Chapter 9

ABIOTIC STRESS AND COTTON FIBER DEVELOPMENT

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INTRODUCTION

Abiotic stresses, particularly water deficit, salinity, and temperature extremes, are the primary factors limiting crop productivity, accounting for more than a 50% reduction in crop yields worldwide (Boyer, 1982). Areas affected by drought are expanding and this trend is expected to accelerate (Burke *et al*., 2006). Growth of the world's population combined with an increase in global prosperity and decrease in arable land are creating increasing demands for food, fiber and biomaterials (Ragauskas *et al*., 2006). More than 80% of available fresh water is consumed by agriculture (Delmer, 2005), and the need for sustainable agricultural methods is ever increasing. Drought is a perennial environmental constraint, affecting an estimated 25 percent of all crops worldwide at enormous cost. Therefore, increasing food and fiber quantity and quality through biotechnology for improved stress tolerance and biomass production has the potential to impact the complex and interrelated issues of globalization, poverty, hunger, population growth, climate change, energy, biodiversity, and environmental degradation. The task of identifying gene functions and developing effective strategies to use these functions for crop improvement is daunting and much more knowledge is needed to achieve the promise of plant biotechnology.

Plant Responses to Water-Deficit Stress

Although cotton is considered to be a drought tolerant plant, like most major agricultural crops, its production is negatively impacted by water-deficit stress. Cotton, being a perennial with an indeterminate growth habit and a complex fruiting pattern, is considered to have the most complicated response to environmental conditions and management practices of the major row crops grown in the United States (Oosterhuis, 1990). Cotton yield is generally proportional to the amount of water available and acceptable yield enhancements from irrigation are typically seen in arid and semi-arid environments such as Arizona, California and West Texas (Radin *et al*., 1992.

Cotton fiber initiation, elongation, secondary cell wall development and maturation are genetically regulated, but are also affected by the environmental conditions faced by the plant during its lifecycle. Throughout cotton development, the plant perceives both internal and external cues that alter the physiological, metabolic, and cellular programs that ultimately determine the final characteristics of the fiber. Understanding fiber biology in terms of these cues has been slow in coming. Water deficit induces a variety of plant responses, including changes in gene

expression, accumulation of the phytohormone abscisic acid (ABA), production of osmotically active compounds, and the synthesis of protective proteins that scavenge oxygen radicals or act as molecular chaperones (Wang *et al*., 2003). These responses are controlled by molecular networks that activate stress responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes (Ramachandra-Reddy, 2004). Comparative analysis of gene expression profiles in cotton leaf and root tissues under well-watered and water-deficit conditions indicated extensive tissue-specific and stress-responsive changes in gene expression (Payton *et al*., 2010). While many of these stress induced genes fall into known functional categories, including, protective factors such as heat shock proteins, desiccation response proteins (dehydrins) and antioxidant enzymes, along with known stress responsive regulatory factors, the majority of stress-responsive transcripts identified in both tissues have functions that are not yet known. Thus, much remains to be learned about abiotic stress responses in cotton.

In recent years, our understanding of the regulatory mechanisms that control stress acclimation mechanisms in the model plant Arabidopsis has dramatically increased. A full review of these findings is outside the scope of this article but readers are directed to Hirayama and Shinozaki (2010) for a recent review. It is clear that research to uncover the basic mechanisms used by plants to respond to stressful environmental conditions will provide a strong foundation for more focused research aimed at understanding comparable mechanisms in cotton and other crops.

METABOLIC ASPECTS OF FIBER DEVELOPMENT

Comparisons between the cotton fiber transcriptome and metabolome at different stages of development have shown that stage-specific events can be characterized by their transcript and metabolite profiles (Gou *et al*., 2007). The up- and down-regulation of genes is dependent on the stage of fiber development as are the metabolic pathways that are utilized. For example, during fiber initiation and elongation, fiber cells must synthesize primary cell walls while maintaining a balance between turgor and extensibility. During the transitional phase from primary to secondary cell wall synthesis, a shift in cell metabolism occurs to meet the demand for cellulose synthesis by re-directing energy to carbohydrate metabolism and secondary cell wall synthesis. This shift in cellular function corresponds with the unique metabolic demands of the two major events in the fiber cell, namely, cell elongation and cellulose deposition.

