities could possibly increase the plasticity of the primary wall, thereby promoting fiber elongation (Cosgrove, 2005). Several XET/XEH genes had expression peaks during cotton fiber elongation (Michailidis *et al.*, 2009; Lee *et al.*, 2010) and/or at the transition stage of fiber development, possibly with a relationship to CFML degradation (Singh *et al.*,

2009a; see below for further discussion of the CFML). Supporting the positive impact of XTH genes on fiber elongation, longer domesticated cotton fiber showed higher expression of several of these genes as compared to wild *G. hirsutum* (Rapp *et al.*, 2010). Proving the importance of XET activity for fiber elongation, analysis of transgenic cotton plants over-expressing *GhXTH1* under the control of the constitutive CaMV 35S promoter showed a positive correlation between inheritance of the transgene and increased fiber length. The transgenic plants had about two-fold increased XET activity and 15-20% longer fiber compared to wild-type cotton or null segregants under greenhouse or field conditions, leading to the conclusion that the ability to transfer xyloglucan chain ends between molecules was a limiting factor for fiber elongation in *G. hirsutum* cv. Coker 312 (Lee *et al.*, 2010).

Existence and effects of a cotton fiber middle lamella

In addition to the primary wall layers that are closest to the protoplast, cotton fiber has an outer primary wall layer called the cotton fiber middle lamella (CFML). The CFML joins adjacent elongating fibers together through forming a unified cell wall between them (Singh *et al.*, 2009a). Fibers are not merely in superficial surface contact; instead they are joined with their neighbors into tissue-like bundles just as other cells form tissues within the plant body. Given that cotton fibers initiate from the ovule as individuals and are subsequently found within the open boll as individuals, the discovery of fused cotton fibers during elongation was surprising. The CFML is enriched in fucosylated xyloglucan and homogalacturonan pectin with a relatively low degree of esterification, and it may contain other unidentified components that help to confer its adhesive properties. The CFML may derive from the pectin-rich outer layer of the primary wall described for 1 to 2 DPA fibers, although xyloglucan was not detected in this outer pectin-rich fiber "sheath" just after initiation (Vaughn and Turley, 1999). The fiber bundles adopt curving paths as elongation proceeds finally creating a tight packet of fibers around each seed. This packet of fiber grows outward until it contacts the boll wall by 3 to 5 DPA. The CFML-mediated formation and packing of fiber bundles explains "spiral structures" described for cotton fiber (Krakhmalev and Paiziev, 2006). This high level of organization of fiber within the boll probably facilitates the elongation of thousands of fibers within a confined space. The CFML is degraded by cell-wall active enzymes during the transition to secondary wall deposition while the inner layer of the primary wall is left relatively intact. This causes the fibers to be released as individuals at the onset of secondary wall deposition (Singh *et al.*, 2009a), although the packing pattern of fiber within the locule does not change until the boll opens.

Transitional Primary Wall Remodeling and Winding Layer Synthesis

The targeted degradation of the CFML, and possibly other primary wall remodeling, at the transition stage provides at least partial explanation for changes in the concentrations of primary wall sugars (Meinert and Delmer, 1977; Singh *et al.*, 2009a). The transition stage of fiber development is often thought of as a period when primary and secondary wall deposition overlap. It

is more accurate to consider it as a separate stage of fiber development that controls cotton fiber quality in important ways. During the transition stage many distinct cellular events occur. The rate of respiration in the fiber/ovule system goes through a trough before rising again during secondary wall synthesis; intra-fiber sugar pools decline; fiber cellulose synthesis occurs at an intermediate rate; the percentage of cellulose in the fiber cell wall increases to ~35%; and the CFML degrades. At the same time, the amount of callose, or ß-1, 3-glucan, in the fiber reaches its peak (Meinert and Delmer, 1977; Maltby *et al.*, 1979; Martin and Haigler, 2004; Guo *et al.*, 2007; Singh *et al.*, 2009a; Abidi *et al.*, 2010b).

In addition, the cytoskeletal microtubules just below the fiber plasma membrane reorient from their transverse state during fiber elongation to adopt a shallow helix relative to the fiber axis. The dynamic behavior of F-actin is a potential regulator of the timing of the transition stage. In control plants, the profilin gene, *GhPFN2*, was highly expressed at 15 to 24 DPA, and GhPFN2 promoted actin bundling in the presence of an arabidopsis formin, AtFH1. Formin proteins interact with PFN and actin to aid F-actin formation. Cotton over-expressing *GhPFN2* had shorter fibers and initiated secondary wall deposition 2 days earlier than control plants (Wang *et al.*, 2010d). Another gene encoding an actin depolymerization factor, *GhADF1*, was expressed at 6 to 27 DPA and is likely to support actin depolymerization at multiple stages of fiber development. Transgenic cotton with down-regulated *GhADF1* expression and less GhADF1 protein showed a heritable increase in fiber length (+5.6%) along with thicker, longer actin cables within fiber. Other phenotypes included higher cellulose content at 24 DPA (~95% compared to ~91% in the control) and a thicker secondary wall (Wang *et al.*, 2009). Together these results show a positive role for F-actin in initiation of secondary wall thickening.

The "winding" cell wall layer, reflecting the first phase of wall thickening, is synthesized with a lattice-like organization of fibrillar elements. The cellulose microfibrils in the winding layer parallel the reoriented microtubules with a shallow angle relative to the fiber long axis (Fig. 1E; reviewed in Seagull, 1993). The change in microfibril angle as compared to the primary cell wall is analogous to creating plywood, and probably explains the fact that a substantial degree of final fiber strength is conferred during the transition phase (Hsieh *et al.*, 1995: Hinchliffe *et al.*, 2011) even though the increase in cell wall mass is not extensive. During the time that the cell wall begins to thicken, changes occur in the degree of esterification of primary wall pectin that may serve to limit elongation potential. In addition, a cuticle that stains darkly in sections viewed in TEM begins to integrate with the primary wall (Singh *et al.*, 2009a). (Singh and coworkers discuss the evidence for synthesis of the cotton fiber cuticle at the transition stage and not during fiber elongation). The primary wall and cuticle are pushed to the perimeter of the fiber as cell wall thickening occurs at the plasma membrane.

