Evaluation of Source Leaf Responses to Water-Deficit Stresses in Cotton Using a Novel Stress Bioassay

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Water-deficit stresses preferentially reduce shoot growth, thereby disrupting the flow of carbohydrates from source leaves to the developing sinks. Here, we use a novel stress bioassay to dissect responses of field and greenhouse-grown cotton (Gossypium hirsutum) source leaves to water-deficit stresses. Fifth main stem leaf samples were harvested at sunrise and subjected to a prolonged elevated respiratory demand in the dark. Sucrose levels are lower in nonstressed cotton at sunrise compared to water-deficit stressed cotton, potentially predisposing the nonstressed tissue to succumb more rapidly. Tissue death was determined initially using the cell viability stain 2,3,5-triphenyltetrazolium chloride, but was determined in subsequent experiments by monitoring the decline in chlorophyll fluorescence yield. Fluorescence yield measurements were obtained within minutes of harvesting and individual samples were monitored over the time course of the treatment. Analyses of the time course and magnitude of chlorophyll fluorescence yield decline in samples from irrigated and dryland plots permitted the detection of stress responses within 24 h of the cessation of irrigation. The rate of fluorescence yield decline during the elevated respiratory demand treatment slowed as the water-deficit stress increased. Upon irrigation, the source leaves of the water-stressed plants recovered to prestress values within 4 d. Well-watered cotton overexpressing heat shock protein 101 had identical rates of fluorescence yield decline as nontransgenic cotton. These results suggest that the delayed decline in fluorescence yield of water-stressed tissue exposed to prolonged elevated respiratory demand can be used as a sensitive indicator of water-deficit stress responses.

Plants respond continuously to changes in available water, temperature, light, salinity, and other abiotic factors (Hasegawa et al., 2000; Turnbull et al., 2001; Chinnusamy et al., 2004; Zheng et al., 2004; Nixon et al., 2005). Physiological responses of plants generally vary with the severity and duration of the stress. The most sensitive processes are altered by a very mild stress and these changes intensify while additional processes become affected in accordance to their sensitivity to stress.

Historically, measurements of leaf or canopy temperatures have been used to evaluate the severity of water-deficit stress in plants (Jackson et al., 1988). Additionally, biomass accumulation and fruiting habit are common measures of field stress responses to water deficits (Burke et al., 1985a; Sharp and Davies, 1989). Physiologists routinely use leaf water potentials (Scholander et al., 1965), relative water contents (Lawlor and Cornic, 2002), and changes in carbon isotope ratios (Fessenden and Ehleringer, 2003) to identify the degree of water-deficit stress in plants.

Sensitivity to water stress depends upon the tissue in question. Under mild water stress, shoot growth is restricted while root growth continues (Sharp and Davies, 1979; Klein et al., 1986). Such restrictions in shoot growth and continuation of root growth are important adaptations to water stress. We have capitalized on the stress-induced restriction of shoot growth to develop a novel bioassay that measures stress responses of source leaves under water-deficit stress. Cotton (Gossypium hirsutum), like most C3 and C4 plants, accumulates photosynthate in the source leaves during the daylight hours and mobilizes these reserves at night to the growing points or sinks of the plant (Warner and Burke, 1993). Water-deficit stress disrupts growth and the flow of carbohydrate resulting in 2- to 3-fold higher Suc concentrations than irrigated controls (Timpa et al., 1986). The research described in this study evaluated the ability of source leaf tissues harvested at sunrise from well-watered and water-deficit stressed cotton to withstand prolonged respiratory demands before experiencing tissue death. The working hypothesis was that stress-induced accumulation of Suc in the source leaf tissues would provide an additional energy supply to help cells withstand prolonged respiratory demands. This differential in stored photosynthate at dawn would predispose the nonstressed leaf tissue to succumb more rapidly than the water-deficit stressed source tissues that contained more stored photosynthate, in addition to the myriad of metabolic changes associated with cellular
water-deficit stress protection. Specifically, we developed a bioassay that identifies source leaf responses to stress-induced cessation of shoot growth, and allows scientists to evaluate 200 to 300 samples at a time. Cotton responses to field and greenhouse imposed water deficits are described in this study.

**RESULTS**

This study evaluated the usefulness of a novel stress test bioassay in detecting the onset and magnitude of cotton water-stress responses under field and greenhouse conditions.