Using a gene expression and GC/MS-based metabolite profiling approach, Gou *et al*. (2007) identified seven metabolic pathways, including secondary metabolites, fatty acid and carbohydrate metabolism, that function during cotton fiber development. At three days post anthesis (DPA), metabolite analysis revealed high levels of sucrose, which correlate with elevated expression of eight aquaporin-like genes. This combination promotes the build-up of turgor by increasing the osmotic potential and accelerating the rate of water uptake, respectively. Aquaporins are present in the plasma membrane (PIPs) and the tonoplast (TIPs) and are essential for cell expansion. Liu, *et al*. (2008), characterized the expression of cotton aquaporin genes *GhPIP1-2* and *GhTIP1*and found these genes to be highly and preferentially expressed at 5 DPA, further supporting their important roles during cotton fiber cell

expansion. Fiber cell elongation also requires that the cell wall be loosened for expansion. α-expansins play a major role in cell-wall weakening and disassembly in processes such as ripening, abscission and certain developmental pathways including pollen-tube growth and xylem formation (McQueen-Mason *et al*., 2007). In cotton fiber, four genes that belong to the α-expansin family were highly expressed during the outgrowth and rapid elongation stages, but were down-regulated when cells entered the secondary cell wall synthesis stage (Gou *et al*., 2007). Similarly, genes encoding putative xyloglucan endotransglycosylases (XTHs), which are involved in cell-wall remodeling, have recently been characterized in cotton and some *XTH* genes were shown to be preferentially expressed during the early stages of fiber elongation (Michailidis *et al*., 2008; Lee *et al*., 2010).

Based on the activity measurements of malate-synthesizing enzymes such as phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH), Thaker *et al*. (1999), demonstrated that the osmolyte, malate, plays an important role during rapid cell elongation. PEPC and MDH activities were elevated during the elongation stage of fiber development, whereas NADPH-MDH activity (an antagonist of PEPC) was reduced. This is consistent with findings that *PEPC* and *MDH* expression levels are higher in fibers from long staple cultivars than in those from short staple cultivars (Basra and Malik, 1983). Other genes implicated in the elongating cell are the plasma membrane proton translocating-ATPase (PM-H^+-ATPase), and vacuolar proton translocating-ATPase (V-ATPase) (Benedict *et al*., 1999). V-ATPase is known to be involved in driving solute movement into vacuoles for maintaining turgor, whereas PM-H^+-ATPase transports H^+ out of the cytosol, acidifying the apoplast and changing the extensibility of the cell wall.

Lipids are an integral part of membrane and cell wall synthesis. Gou *et al*. (2007) reported the upregulation of lipid biosynthetic genes and lipid metabolism at 6 DPA that was maintained throughout the elongation phase. In accordance with the amounts of fatty acids in fiber cells, genes that encode enzymes such as acyl-CoA-binding protein, fatty acid elongase, 3-keto-acyl-CoA synthase, β-ketoacyl-CoA synthase, and ω-3 fatty acid desaturase and very-long-chain fatty acid condensing enzyme, were upregulated at this stage and greatly reduced at 21 DPA. This is consistent with findings that lipid metabolizing enzymes and lipid transfer proteins, which have recently been shown to induce cell wall extension in in vitro assays (McQueen-Mason *et al*., 2007), are particularly highly expressed in fiber cells (Song and Allen, 1997; Orford and Timmis, 1998; and Ji *et al*., 2003). During fiber elongation, two predominant respiratory pathways, the oxidative pentose phosphate pathway (OPPP) and glycolysis, provide energy and the conversion of substrates to intermediates required for biosynthesis. The enzyme activity levels in these pathways vary with the demand for respiratory products (Thaker *et al*., 1999). For example, measured activity of glucose-6 phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) was high during the cell elongation up to 15 DPA, before falling to negligible levels at 24 DPA and 30 DPA, respectively. Thus, increased activity of OPPP enzymes could reflect the demand for NADPH and intermediates in the regulation of carbon channeling during the elongation phase and this is further supported by increased hexose kinase activity (Thaker *et al*., 1999).