As expected, significant changes in gene expression and potential regulatory processes occur at the transition stage. In *G. hirsutum*, there is a decline in the expression of primary wall-related genes and the increased expression of secondary wall-related genes (Hinchliffe *et al.*, 2010 and other references therein). In addition, some genes have distinct peaks of expression at the transition stage. These may encode proteins that affect cellular processes directly (*e.g.*, Singh *et al.*, 2009a) or underpin the signaling events that control the timing of the transition phase. For example, GhRAC13, a small GTPase, may contribute to an oxidative burst that stimulates the

onset of secondary wall deposition (Potikha *et al.*, 1999; Yang *et al.* 2008). This signaling event may be further controlled through transition stage up-regulation of SOD to act as a scavenger of ROS in the cell wall. The expression patterns of four Cu/Zn-SOD genes have been analyzed in fiber. Two of these, *GhCSD3* and *GhCSD3a*, were expressed two-fold higher only in 16 DPA fiber, SOD specific activity peaked at 24 DPA, and SOD was localized in the primary and secondary wall. The authors proposed that altered levels of ROS could signal changes in gene expression related to cell wall synthesis and/or affect cell wall properties directly through effects on cell wall rigidity (Kim *et al.*, 2008; Kim and Triplett, 2008).

The timing of the transition phase can be changed by genotype or environment and impacts cotton fiber quality. Microscopic and gene expression analysis in cultured fiber showed that natural auxin (IAA) accelerates entry into the transition phase and the onset of high-rate cellulose synthesis as compared to synthetic auxin (NAA) (Singh *et al.*, 2009b). Thermogravimetric analysis (fiber weight vs. exposure to high temperature) showed that the transition phase started either at 17 to 18 DPA or 21 to 24 DPA in two greenhouse-grown *G. hirsutum* cultivars (Abidi *et al.*, 2010a). In a highly regulated greenhouse with a relatively cool 26/22°C day/night cycle, secondary wall-related genes were up-regulated by 19 DPA in fiber of *G. hirsutum* cv. Deltapine 90 (Singh *et al.*, 2009b), which is later than often observed in the field. A study of two *G. hirsutum* near-isogenic lines, one with consistently higher (~15%) fiber bundle strength attributable to one or two genes, clarified a major reason why the timing of the transition stage varies (Hinchliffe *et al.*, 2010; Hinchliffe *et al.*, 2011). Over several field seasons, fiber of these lines showed > two-fold higher expression of genes related to secondary wall synthesis as early as 12 DPA or later than 18 DPA (in a season with cool night temperature). Hinchliffe and coworkers determined that the timing was governed by temperature: the transition started when 160 degree day heat units were accumulated after anthesis (calculated from daily high and low temperatures using a threshold of 15.5°C). This relationship held true for plants grown in the greenhouse by Singh and coworkers (Singh *et al.*, 2009a). Furthermore, entering the transition stage earlier resulted in higher fiber bundle strength, for reasons that remain to be discovered. These results show the importance of identifying potential master control genes for the onset of the transition stage in cotton fiber (Hinchliffe *et al.*, 2010; Hinchliffe *et al.*, 2011).

Secondary Wall Synthesis

Cotton fiber secondary wall thickening occurs through the deposition of nearly pure cellulose. So far no other component has been characterized that might exist between the cellulose fibrils in the unique cotton fiber secondary wall. The percentage of crystalline cellulose was 90% in wild-type fiber with a maturity ratio of 0.89 as measured by image analysis of fiber cross-sections. In transgenic fiber with similar fiber perimeter, the percentage of crystalline cellulose increased to nearly 92% along with an increase in the maturity ratio to 0.95-0.99 (Haigler *et al.*, 2007). After chemical-dehydration and sulfuric-acid extraction, ~85% or 90% percent cellulose was found for the fiber of two other *G. hirsutum* cultivars (Abidi *et al.*, 2010b). All of these values may under-estimate the crystalline cellulose content of the isolated secondary wall because: (a) the whole fiber including the cuticulated primary wall was included in the fiber weight; and

(b) the acids used as extractants may have dissolved some less ordered cellulose. Therefore, the cotton fiber secondary wall likely contains >95% cellulose, making it the purest cellulose synthesized by plants. Secondary wall thickening is characterized by an increased rate of cellulose synthesis, greater length of individual cellulose chains, and orientation of closely packed cellulose fibrils in an increasingly steep helix relative to the fiber axis (Fig. 1F). The cellulose microfibrils also reverse their direction of helical travel at intervals, creating "reversals" within the secondary wall structure (reviewed in Seagull, 1993).

The cellular, biochemical, and genetic regulation of cellulose synthesis are key aspects of cotton fiber differentiation, but the details are beyond the scope of this review. Broadly, the cellulose synthase genes and other co-functional genes that support cotton fiber secondary wall cellulose synthesis are orthologs of those required for secondary wall deposition in the xylem of vascular plants, whereas cotton fiber elongation and the wall thickening of arabidopsis leaf trichomes are supported by genes related to primary wall synthesis (Betancur *et al.*, 2010). This and other evidence supports the idea that a process for xylem secondary wall cellulose synthesis at the base of the land plant lineage was widely adapted in other cells as plants evolved (Haigler *et al.*, 2009; Zhong *et al.*, 2010; Hinchliffe *et al.*, 2010). The conservation of the secondary wall cellulose synthesis genetic program is also indicated by comparing gene transcription in wild vs. domesticated *G. hirsutum*, which showed less difference between genotypes during secondary wall synthesis as compared to elongation (Rapp *et al.*, 2010). However, cotton fiber employs the basal xylem secondary wall thickening program in a unique way given that hemicellulose and lignin synthesis do not occur while nearly pure cellulose is synthesized in cotton fiber. Nonetheless, rather than leaf trichomes, the best models in arabidopsis for cell wall thickening in cotton fiber are xylem conducting cells or other cellulose-rich schlerenchyma cells (Betancur *et al.*, 2010).

How the cotton fiber supports and regulates the strong irreversible carbon sink represented by secondary wall cellulose synthesis has been reviewed previously (Haigler *et al.*, 2001; Delmer and Haigler, 2002; Haigler, 2007). A particular focus of research has been the possibility that the UDP-glucose substrate for cellulose synthesis is generated as sucrose is cleaved by sucrose synthase. Cryogenic TEM sample preparation methods that disallowed protein movement showed that Sus was located near the plasma membrane and in cotton fiber secondary walls (Salnikov *et al.*, 2003). The cell wall location was unexpected but subsequently confirmed by others (Ruan, 2007; Brill *et al.*, 2011). Brill and coworkers identified and characterized a novel Sus isoform, SusC, with divergence outside the catalytic region from other Sus proteins. SusC gene expression was up-regulated during cotton fiber secondary wall cellulose synthesis, and SusC together with other Sus isoforms were found in the apoplast as well as intracellularly. The function of apoplastic Sus in cotton fiber remains to be determined. It is potentially involved in synthesis of apoplastic callose that was co-distributed with Sus (Salnikov *et al.*, 2003) and/or cellulose synthesis (Brill *et al.*, 2011). Additional evidence for the importance of sucrose in cotton fiber secondary wall deposition is provided by transgenic plants constitutively overexpressing spinach sucrose phosphate synthase, a key regulator of the rate of sucrose synthesis (Haigler *et al.*, 2007). Several independent transgenic cotton lines had higher leaf sugar content. One line with the highest level of spinach sucrose phosphate synthase protein in leaf and fiber