**Weather Variability**

The study was conducted in Lubbock, Texas during the 2003, 2004, and 2005 growing seasons. The weather patterns during the 3 years of this study represented the driest year on record (2003), the wettest year on record (2004), and the average year (2005; Fig. 1). The 2003 growing season began with sufficient moisture to start the crop but failed to have any physiologically significant rainfall throughout the remainder of the season. The 2004 growing season had rain events throughout the season with four events of 15 mm or greater, and six events of approximately 10 mm. The 2005 growing season had rain events evenly spaced throughout the season with five events of 15 mm or greater.

**Comparison of a Cell Viability Assay with the Stress Test Bioassay**

A stress test was developed that easily allowed the evaluation of physiological responses of 200 to 300 field-grown plants per day per person. The stress test capitalized on the plant’s inherent sensitivity to stress, the reduction in shoot growth associated with stress, and the impact of stress-induced growth reductions on source-sink relations. Initial studies collected leaf punches from source leaves at dawn, increased leaf disc respiratory demands by placing the tissue under elevated temperatures in the dark, and monitored the rate of tissue death using the 2,3,5-triphenyltetrazolium chloride (TTC) viability assay. TTC reduction has been used as a viability assay for over 50 years (Bennett, 1949) and represents a simple means of assessing viability when dealing with large numbers of samples. Unfortunately, the TTC procedure provides only a point-in-time measurement of cell viability. If determining changes in the rate of tissue death is needed, then additional samples are required for each time point desired. In an effort to avoid significant increases in the number of tissue samples needed to determine the rate of tissue death, a novel stress test was developed for use in this study. We used the repeated chlorophyll fluorescence yield (CFY) measurement of individual tissue samples to provide a relative measure of the rate of tissue death during the dark incubation at elevated temperatures. Figure 2 is a graph showing the relationship between cotton leaf cell viability measured using TTC reduction and CFY following extended dark incubation of leaf samples at 39°C. The results indicated that the CFY measurements were an excellent surrogate for the TTC reduction determinations. It is interesting to note that TTC reduction increased within minutes of moving the leaf discs from room temperature (Fig. 2, white circle) to the 39°C dark incubation treatment. The increased reduction in TTC upon heat treatment may indicate either an uncoupling of electron transport resulting in an increased rate of TTC reduction or a change in the rate of TTC uptake by the tissue. In either case, the TTC fluorescence yield comparison showed a linear relationship for the incubated tissues.

**Impact of Sampling Time on the Stress Test Determination**

The initial premise for the stress test bioassay was that the large nighttime mobilization of photosynthate from source leaves to developing sinks of nonstressed plants would result in significantly less photosynthate remaining in the source leaves at dawn compared with the source leaves of stressed plants exhibiting reduced shoot growth. Figure 3 is a graph showing the time course of fluorescence yield decline at 39°C of irrigated (white symbols) and dryland (black symbols) cotton source leaf samples harvested at sunset (7:30 PM) on day of year (DOY) 199 in 2004. Circles represent the samples harvested at sunset and the squares represent the samples harvested at sunrise. A significant difference in the rate of tissue decline between irrigated and dryland samples harvested at 7:30 AM was apparent within 30 min. The samples from the irrigated treatment declined from the initial CFY reading of 0.8 to a reading of 0.12 during the 6 h incubation. The samples from the dryland treatment, however, only declined from a CFY reading of 0.81 to a final reading of 0.33. Samples from the irrigated and dryland treatments harvested at 7:30 PM exhibited CFY values similar to one another during the first 2.5 h. The CFY of the sample from the irrigated treatment then declined rapidly between the 2.5 and 6 h readings resulting in a CFY reading similar to the CFY of the sample harvested from the irrigated treatment at 7:30 AM. The CFY of the samples from the dryland treatment harvested at 7:30 PM remained elevated compared with the sample from the irrigated treatment, ending with a CFY similar to the sample from the dryland treatment harvested at 7:30 AM. Because of these results, subsequent samples were harvested at dawn to enhance the rate at which treatment differences could be detected.

**Carbohydrate Analyses**

Leaf discs harvested in the morning from the fifth mainstem leaves of irrigated and dryland cotton were...
evaluated for Suc and starch contents to determine if differential levels of photosynthate could be detected between the treatments. Figure 4 shows the levels of Suc (black bars) and starch (white bars) in leaves of irrigated and dryland cotton. Suc levels increased and starch levels decreased in leaves of dryland cotton compared to the irrigated samples. Similar increases in Suc levels have been reported previously for water-deficit stress sensitive cotton lines (Timpa et al., 1986). Greenhouse-grown cotton from which water was withheld for 24 h also showed increased Suc levels (black circles) compared with the well-watered plants (white circles). Suc levels declined with the prolonged elevated respiratory demand brought on by incubating the tissue in the dark at 39°C. Similar rates of Suc decline were observed during the first 3 h of heat treatment; however, the rate of Suc decline increased in the dryland samples and decreased in the irrigated samples between 3 and 6 h of incubation. The decreased rate of Suc decline in the irrigated samples between 3 and 6 h is most likely the result of the increased tissue death indicated by the decline in the fluorescence yield and TTC reduction.