At the onset of secondary cell wall formation, data gathered from transcript and metabolite profiles clearly demonstrate dynamic changes in metabolism that center on cellulose synthesis (Gou *et al*., 2007). Thus, metabolic pathways that are active during fiber elongation are down-regulated with the onset of secondary wall formation. This is evident in the reduction of G6PDH and 6PGDH activity, indicating a transition in metabolic priorities (Thaker *et al*., 1999). To illustrate this, pectin, a polysaccharide component of primary cell walls, is synthesized in part by UDP-glucose-6-dehydrogenase and UDP-D-glucuronate 4-epimerase. These enzymes, which convert UDP-glucose into UDP-D-glucuronate and then UDP-galacturonate, are down-regulated during the secondary wall synthesis stage. In light of the view that UDP-glucose serves as an immediate substrate for cellulose polymerization in cotton fiber, down-regulation of enzymes that compete for UDP-glucose makes metabolic sense (Guo *et al*., 2007). Interestingly, the activity of the glycolytic enzymes, aldolase and pyruvate kinase increase upon the shift to secondary cell wall deposition, indicating a role in cellulose synthesis (Thaker *et al*., 1999). Metabolite profiling showed that glucose, and to some extent, fructose accounts for about 50% of the total polar phase metabolites in rapidly elongating fiber cells, but decreases to 9% at 21 DPA, indicating an increase in carbohydrate utilization for cellulose synthesis (Guo *et al*., 2007). The demand for carbon in secondary cell wall synthesis is further supported by an increase of both gene expression and activity of pectin degrading enzymes, such as β-galactosidase and β-arabinosidase.

In-depth reviews by Delmer (1999) and, more recently, Haigler (2007), discussed the carbon flux into cellulose. In the models presented by these authors, UDP-glucose, derived from a variety of enzymatic reactions, is the immediate substrate for cellulose synthesis. One source of UDP-glucose is the hydrolysis of sucrose by sucrose synthase (SuSy). Although it is not conclusively determined whether the cytosolic (S-SuSy) or the membrane-associated (M-SuSy) enzyme supplies the substrate for cellulose synthesis, substantial evidence indicates that M-SuSy is likely to be the predominant enzyme that channels UDP-glucose to cellulose while S-SuSy partitions carbon for general metabolic needs (Haigler, 2007). This evidence comes from the observation that more than 50% of total SuSy protein is tightly associated with the plasma membrane, paralleling the patterns of cellulose deposition during secondary wall synthesis (Amor *et al*., 1995; Salnikov *et al*., 2003). Furthermore, it was shown that sucrose and not UDP-glucose was the preferred substrate for cellulose synthesis, indicating that a direct, energy-saving mechanism for channeling UDP-glucose to cellulose synthase is in place. However, UDP-glucose for cellulose synthesis could also be supplied by UDP-glucose pyrophosphorylase (Carpita and Delmer, 1981; Waefler and Meiser, 1994). It should be noted, however, that production of UDP-glucose through this reaction requires more energy input than from SuSy (Haigler, 2007).

Ultimately, all carbon comes from imported sucrose or re-synthesized sucrose within the cell. Besides SuSy, cell wall and vacuolar invertases also catalyze the break-down of sucrose into glucose and fructose. On the other hand, sucrose-phosphate synthase (SPS) can exert control over carbon allocation by irreversibly re-synthesizing sucrose-6-phosphate followed by the production of sucrose by sucrose-phosphate phosphatase (SPP). This sucrose cycling may be useful for efficiently controlling metabolic processes at the different stages of fiber development (Haigler *et al*., 2001; 2007).

ENVIRONMENTAL EFFECTS ON FIBER DEVELOPMENT

Cotton plants grown under unsuitable environmental conditions such as temperature extremes, water deficit, and salinity stress face reduced growth and productivity resulting from loss of fruit and altered fiber development. McMichael *et al*. (1973) found that water-deficit stress before 14 DPA leads to boll abscission, but beyond that "window of susceptibility" abscission generally does not occur. However, water-deficit stress during fiber elongation or secondary wall synthesis leads to decreased fiber length and maturity, respectively (reviewed in Cothren, 1999). Although it is not fully known how fiber quality is affected by stress, it could be due, at least in part, to the accumulation of signaling molecules such as abscisic acid (ABA). In cotton, as in other plants, ABA produced in response to water deficit and heat stress, for example, induces stomatal closure and lowers leaf water potential, these responses negatively affect photosynthesis and accumulation of carbon assimilate (Cothren, 1999; Finkelstein *et al*., 2002). Many of these biochemical and physiological changes result from ABA-induced changes in gene expression patterns. Moreover, Dasani and Thaker *et al*. (2006) reported an inverse correlation between final fiber length and ABA levels in three different cultivars. In a long staple cultivar, rapid ABA accumulation started after the fiber had attained peak elongation while, in a short staple cultivar, ABA accumulation was observed during elongation. Greater inhibition of fiber elongation was observed in cultured ovules of short and middle staple cultivars than in ovules of a long staple cultivar when the media were supplemented with ABA. It is yet to be determined if these changes ultimately affect cellulose synthesis in the fiber.