produced fiber with thicker secondary walls (compared to wild-type and a null segregant) when grown in the growth chamber with cool nights. Cool temperatures are well known to hinder the rate of cellulose synthesis and fiber wall thickening in wild-type cotton (Haigler *et al.*, 2007 and references therein). In related results, several lines of transgenic cotton over-expressing mustard annexin had higher fiber cellulose content compared to wild-type under salt stress conditions. The mature fiber phenotype was correlated with salt-induced up-regulated expression in leaves and fiber of genes encoding sucrose phosphate synthase, sucrose synthase, and cellulose synthase (Divya *et al.*, 2010).

Fiber Maturation

The signal to end secondary wall cellulose synthesis is unknown, but afterwards the terminal, ill-defined maturation stage of fiber development begins. Difficulty in extracting protein and nucleic acids from late stage fiber hinder mechanistic studies (Kim and Triplett, 2001). Analogously to xylem tracheary elements, programmed cell death mechanisms may also operate during this time, at least partly controlled by ROS (Potikha *et al.*, 1999; Kim and Triplett, 2001). Cellular hallmarks of programmed cell death mechanisms include nuclear blebbing, DNA and cytoplasmic degradation, and caspase-like activity (Love *et al.*, 2008); however, attempts to document DNA degradation were inconclusive for cotton fiber (Roche, 2009). Soon after boll opening, the cotton fiber dries and collapses into the kidney bean cross-sectional shape that aids spinning into yarn, assuming that the fiber has an optimal maturity ratio. The fiber of many seeds fluffs into the mass typical of the open cotton boll (Fig. 1I, J).

SUMMARY

The cotton fiber is economically important and a significant tool for understanding cellular, biochemical, and molecular processes at the single-cell level. Advances in understanding fiber development are being made more rapidly now through research centered on functional genomics and cell biology. Virus induced gene silencing in cotton fiber should help future research of this type to progress faster (Tuttle *et al.*, 2008; Idris *et al.*, 2010; Tuttle and coworkers, in preparation for publication). Results from proteomics and metabolomics are starting to appear and will undoubtedly increase in the future. The description of transcriptional regulators such as miRNA and transcription factors could soon lead to an integration of their effects into a larger model of fiber gene expression regulation. Likewise, the genetic and physiological data on the roles of individual growth regulators such as ET and BR should be integrated to highlight their mechanisms. Further study of nearly 100% cellulose synthesis in cotton fiber is expected to be applicable to altering cell wall properties in cotton and other plants, including biomass crops. Each proof of a control point of fiber morphogenesis increases the potential to devise sophisticated strategies to improve fiber quality beyond the remarkable characteristics already conferred through evolution, domestication, and traditional breeding. Next generation cotton germplasm with higher fiber yield and quality, as well as potentially novel fiber characteristics, will be an important part of providing renewable, sustainable, resources to a growing human population.

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Chapter 11

FLORIGEN AND COTTON: MANIPULATING PLANT ARCHITECTURE TO IMPROVE PLANT PRODUCTIVITY

Roisin C. McGarry and Brian G. Ayre
Department of Biological Sciences
University of North Texas, Denton, TX 76203

INTRODUCTION

Plant architecture and the timing and distribution of reproductive structures are fundamental agronomic traits. The functions of members of the phosphatidylethanolamine binding protein (PEBP) family, specifically *FLOWERING LOCUS T* (*FT*), are important for regulating plant architecture, and manipulating *FT* expression has consequences for agriculture. Ectopic expression of *FT* in perennial, photoperiodic cotton increases determinate plant growth and overcomes photoperiodism, facilitating crosses with domesticated accessions. Thus, judicious manipulation of *FT* expression in cotton provides new tools for cotton breeding programs and crop management.

PLANT ARCHITECTURE IS THE PRODUCT OF MERISTEMATIC ACTIVITIES

The architecture of each plant species is uniquely specified through the activities of indeterminate and determinate meristems (Sussex and Kerk, 2001). Indeterminate meristems are replenishing reservoirs of undifferentiated plant cells needed for continued plant growth. In aerial tissues, these indeterminate meristems establish the placement of leaves, position of nodes and branches, and internode distances. This reiterative vegetative growth arises from a single point, and is referred to as monopodial growth. Cells of determinate meristems differentiate to form the reproductive structures of inflorescences and flowers. Because the apical meristem terminates in this case, the most proximal axillary bud must be released from apical dominance to continue the species-specific body plan. This is referred to as sympodial growth. Plant architecture then is a basic agronomic trait, and, not surprisingly, architecture regulation has a major impact on the agronomic success of crop plants. For example, the Green Revolution brought dramatic increases in crop yields as a result of introducing semi-dwarf varieties of wheat and rice (Borlaug, 2000; Peng *et al.,* 1999).

Cotton (*Gossypium spp*.), the world's most important textile crop, is grown primarily for fiber, which are the cell wall remains of individual cells that develop on the epidermal surface of the seed coat. The remainder of the seed is predominantly embryo and is a valuable source of oil and protein (Ruan *et al.,* 2005; Stewart and Mauney, 1986). The entire seed is therefore a valuable

commodity, and enhancing yield would have great impact on producers and subsistence farmers alike. Historically, cotton yield increases per acre have paralleled advances in technology and production practices (http://www.ers.usda.gov/Briefing/Cotton/; Meyer *et al.,* 2007). However, further investment in developmental biology and biotechnology is required to enhance production for an expanding world population and an increasingly competitive world market. In this chapter, we will discuss how the principles of plant architecture gleaned from model systems can be translated to cotton to further improve yields. Specifically, we will address how manipulating the timing and position of floral meristems have the potential to increase yields, reduce producer inputs, and benefit crop management.