Comparison of Varietal Water-Deficit Stress Responses in 2003

Six commercial cotton varieties were evaluated during the first year of this study. Figure 5 shows the graph of the time course of fluorescence yield decline of irrigated (white circle) and dryland (black circle) cotton source leaf samples on DOY 218 in 2003 harvested at sunrise and dark incubated at 39°C. Varietal differences were apparent using this bioassay in how the plants had responded to their environments prior to sampling. All of the varieties evaluated from the irrigated and dryland treatments exhibited an initial CFY value of 0.8 prior to the 39°C metabolic challenge and then exhibited a decline in CFY over the next 7 h of treatment. Suregrow 215 samples from the irrigated plants showed a gradual decline in CFY over the 7 h treatment with a final value of 0.256 being observed. The dryland sample showed a decline in CFY during the first 3 h of treatment, and then CFY values leveled off at a value of 0.575. PM2326 samples from the irrigated plants showed a gradual decline in CFY over the 7 h treatment with a final value of 0.314 being observed. The dryland sample showed a decline in CFY during the first 6 h of treatment, and then CFY values leveled off at a value of 0.501. FM989 samples from the irrigated plants declined in CFY over the 7 h treatment to a final value of 0.348. The dryland sample showed a decline in CFY during the first 4 h of treatment, and then CFY values leveled off at a value of 0.630. PM2280 samples from the irrigated plants declined in CFY over the 7 h treatment to a final value of...
The dryland sample showed a decline in CFY to a value of 0.538. Beltwide 24 samples from the irrigated plants declined in CFY over the 7 h treatment to a final value of 0.275. The dryland sample showed a decline in CFY to a value of 0.568. Finally, DP555 samples from the irrigated plants declined in CFY over the 7 h treatment to a final value of 0.258. The dryland sample showed a decline in CFY to a value of 0.593.

Graphs of dryland Paymaster 2326 responses to watering and subsequent drying between DOY 219 and DOY 239 during the 2003 season are shown in Figure 6. Initial readings of the irrigated (white circle) and dryland (black circle) cotton were taken on DOY 219 immediately before irrigating the dryland treatment for 4 d with 5 mm of irrigation per day via subsurface drip lines. The irrigation of the dryland plot was stopped on DOY 223 and the plot was allowed to dry during the next 2 weeks. The solid black line in the DOY 223, 224, 226, 231, and 239 graphs shows the response of the original dryland plants to watering and subsequent drying. The gray lines show the data obtained on DOY 219 for comparison. Within 4 d of irrigation, the previously water-stressed plants exhibited CFY declines identical to plants that had always received irrigation. One day following the cessation of irrigation the plants exhibited CFY declines that were slower than observed the day before. These results suggest that the plants sensed differential water availability and had responded to the developing water-deficit stress. The water-deficit stress intensified over the next 2 weeks with the plants receiving no additional irrigations and only 7 mm of rainfall.

The transition of the cotton plants from nonstressed to water-deficit stressed to nonstressed was observed in 2004 in response to seasonal rainfall events. Figure 7 shows the time course of fluorescence yield decline of irrigated (white circles) and dryland (black circles) Suregrow 215 cotton between DOY 170 and 188 so that the dryland cotton evaluated on DOY 190 exhibited only a moderate level of stress compared with the irrigated plots. No precipitation over 1 mm occurred between DOY 190 and 204, and

**Figure 3.** Graph of the time course of fluorescence yield decline of irrigated (white symbols) and dryland (black symbols) cotton source leaf samples on DOY 199 in 2004 harvested in the morning (7:30 AM) and at sunset (7:30 PM) and dark incubated at 39°C for 6 h. Circles represent the samples harvested in the morning and the squares represent the samples harvested at sunset.