There are substantial data that show cotton fiber cellulose deposition and the degree of polymerization are affected by cool temperatures and, furthermore, that this process might be more sensitive than respiration (Haiger, 2007). Fibers exposed to cool temperatures have a prolonged period of elongation and reduced rate of secondary wall thickening, giving rise to growth rings (Basra and Saha, 1999). Temperatures below 27º C can negatively affect cellulose deposition in the secondary wall through the disruption of photoassimilate production, transport and uptake, the availability of respiration-derived energy, or direct and/or indirect effects on enzyme activity and kinetics (Roberts *et al*., 1992). The decrease in cell wall synthesis during cool nights could relate to the metabolic pathways that partition the substrate for cellulose synthesis at different developmental stages (Haigler, 2007). For example, Haigler *et al*. (2001) proposed a model to indicate that, under stress conditions, cells could shift from a M-SuSy (thought to channel UDP-glucose to cellulose synthase) to the soluble isoform (S-SuSy), reflecting a down-regulation of cellulose synthase.

IMPROVEMENT OF ABIOTIC STRESS TOLERANCE USING BIOTECHNOLOGY

The development of more stress-tolerant crops has been hindered by our limited knowledge of the precise physiological parameters that reflect the genetic potential for improved productivity under water-limited and thermally stressful environments. The potential to identify key traits that limit yield under abiotic stress conditions hinges upon an understanding of the crop at

the physiological and molecular levels. Moreover, an understanding of physiological processes that result in crop yield is paramount to accurate identification and introgression of candidate genetic material for yield improvement. Identification and characterization of quantitative trait loci (QTL) associated with improved fiber quality and yield under stressful growing conditions and selective introgression of QTL into elite cotton cultivars using a molecular breeding approach is underway (Paterson, *et al*., 2003; Saranga *et al*., 2004). While this approach is likely to bring improvements in stress tolerance, it only allows breeders to tap the genetic diversity existing within the species and, perhaps, its close relatives. QTL introgression can also introduce undesirable agronomic characteristics from the donor parents. Therefore, the development of transgenic plants by the introduction of selected genes provides a more focused approach for the creation of plants with improved abiotic stress tolerance and use of transgenes allows for the transfer of genes from any source, including non-plant species. Transgenic technology also allows for the expression of the introduced gene to be precisely controlled both temporally and spatially. This capability can be critical if expression of a given gene is needed only at a specific developmental stage, in a specific organ or tissue, or in response to specific environmental conditions. Although promoters that are constitutively expressed at high levels are still widely used, they are not appropriate for all transgenes. This is especially true for genes that encode stress responsive regulatory factors, which can have serious deleterious effects when constitutively expressed. Generation and testing of transgenic cotton plants that express gene cassettes controlled by stress-inducible promoters is now underway and it seems possible that this approach will allow for the enhancement of stress tolerance phenotypes without negative agronomic consequences. Therefore, while we are likely to see steady progress using traditional and molecular breeding strategies, transgenic modifications will provides a wider variety of options for the improvement of stress tolerance in crop plants.

More than a decade has passed since the first commercially successful transgenic agricultural crops were launched. These first products were based, in large part, on simple monogenic traits, such as herbicide tolerance or insect resistance, which did not require manipulation of complex molecular pathways in the transgenic plant (Century *et al*., 2008). Engineering crops with improved abiotic stress tolerance has proven to be much more difficult due to the multiple complex pathways involved in controlling the native stress responses. Most strategies for engineering abiotic stress tolerance in plants used so far have relied primarily on the expression of genes that encode protective molecules, such as dehydrins and antioxidant enzymes or enzymes involved in the synthesis of functional metabolites and ion pumps (for examples see Roxas *et al*., 2000; Kornyeyev *et al*., 2001; Payton *et al*., 2001; Yan *et al*., 2003; Park *et al*., 2004; He *et al*., 2005). More recently, strategies that employ genes involved in signaling and regulatory pathways for engineering plant stress responses have been developed and show great promise (Umezawa *et al*., 2006). Manipulation of these types of genes can affect a broad range of downstream events, which may result in superior tolerance to multiple stressful environments. An attractive target for manipulation and gene regulation is transcription factors (TFs) that bind to promoter regulatory elements and activate cascades of genes that act together in response to internal or external signals (Bhatnagar-Marthur *et al*., 2008). One the most well studied groups of TFs involved in drought and cold tolerance are the *CBF* (C-repeat binding factor) genes (also known