COTTON PLANT ARCHITECTURE: THE TRANSITION TO FLOWERING

In cotton, the apical meristem of the main stem is indeterminate and monopodial, meaning that it remains meristematic and produces vegetative structures (nodes, internodes, leaves and axillary buds) for the life of the plant. In domesticated, day-neutral cultivars, the axillary buds of the first four nodes may remain dormant or may form monopodial vegetative branches that reiterate the main stem. Axillary buds of later-forming nodes grow out as fruiting branches and node of first fruiting branch (NFFB) is a measure of a variety's 'earliness' (Guo *et al.,* 2008). A fruiting branch is a sympodial, cymose inflorescence. The apical meristem of a fruiting branch (inflorescence apical meristem, IAM) produces a single node, internode, leaf and two axillary buds, and then transitions from a vegetative meristem to a floral meristem, forms a flower, and ultimately a boll. The leaf produced is a subtending leaf (subtends the flower); one of the axillary buds usually becomes dormant while the second axillary bud grows out to form the next sympodial unit. It in turn produces a node, internode, leaf and axillary buds, and transitions to a floral meristem. This pattern repeats for the life of the plant, giving fruiting branches a 'zigzag' appearance instead of being straight like main-stems and vegetative branches (Gore, 1935; Oosterhuis, 1990).

Once the signal to flower is received by the meristem, the meristem can differentiate into a terminal flower, but commonly forms an inflorescence. Inflorescence architecture is controlled by the distribution of indeterminate inflorescence meristems (IM) and determinate floral meristems (FM). Prusinkiewicz *et al.* (2007) presented an elegant, unifying model to explain inflorescence architecture (Prusinkiewicz *et al.,* 2007). An inflorescence has an inflorescence apical meristem (IAM) which produces lateral meristems from its flanks (inflorescence lateral meristem, ILM). The fate of these meristems is determined by a quantitative character called '*vegetativeness*' (*veg*). *Veg*, is not a compound or a gene, but a 'state of being'. If *veg* is high, IAMs will produce new growth with new ILMs, which may themselves produce new growth and more ILMs. If *veg* drops below a threshold, the IMs convert to determinate FMs and form flowers. In a young plant, *veg* is initially high but drops with age. In panicles such as mountain ash, *veg* drops uniformly throughout the inflorescence, resulting in relatively synchronized flowering and termination of growth. If *veg* drops quickly after formation of an inflorescence, a simple panicle is formed, but if *veg* drops slowly, a compound panicle is formed because the

lateral meristems are able to reiterate the inflorescence pattern before switching to floral identity. During the formation of cymes and racemes, loss of *veg* is not uniform. In racemes, *veg* stays high in the IAM and drops in the ILMs so that the IAM continues growth and the ILMs form flowers (monopodial inflorescence; *Arabidopsis thaliana*, snapdragon, *Antirrhinum majus*). In cymes, *veg* drops in the IAM but remains high in ILMs, such that the IAMs form a flower and growth continues from the ILMs (sympodial inflorescence; tomato, *Solanum lycopersicon*, cotton) (Prusinkiewicz *et al.*, 2007) (Fig. 1).

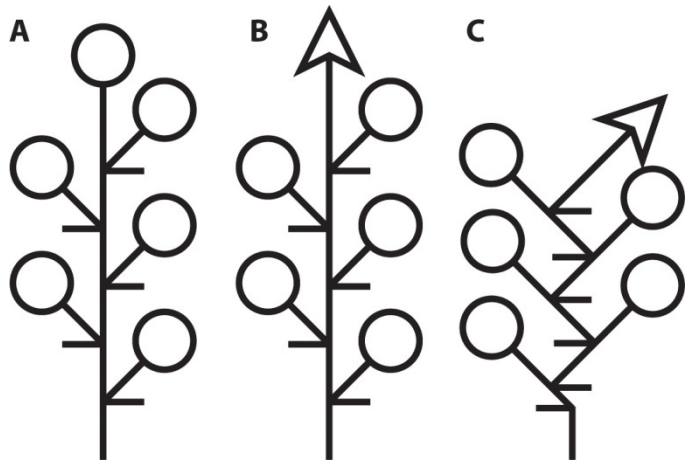

Figure 1. Simplified representation of (A) a panicle, (B) a raceme, and (C) a cyme, after (Prusinkiewicz *et al.* 2007). In arrowheads, *vegetativeness* (*veg*) is above a threshold, and meristem retains an indeterminate identity (e.g. continued vegetative growth); in circles *veg* has dropped below a threshold and the meristem has converted to a determinate fate (e.g., a flower). In panicles (A), *veg* drops uniformly in all buds resulting in a synchronized transition. In racemes (B), *veg* stays high in the apical meristem and drops in the lateral buds to give a monopodial main axis. In cymes (C), *veg* drops in the apical meristem and remains high in the axillary / lateral buds, resulting in a sympodial axis.

Experiments in Arabidopsis, snapdragon, various Solanaceae and other model systems have illuminated paradigms for controlling *veg* levels. Floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) suppress *veg* to specify a flower. When either is over-expressed in Arabidopsis, the transition to flowering is accelerated and the IAM of the raceme loses its indeterminate character and terminates as a single flower. Conversely, *lfy* and *ap1* mutants have excessive *veg* phenotypes: flowering is delayed, inflorescences have more branches and bract leaves, and flowers that do form have stem-like characteristics and form late on the inflorescence, consistent with the model that *veg* reduces with age. On the contrary, *TERMINAL FLOWER 1* (*TFL1*) maintains *veg*. *tfl1* mutants have solitary flowers where inflorescence branches would normally be and the IAM terminates as a solitary flower (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992). This phenotype is nearly identical to *LFY* over-expression. *TFL1* over-expression

results in late flowering and a phenotype similar to *lfy* mutants (Benlloch *et al.,* 2007). The tomato paralog of *TFL1* is *SELF PRUNING* (*SP*). The *sp* mutant of tomato has accelerated termination of sympodial growth, and results in a more compact, determinate plant with nearly homogeneous fruit set. Identifying the *sp* phenotype "was the single most important genetic trait in the development of modern agrotechniques for this crop plant because the 'determinate' growth habit facilitates mechanical harvest" (Rick, 1978). Consequently, appreciating how to control or manipulate *veg* levels in IMs can directly impact plant architecture and productivity.

FLORIGEN AND PHOTOPERIODISM

For over seventy years, the flowering factor, termed florigen, was the elusive "Holy Grail" of plant biology (Zeevaart, 2008). Abundant physiological data characterized florigen as a substance perceived by leaves and transmitted to the shoot apex to stimulate flowering yet the nature of that signal remained unknown (Chailakhyan, 1968). Extensive genetic and biochemical research, largely in model plants such as Arabidopsis, identified a number of genes involved in different flowering response pathways, and from these, *FLOWERING LOCUS T* (*FT*) emerged as a common element. The FT gene product is recognized as florigen (Turck *et al.,* 2008; Zeevaart, 2008).