**Figure 4.** Graph of the Suc (black bars) and starch (white bars) contents of irrigated and dryland field-grown cotton source leaf samples, and graph of changes in Suc content of water-stressed and well-watered leaf discs harvested from greenhouse-grown cotton.
the graphs for DOY 194, 201, and 204 illustrate the development and maintenance of water-deficit stress over this time period. Rain events on DOY 205 and 207 provided 22 mm of water to the dryland plants. The DOY 208 graph shows a dramatic change in the CFY decline compared with DOY 204. Additional rainfall between DOY 209 and 222 provided an additional 48 mm of water to the dryland plants. Samples harvested on DOY 223 exhibited similar CFY declines between the irrigated and dryland plants.

A second study evaluated the transition from irrigated to water-deficit stressed cotton between DOY 236 and 244 of the 2004 growing season. Figure 8 shows the time course of fluorescence yield decline of irrigated (white circles) and dryland (black circles) Suregrow 215 during this evaluation period. Irrigation was turned off the morning of DOY 236 and the plots were allowed to develop a water-deficit stress over the next week. Samples taken from the previously irrigated plots on DOY 238 exhibited similar CFY declines to those of DOY 236. The rate of the CFY decline was somewhat slower initially, however, the final CFY value obtained after 6 h of treatment was the same as that seen for DOY 236. Samples from the previously irrigated plots taken on DOY 239 showed an even slower initial decline in CFY compared with either DOY 236 or 238, and the final CFY values were significantly above those values obtained on DOY 236 and 238. The differences in CFY decline between the irrigated and dryland plots became smaller as the plants from the previously irrigated plot became more severely water-deficit stressed. No differences in the CFY decline were observed between the previously irrigated and dryland plots on DOY 244.

Spatial Variability Effects on Subsurface Drip Irrigated Cotton

The sensitivity of the new stress bioassay provides a useful tool in evaluating the impact of spatial variability within an irrigation treatment. Figure 9 shows the time course of fluorescence yield decline of irrigated (white circles) and dryland (black circles) Suregrow 215 cotton between DOY 236 and DOY 238 of the 2004 season. The photograph shows the location of samples harvested from the irrigated plot on DOY 236 (top arrow), DOY 237 (bottom arrow), and DOY 238 (both arrows). Dryland samples harvested from a single location on DOY 236, 237, and 238 showed similar CFY declines across all dates. The samples from the irrigated plots, however, showed CFY decline characteristics of a well-watered crop on DOY 236, and a more stressed appearance in the DOY 237 samples. The samples were harvested from a single row and the sampling locations were approximately 5 m apart. The samples harvested on DOY 238 from the same location within the irrigated plot as the samples shown in the graph for DOY 236 showed CFY declines similar to the DOY 236 samples. The samples harvested on DOY 238 from the same location within the irrigated plot as the
samples shown in the graph for DOY 237 showed CFY declines identical to the DOY 237 samples. These results show the high degree of reproducibility of the bioassay and illustrate the need for caution when analyzing field-grown materials within a single irrigation treatment.

Comparison of Varietal Water-Deficit Stress Responses in 2005

In light of the findings that spatial variability can have significant impact on the outcome of the stress bioassay, the evaluation of genetic diversity in the stress responses of commercial cotton lines was reevaluated. Samples were harvested randomly along the treatment rows, and plots were alternated within the field. Figure 10 shows the time course of fluorescence yield decline of irrigated (white circles) and dryland (black circles) FM989, SG215, DP444, and FM800 cotton sampled on DOY 237 of the 2005 season. Clear varietal differences in plant status are observed in both the irrigated and dryland treatments. The three broad-leaf cotton varieties from the dryland treatment (FM989, SG215, and DP444) had higher CFY values after the 5.5 h heat treatment than that of the okra leaf variety (FM800). The CFY decline in the DP444 irrigated samples was greater than that of the FM989 and SG215 irrigated samples. The okra leaf variety CFY decline in the irrigated treatment was less than any of the broad leaf samples.

Evaluation of Water-Deficit Stress Responses in Greenhouse-Grown Cotton

The responses of cotton to imposition of and release from water-deficit stress in the field revealed physiological responses occurring over the time course of days to weeks. A series of experiments were performed to determine if similar temporal changes in stress responses occurred in greenhouse-grown cotton grown with more restricted rooting volumes than that of the field. Figure 11 shows that greenhouse-grown cotton grown under well-watered and limited-water conditions exhibit CFY declines similar to those of irrigated and water-deficit stressed field-grown plants. To evaluate the time course of the plant’s response to a sudden severe water-deficit stress and recovery from the stress, cotton plants were grown to the 8 to 10 main