as dehydration-responsive element-binding protein [*DREB1*] genes). These ABA-independent transcription factors belongs to the ERF/AP2 family that binds to the DRE/CRT motif with a conserved (A/G)CCGACNT sequence within the promoters of a suite of genes known to establish stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007). As reviewed by Century *et al*. (2008), overexpression of these genes, specifically *CBF3*, in Arabidopsis and ectopic expression in wheat (Pellegrineschi *et al*., 2004), tomato (Wang *et al*., 2003; Chaves and Oliveira, 2004), tobacco (Kasuga *et al*., 2004), rice (Oh *et al*., 2005), and potato (Benham *et al*., 2005; Pino *et al*., 2007) produced enhanced tolerance to one or more types of abiotic stress. However, in some of these examples, the benefits of constitutively over-expressing *CBF3* were overwhelmed by undesirable side effects such as growth retardation. In some cases, these negative effects were mitigated with the use of the stress-inducible promoters while still providing increased stress tolerance (Bhatnagar-Marthur *et al*., 2008).

Another group of TF shown to confer multiple stress resistance is the ABA-dependent TF from Arabidopsis, *ABF3*. ABA regulates seed desiccation tolerance and dormancy and inhibits the phase transition from embryonic to germinative growth and from vegetative to reproductive growth. In addition, ABA acts as an internal signal to mediate some physiological responses to environmental stresses. ABA has been shown to regulate plant responses to drought, cold, and high temperature (reviewed in Marion-Poll and Leung, 2006). ABA levels increase in vegetative tissues during exposure to these stresses, triggering adaptive responses that are essential for their survival and productivity. For example, under drought conditions, ABA induces stomatal closure, minimizing water loss through transpiration (Finkelstein *et al*., 2005). Many of the biochemical and physiological changes that result from ABA-induced changes in gene expression patterns are dictated, in part, by a family of ABRE (Abscisic acid response elements)-binding transcription factors, or ABFs (ABRE-binding factors). Their expression is induced by ABA and by high salinity, cold or drought. Thus the ABF family of transcription factors is likely to be involved in ABA-dependent stress responses. Analysis of Arabidopsis that constitutively overexpress *ABF3* demonstrated that they are tolerant to chilling, freezing, heat and oxidative stress, with minimal inhibitory effect on germination and seedling growth (Kim *et al*., 2004). Moreover, Oh *et al*. (2005) and Vanjildorj *et al*. (2005) showed that constitutive ectopic expression of ABF3 in rice and lettuce resulted in increased tolerance to drought with normal growth in terms of whole plant morphology and seed development. These results indicate that tansgenes that express stress responsive transcription factors such as CBF3 and ABF3 may be good candidates for engineering multiple stress tolerance in cotton.

In addition to the technology used to generate transgenic plants that express their introduced genes in an appropriate way, it is also important to consider how these transgenic plants are evaluated to determine the effects of the introduced gene on stress tolerance characteristics. In most cases, transgenes have been tested only in model system plants such as *Arabidopsis* or tobacco. While these "proof-of-concept" experiments can give important clues about the potential usefulness of specific genes in crop plants such as cotton, in many cases the published work has depended on the assessment of transgenic plants under artificial environments that are unlikely to be faced by crops under field conditions. In addition, the physiological characterization in many of these studies does not extend beyond evaluation of growth or survival under

severe conditions. Therefore, rigorous physiological evaluation of the tolerance of transgenic crop plants to abiotic stresses and the effects of specific transgenes on agronomic traits such as yield and quality are generally lacking. Thus, as research in this area progresses and more stress tolerance candidate genes are tested, evaluation of plants that contain these genes in the field under "real world" conditions will, of course, become a priority. The effects of candidate stress tolerance genes on fiber yield and quality and the ability of these genes to provide agronomic improvements when introgressed into current cultivars will be critical to their eventual adoption by the cotton industry.

SUMMARY

Abiotic stresses, including water deficit and extreme temperatures, limit the yields and quality of cotton produced around the world. Efforts to develop new biotechnologies to improve abiotic stress tolerance in plants such as cotton are underway. While stress tolerance mechanisms are genetically and biochemically complex, tremendous progress is being made in our understanding of the regulatory pathways that regulate these mechanisms in model plants such as *Arabidopsis*. This research will undoubtedly uncover dozens, if not hundreds, of new candidate genes with the potential to provide improved stress tolerance characteristics in crop plants, including cotton. Evaluation of these genes in crop plants may take many years and the development of commercial cultivars that incorporate the most successful of these technologies is likely to take decades.

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