The Arabidopsis *FT* is part of a small gene family whose gene products share similarity with mammalian phosphatidylethanolamine binding proteins (PEBP; (Kardailsky *et al.* 1999; Kobayashi *et al.,* 1999). The other members of the gene family include *TWIN SISTER OF FT* (*TSF*), *TERMINAL FLOWER 1* (*TFL1*), *CENTRORADIALIS* (*ATC*), *MOTHER OF FT AND TFL1* (*MFT*), and *BROTHER OF FT AND TFL1* (*BFT*). *TSF* is a paralog of *FT* and also promotes flowering (Jang *et al.,* 2009; Yamaguchi *et al.,* 2005). *TFL1*, on the other hand, encodes a protein of similar sequence yet antagonistic function to FT (Kardailsky *et al.* 1999; Kobayashi *et al.,* 1999), and a single amino acid change can convert FT into a functional TFL1-like molecule (Hanzawa *et al.,* 2005). While FT and TSF promote flowering at meristems, TFL1 maintains the indeterminate state of the meristem, effectively repressing flowering. Appreciating the antagonistic activities encoded by these two flowering genes has strong implications for understanding and manipulating plant architecture, as reviewed by McGarry and Ayre (2012).

Changes in day length, or photoperiod, have long been recognized to impact flowering among different plant species (Garner and Allard, 1920). The "external coincidence model", the genetic basis of photoperiodic flowering, was proposed from research in Arabidopsis (Abe *et al.,* 2005; Ayre and Turgeon, 2004; Corbesier *et al.,* 2007; Turck *et al.,* 2008) and is supported by research in tomato and rice (Kojima *et al.,* 2002; Lifschitz *et al.,* 2006; Tamaki *et al.,* 2007). As a facultative long-day plant, Arabidopsis initiates reproductive development when grown in long days (16 hour photoperiod), but will also flower when grown for an extended time under a short 12 hour photoperiod. *CONSTANS* (*CO*) mRNA accumulates in leaves late in the day (Liu *et al.,* 2008; Suarez-Lopez *et al.,* 2001). In short days, *CO* mRNA accumulates after dusk but the encoded CO protein is degraded in the absence of light. In long days, the *CO* mRNA accumulates while plants are still illuminated, and light signaling complexes stabilize the CO protein (Jang *et al.,* 2008). The CO protein is a transcription factor which turns on the expression of *FLOWERING LOCUS T*

(*FT)* in the companion cells of leaves (Abe *et al.,* 2005; Wigge *et al.,* 2005). The FT protein enters the phloem, moving from mature leaves to the meristematic regions of the plant, where it forms a heterodimer with the transcription factor FD (Abe *et al.,* 2005). In the nuclei of apical cells, the FT/FD complex turns on the expression of two meristem identity genes, *APETALA 1* (*AP1*) (Abe *et al.,* 2005; Wigge *et al.,* 2005) and *LEAFY* (*LFY*) (Schultz and Haughn ,1991; Weigel and Nilsson, 1995), and the activities of these gene products yield a flower (Fig. 2).

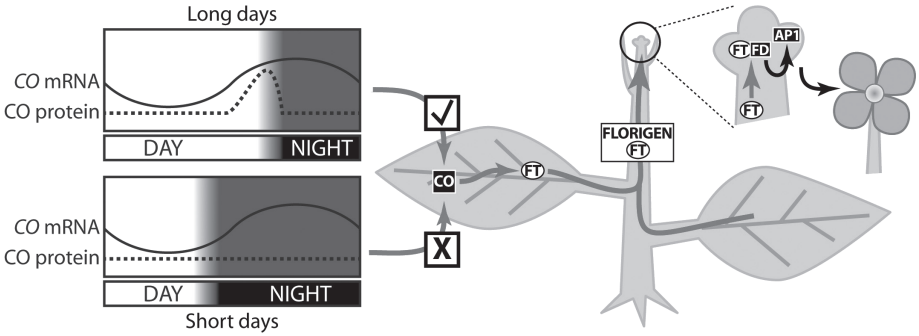

Figure 2. Coincidence model for a generic long-day plant. *CONSTANS* (*CO*) is expressed with a circadian rhythm, and begins accumulating late in the day. CO protein is stabilized in the light, but rapidly degraded in the dark. Under long-day conditions, when circadian expression of *CO* and light stabilization coincide (top left), CO protein accumulates to promote expression of *FLOWERING LOCUS T* (*FT*), encoding florigen. In short day conditions, *CO* expression and light stabilization do not coincide (bottom left), and CO protein does not accumulate to activate *FT*. FT protein is phloem mobile and migrates entirely through the symplasm (presumably) to reach the meristem (right) to interact with FD and promote flowering by activating *APETALA1* (*AP1*).

The FT signal appears to be conserved among flowering plants (Kojima *et al.,* 2002; Lifschitz *et al.,* 2006; Mathieu *et al.,* 2007). Indeed, *FT* orthologs from an array of monocots and eudicots, such as poplar (*Populus* spp.), tomato, citrus (*Poncirus trifoliata* L. Raf), and wheat (*Triticum aestivum*), have been expressed in heterologous species and induced early flowering (Bohlenius *et al.,* 2006; Endo *et al.,* 2005; Hsu *et al.,* 2006; Lifschitz *et al.,* 2006; Yan *et al.,* 2006; Zeevaart, 2008). Furthermore, expression from *FT* orthologs over-rides the endogenous photoperiod of the host plant (Kojima *et al.,* 2002).

COTTON IS A PERENNIAL, SHORT-DAY PLANT

Two allotetraploids (AADD), *Gossypium hirsutum* (Upland Cotton, ~90% of USA cultivation) and *G. barbadense* (Pima or Extra-Long Staple Cotton), are cultivated in the USA. Wild accessions have diverse morphologies, but 6000 years of independent domestication has led to convergent traits that allow these tropical, short-day photoperiodic perennials to be grown and harvested as compact, day-neutral annual crops (Lubbers and Chee 2009; Percy 2009; Wendel *et al.,* 2009).

Perennials and annuals have fundamentally different life strategies: annuals focus end-of-season resources on reproduction to ensure the success of the next generation while perennials will compromise reproductive growth to ensure survival of the parent to the next season. *Gossypium* species experience repeated, yearly cycles of vegetative growth in long-day seasons with reproductive development triggered by short-day photoperiods. Despite its inherent perennial nature, cotton varieties domesticated for temperate climates have been bred for day-neutrality and are cultivated and harvested as an annual crop (Oosterhuis, 1990): Seed is planted each spring, plants flower early in their life cycle and bolls are harvested late in the season before cold temperatures terminate the crop. This management strategy is well-suited to highly mechanized production practices but is at odds with the plant's natural growth habit and can complicate breeding and crop management, and reduce the quantity and quality of yields (Oosterhuis, 1990). In addition, flowering and fruit set in both ancestral and modern lines are not synchronous but continue throughout the season, encouraging producers to extend the growing season to maximize yield. But the highest quality fibers are from bolls that form at the first fruiting position of the first 10 fruiting branches, and poor quality fiber from later-forming bolls can discount value despite contributing to yield (Kerby *et al.,* 2010; Oosterhuis, 1990). Extending the growing season also increases producer costs for irrigation, fertilization, pesticides and herbicides (Jost *et al.,* 2006). Further still, both modern and ancestral lines continue vegetative growth after initiating reproductive growth. This perennial trait diverts resources away from fiber and seed production, and late season rain can complicate harvest by causing a flush of vegetative growth (Oosterhuis, 1990). To control growth habit, growth inhibitors are used during the growing season to make the crop pseudo-determinate and defoliants are used at the end of the season in preparation for mechanical harvest (Cothren and Oosterhuis, 2010; Jost *et al.,* 2006; Shurley *et al.,* 2004). These treatments further increase producer costs and also have negative environmental consequences (2009 Georgia Cotton Production Guide, http://www.ugacotton.com).

Because breeding has focused primarily on fiber yield and quality among domesticated, day-neutral lines, modern cultivated cotton suffers from restricted genetic diversity (Paterson *et al.,* 2004). This highly vulnerable gene pool is in fact attributed to several domestication bottlenecks. For instance, polyploid cottons arose from only two of eight extant diploid genomes, and only a small subset of wild genotypes was domesticated (Paterson *et al.,* 2004). Moreover, tetraploid genotypes were trafficked from their center of diversity in Mexico and central America to the USA, Australia, China and other countries (Paterson *et al.,* 2004). Ancestral accessions, however, including heirloom cultivars, landraces, natural *G. hirsutum* and *barbadense* isolates and their diploid progenitors, are a rich but generally untapped source of natural variation (Iqbal *et al.,* 2001) affecting fiber quality and yield, and resistance to biotic and abiotic stresses (Guo *et al.,* 2008; Robinson, 2007; Saha *et al.,* 2006).

One solution to counter genetic vulnerability is to introduce exotic germplasm (Myles *et al.,* 2011). Introgressing the diversity exhibited among ancestral accessions into elite lines has potential for crop improvement; however, ancestral lines are photoperiodic short-day plants and do not flower until the short days of fall. Domesticated day-neutral cultivars, on the other hand, flower early in their life cycle irrespective of day length, and have already reached cutout (*i.e.,* the point at which the resource demand of existing bolls ostensibly prevents new growth) by autumn. These differences in the onset of flowering complicate crossing and increase costs by

necessitating growth in greenhouses or tropical territories and limit breeding to annual cycles unless photoperiod is artificially shortened in specialized growth facilities (Paterson *et al.,* 2004; Robinson, 2007; Saha *et al.,* 2008). Furthermore, some accessions require additional environmental cues, such as specific temperatures, to initiate reproductive growth and the specific conditions required for flowering are difficult to replicate. Therefore, any practical introduction of exotic germplasm requires a mechanism to uncouple desired parent lines from photoperiodism.

In addition, the cotton research community is interested in generating nested association mapping populations, in which numerous ancestral lines are crossed to a single domesticated line, and progeny of these crosses are then backcrossed to the domesticated parent to obtain recombinant inbred lines (Bergelson and Roux, 2010; Kump *et al.,* 2011; Yu *et al.,* 2008). The goal is to develop a population of lines homozygous for stretches of ancestral DNA in an otherwise modern genome and associate traits to these segments. This effort is hampered by photoperiodism in the ancestral lines: either the populations need to be created under short-day conditions, or homozygous regions linked to photoperiodic QTLs will be lost from the population. The former will be demanding in time and resources, and the latter will compromise the value of the population. A mechanism to promote flowering and accelerate the life cycle of ancestral lines would alleviate these limitations.

MANIPULATING FLOWERING TIME: A TRANSGENIC APPROACH

Enhancing plant productivity is intimately linked with improving the time to flower. Trees are perennial plants that often experience an extended juvenile phase, sometimes years, before becoming competent to flower, and this delay poses a significant challenge for biotechnology and breeding programs. In aspen, for example, the onset of reproductive growth usually requires between 8 - 20 years. However, when the Arabidopsis floral meristem identity gene *LEAFY* was introduced in aspen, the transgenic plants flowered within months (Weigel and Nilsson, 1995). This was an excellent demonstration of how manipulating a heterologous gene could dramatically shorten generation time, a boon for breeding and trait introgression programs in crop species.

With the subsequent identification of *FT* as the mobile floral signal (Corbesier *et al.,* 2007), this gene became a target for manipulating flowering time. Over-expression of an *FT* ortholog in transgenic poplar induced juvenile trees to produce inflorescences (Bohlenius *et al.,* 2006) instead of solitary flowers (Weigel and Nilsson, 1995). Interestingly, functionally diverged paralogs *FT1* and *FT2* work in contrasting seasons to coordinate cycles of reproductive and vegetative growth in perennial poplar (Hsu *et al.,* 2011). Thus, FT determines flowering time, even in an adaptive perennial with a duplicated genome (Hsu *et al.,* 2011). Consequently, flowering time could be accelerated in plants amenable to transformation which held particular promise for biotechnological applications in species with long life cycles.

VIRUS-INDUCED FLOWERING IN COTTON

Generating transgenic cotton is a time-consuming labor that requires extensive tissue culture (Wilkins *et al.,* 2004). A significant drawback to transformation of cotton is that, while cotton species can be infected with *Agrobacterium tumefaciens* (the standard method for introducing

foreign DNA into plant cells to generate stable transgenics), the subsequent regeneration from callus to fertile plants through tissue culture is very limiting (John and Stewart, 2010). Indeed, consistent regeneration has been observed only among Coker varieties (Trolinder and Goodin, 1987). Thus, ectopically expressing *FT* in transgenic ancestral and/or diploid photoperiodic lines of cotton may require herculean effort.

Because some plant species remain recalcitrant to transgenic approaches, virus-derived technologies offer a practical alternative. Virus-derived vectors are most commonly used for virus-induced gene silencing (VIGS) (Robertson, 2004), which in cotton, has particular promise because the major cultivated lines are allotetraploids: VIGS would be expected to silence both homoeologs (unless the silencing sequence was specifically designed not to), whereas loci disrupted by mutagenesis would likely be complemented by the homoeolog. Both *Cotton leaf crumple virus* (*CLCrV*) (Idris *et al.,* 2010; Tuttle *et al.,* 2008) and *Tobacco rattle virus* (Gao *et al.,* 2011) have been adapted for VIGS in cotton.

CLCrV is a whitefly- (*Bemisia tabaci*) transmitted *Begomovirus* (family Geminiviridae) endemic to the southwestern United States and northwestern Mexico with benign infection symptoms (Idris and Brown, 2004). In the disarmed CLCrV (dCLCrV) system, a multiple cloning site replaces sequences between the start and stop codons of the gene encoding the coat protein (Tuttle *et al.,* 2008). Deleting the coat protein gene sequence disarms the vector since the coat protein is required for whitefly transmission (Azzam *et al.,* 1994; Briddon *et al.,* 1989) and whiteflies are the only natural vector for transmission. In addition, the virus is not transferred through the pollen or egg (Mink, 1993; Sudarshana *et al.,* 1998) and seeds are thus free of virus. Tuttle and colleagues (2008) cloned up to 500 nt of sequence antisense to the *G. hirsutum* magnesium chelatase subunit 1 (*Chl1*) or phytoene desaturase (*PDS*) gene into dCLCrV and delivered these by biolistic bombardment to cotton seedlings. Infected plants demonstrated systemic and sustained silencing of *Chl1* or *PDS*, clearly visualized as sectors of chlorotic tissues (Tuttle *et al.,* 2008). Virus-based vectors can also be used for gain-of-function analysis in cotton; however, geminiviruses such as dCLCrV have size constraints, and sequences larger than the deleted coat-protein gene (~800 nucleotides) tend to be quickly lost (Timmermans *et al.,* 1994). Notwithstanding, dCLCrV was engineered to express the green fluorescent protein marker to visualize viral movement through the plant vasculature (Idris *et al.,* 2010; Tuttle *et al.,* 2008).

"Virus-induced flowering" (VIF) is an emerging tool to promote transient flowering and obviates the time and labor of generating stable transformants (McGarry and Ayre, 2012; (Yamagishi *et al.,* 2011). Recently, the arabidopsis *FT* gene was cloned into dCLCrV and used to infect cotton (McGarry and Ayre, 2012), and into *Apple latent spherical virus* (*ALSV*) and used to infect apple (Yamagishi *et al.,* 2011) and soybean (Yamagishi and Yoshikawa 2010) varieties. When *FT* was expressed from ALSV in apple, it reduced the juvenile phase such that plants flowered within months after infection instead of the usual span of several years to reach reproductive maturity (Yamagishi *et al.,* 2011). When the same virus was used to infect indeterminate varieties of soybean, VIF yielded early flowering and reduced vegetative growth among indeterminate short-day soybean plants (Yamagishi and Yoshikawa 2010). Because the function of FT is demonstrated to be highly conserved across angiosperms, VIF does not require isolating florigen from non-model plants. Since almost all

viruses and the FT protein move through the phloem vasculature (Corbesier *et al.,* 2007), VIF further amplifies the florigenic signal from infected regions of the plant to meristems where the transition from vegetative to reproductive growth occurs (Corbesier *et al.,* 2007).

We engineered dCLCrV to express the arabidopsis *FT* gene, and used this to infect cotton varieties (McGarry and Ayre, 2012). The day-neutral cultivar DeltaPine 61 (DP61) and photoperiodic line Tex 701 (USDA GRIN accessions PI 607174 and PI 165329, respectively) were chosen for initial experiments because they were previously used to map QTLs related to photoperiodic flowering (Guo *et al.,* 2008). *AtFT* cDNA was cloned downstream of the viral coat protein promoter in dCLCrV, generating dCLCrV::FT, and, along with control constructs dCLCrV (*i.e.*, empty-vector control) and a vector containing antisense sequence to the *G. hirsutum* magnesium chelatase subunit 1 gene (dCLCrV::αChl1; Tuttle *et al.,* 2008), were used to inoculate DP61 and Tex 701 seedlings by biolistic bombardment. Although not transmitted plant to plant, dCLCrV can move throughout the whole plant, and systemic *Chl1* silencing was observed as chlorosis 14 days after bombardment and was still active in 90 d old plants. Plants bombarded with dCLCrV (*i.e.,* no insert) showed mild symptomology as expected, demonstrating that dCLCrV delivering foreign DNA does not overtly impact plant growth. Our bombardment protocol was optimized to achieve 80% infection efficiency.

When grown in a greenhouse with supplemental light (16/8 hr day/night, non-inductive long-day conditions), the photoperiodic dCLCrV::FT-infected Tex 701 plants transitioned to reproductive growth as early as 33 days post-germination (dpg) at node 5 and the first flowers reached anthesis at 71 dpg, showing successful VIF (Fig. 3).

This compares favorably to uninfected, day-neutral DP61 plants which produced fruiting branches at node 5 with flowers reaching anthesis by 64 dpg. None of the uninfected Tex 701, nor dCLCrV- or dCLCrV::αChl1-infected Tex 701 flowered under these non-inductive conditions.

Figure 3. "Virus-induced flowering" (VIF) in photoperiodic cotton accession TX701. Both plants were grown under long-day conditions (16 hr light) in a greenhouse with supplemental lighting. The plant on the left was infected with a disarmed cotton leaf crumple virus carrying *FT* from arabidopsis in place of the coat protein gene, and arrows point to a few of the many reproductive structures on the plant. The plant on the right was not infected with an *FT*-carrying virus, and is complete vegetative.

FT-induced Tex 701 flowers were used as pollen donors in crosses with uninfected DP61 (McGarry and Ayre, 2012). The cross-pollinated flowers formed healthy bolls with good seed yields (21.3 ± 11.0 seeds per boll, $n = 20$ bolls compared to 30 ± 3.9 seeds per boll of self-pollinated DP61 plants, $n = 9$ bolls). The F_1 generation was scored for three traits: leaf shape, node of first fruiting branch, and presence/absence of floral spots. All 46 F_1 seedlings had leaf shape intermediate between the extreme lobing or "okra leaf" phenotype of the Tex 701 and normal cotton leaves of DP61. NFB among the F_1 (14.7 ± 2.2, $n = 46$) was intermediate between day-neutral DP61 (5.1 ± 0.9, $n = 10$) and photoperiodic Tex 701 (no floral buds detected by node 24, $n = 8$). Finally, F_1 flowers had floral spots characteristic of the Tex 701 pollen donor rather than the absence of spots characteristic of the DP61 pollen recipient. Importantly, the F_1 did not harbor viral sequences when screened by PCR. Thus, VIF is an effective technology for facilitating crosses between ancestral and modern accessions, and the progeny of these crosses do not carry viral DNA and should not be derisively labeled as "genetically modified organisms".

We demonstrate that VIF can convert vegetative meristems to floral meristems in cotton. Occasionally, dCLCrV::FT-infected Tex 701 fruiting branches ceased vegetative growth and terminated in floral clusters (Fig. 4).

We interpret these morphologies as ILMs that have transitioned to floral identity prior to forming a new sympodial unit (*i.e.,* node, internode, subtending leaf and axillary bud with a new ILM), or to describe this phenomenon in terms of the Prusinkiewicz model (Prusinkiewicz *et al.,* 2007), *veg* in the ILM decreased rapidly such that the IAM and ILM transitioned to a determinate floral fate at roughly the same time. Furthermore, we found that dCLCrV::FT infection phenocopied the effect of inductive short days on leaf growth in Tex 701. Leaves from fruiting branches of Tex 701 assume a determinate lanceolate shape instead of the characteristic lobing of main-stem

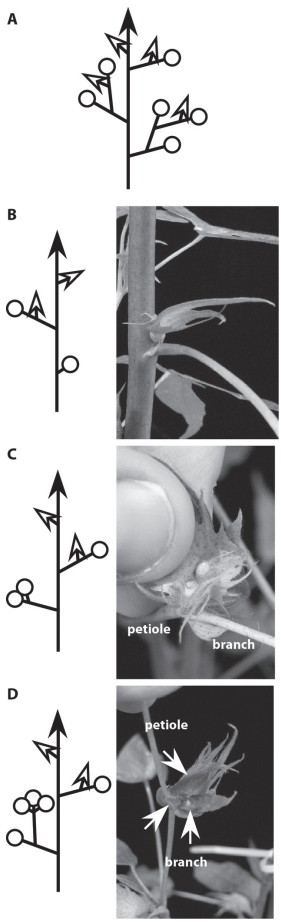

Figure 4. VIF in wild accession TX701 frequently caused fruiting branches to terminate in a floral cluster rather than continue sympodial reiterations. (A) A schematic of canonical flowering in cotton is shown. White circles are determinate floral buds and white arrows are the terminal axillary buds forming the next sympodial reiteration of the fruiting branch; black arrows are the monopodial main stem apical bud. (B, C, D) Schematics and pictures of fruiting branches that terminated with a floral structure or floral cluster rather than continuing sympodial growth. (B) Floral structure directly on the main stem *in lieu* of a fruiting branch. (C) Two floral buds in the same bract whorl. (D) A cluster of three independent flowers (arrows). In (C) and (D) the fruiting branch and petiole of the subtending leaf are labeled.

leaves. Tex 701 plants infected with dCLCrV::FT similarly demonstrated this determinate leaf shape transition along fruiting branches whereas dCLCrV-infected or untransfected Tex 701 grown under long days maintained the heavily-lobed "okra" leaf shape (McGarry and Ayre, 2012). In addition to these determinate features, our work with VIF in day-neutral cotton accession DeltaPine 61 showed that *FT* promoted determinate growth distinct from flowering. While dCLCrV::FT-infected DP61 flowered slightly earlier than uninfected controls (NFB 3 ± 0, n = 3 vs 5.1 ± 0.9, n = 10, respectively), dCLCrV::FT-infected DP61 plants exhibited fewer and shorter sympodial units per fruiting branch than uninfected or mock-inoculated controls (McGarry and Ayre, 2012). Our findings suggest that over-expression of *FT* accelerates determinate growth to yield a more compact plant architecture.

The maize (*Zea mays*) *FT* ortholog, *ZCN8*, also exhibits pleiotropic functions in plant growth (Danilevskaya *et al.,* 2011). Down-regulating *ZCN8* expression with an artificial microRNA not only delayed the floral transition, but the same transgenic plants had larger leaves and stems and more tassels (Danilevskaya *et al.,* 2011). Conversely, over-expression of *SFT* and *FT* in day-neutral tomato and tobacco caused early flowering, and plants displayed fewer leaflets per compound leaf, shorter internodes, and thinner stems (Lifschitz *et al.,* 2006). Taken together, these data extend the function of *FT* from "flowering gene" to more generally promoting the transition from indeterminate (vegetative) to determinate (floral) plant growth.

FUTURE CONSIDERATIONS

Although VIF provides valuable results, we cannot control the timing, duration or strength of the floral signal and the dCLCrV vector is not completely without symptomology. An inducible system for controlling *veg* levels would permit more meaningful analysis of the potential of manipulating plant architecture to increase yields and synchronize the crop. Alternatively, identifying the *GhFT* orthologs and manipulating the expression of the native genes may also reduce pleiotropic effects.

In plants with significantly larger genomes, the PEBP family is substantially expanded from that of Arabidopsis, and the functions of the gene family members are more complex. The *FT* family in pea and other legumes has been classified into three subclades, with members demonstrating differences in expression patterns and tissue specificity, timing of flowering, and response to photoperiod (Hecht *et al.,* 2011). Indeed, the cooperative activities of several different pea FT members are required for floral induction (Hecht *et al.,* 2011). In the biennial *Beta vulgaris* (beet), flowering time is controlled by two *FT* paralogs: one is essential for flowering while the other is a repressor of flowering necessary for the vernalization response (Pin *et al.,* 2010). This finding was in contrast to work in sunflower (*Helianthus annus*) in which a frameshift mutation in *HaFT1*, an allele that experienced selection during early domestication, delays flowering by interfering with the action of *HaFT4* (Blackman *et al.,* 2010). More recently it was shown that divergent *FT* paralogs in poplar, *FT1* and *FT2*, determined the annual cycles of reproductive and vegetative growth in this woody perennial (Hsu *et al.,* 2011). In conclusion, control of flowering time is of critical importance to plants, and the strategies employed by annuals and perennials may invoke different regulatory points. The redundancy observed among

the PEBP gene family raises questions about their functional diversification. Further focus on the identification and functional characterizations of the cotton PEBP family may elucidate aspects of indeterminate and determinate growth regulation in perennial cotton. Such insight could prove invaluable for enhancing cotton productivity and improving crop management.

SUMMARY

Manipulating expression of *FT* in cotton holds promise for modifying cotton plant architecture by reducing indeterminate and vegetative growth and promoting flowering and determinate plant growth. These alterations in growth habit may have tangible consequences for cotton production and management. Moreover, we demonstrate the utility of VIF, virus-induced flowering, as a tool for cotton breeding to facilitate the introgression of desirable germplasm from ancestral cotton accessions into domesticated lines without genetically modifying the germline.

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INDEX

Symbols

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z