Figure 6. Graphs of dryland Paymaster 2326 showing source leaf responses to watering and subsequent drying between DOY 219 and DOY 239 during the 2003 season. Initial readings of the irrigated (white circles) and dryland (black circles) cotton were taken on DOY 219 immediately before irrigating the dryland treatment for 4 d. The irrigation of the dryland plot was stopped on DOY 223 and the plot was allowed to dry during the next 2 weeks. The solid black line in the DOY 223, 224, 226, 231, and 239 graphs shows the response of the dryland plants to watering and subsequent drying. The gray lines show the data obtained on DOY 219 for comparison.
stem leaf stage in 16-cm diameter pots. Samples were harvested from the well-watered plants and the CFY declines were determined (Fig. 12A). Immediately after the samples were harvested the watering system was turned off and the plants were allowed to undergo a rapid and severe water-deficit stress for 24 h. All fully expanded leaves on the plants exhibited severe wilting after the 24 h water-deficit stress. Samples were harvested and the CFY decline analyzed (Fig. 12B). The plants were rewatered immediately after harvesting the samples and the response of the cotton plants monitored for an additional 7 d.

The pattern of CFY decline from five replicate plants showed the rapid decline in the well-watered plants similar to the irrigated field-grown plants (Fig. 12A). The severely wilted leaves sampled after a 24 h water-deficit stress showed only a slight change in the rate of the CFY decline compared with the well-watered plants (Fig. 12B). The characteristic response to water stress exhibited by a slow CFY decline in the bioassay was not apparent until the next morning (Fig. 12C), 24 h after the plants had been rewatered. A CFY decline identical to the 24 h recovery samples was observed after 48 h of recovery from the water stress (data not shown). It was only after 72 h post watering that the rate of CFY decline increased (Fig. 12D), suggesting that physiological recovery was under way. The plant’s further recovery from the stress was suggested by the enhanced rate of CFY decline 96 (Fig. 12E) and 120 h (Fig. 12F) after rewatering.

Evaluation of Heat Shock Protein 101 Overexpression in Cotton on the Stress Bioassay

Enhanced heat tolerance has been attributed to the constitutive expression of heat shock protein 101 (hsp101) in Arabidopsis (Arabidopsis thaliana; Queitsch et al., 2000). Because of the potential for hsp enhancement of heat tolerance and an association between water-deficit stress in the field and accumulation of hsps in cotton (Burke et al., 1985b), we studied the effect of overexpression of Arabidopsis hsp101 in cotton on the CFY decline bioassay. Figure 13 shows the PCR, western blot, and time course of fluorescence yield decline of well-watered Coker 312 cotton constitutively expressing hsp101 (white circles) and well-watered nontransgenic Coker 312 (black circles). The western-blot analysis, using antibodies kindly provided by Dr. Elizabeth Vierling, showed the presence of hsp101 in the non-heat-stressed transgenic Coker 312. The CFY decline for the transgenic and nontransgenic plants was identical (Fig. 13), indicating that the overall tissue death monitored using this assay was not affected by the presence of the hsp101 protein.
DISCUSSION

Throughout the day as much as one-half of the carbon assimilated by photosynthesis is stored in the chloroplast as starch. At night, this transitory starch is degraded, the degradation products exported to the cytosol, converted to Suc, and exported from the leaf (Geiger and Batey, 1967). In nonstressed plants only a small amount of starch is left at the end of the night (Warner and Burke, 1993). Under mild water stress, shoot growth is restricted while root growth continues (Sharp and Davies, 1979; Klein et al., 1986). We have capitalized on these characteristic plant responses to water-deficit stress in the development of a novel bioassay for evaluating plant stress. Leaf punches from source leaves of well-watered and water-deficit stressed plants were placed on a metabolic treadmill by prolonged exposure to elevated temperatures in the dark. Increases in temperature are known to increase plant tissue respiration, as exemplified by a recent study of soybean (Glycine max) leaves that showed that respiration increased by a factor of 2.5 between 18°C and 26°C average night temperatures (Bunce, 2005).

Comparison of a Cell Viability Assay with the Stress Test Bioassay

TTC reduction has been used as a viability assay for over 50 years (Bennett, 1949) and represents a simple means of assessing viability when dealing with large numbers of samples. Steponkus (1971) concluded that the TTC procedure gives a reasonably accurate estimate of viability for leaf discs, stem sections, and tissue cultures. Unfortunately, the TTC procedure provides only a point-in-time measurement of cell viability. If determining changes in the rate of tissue death is needed, then additional samples are required for each time point desired. We used the repeated CFY measurement of individual tissue samples to provide a relative measure of the rate of tissue death during the dark respiratory challenge. CFY measurements used in this study were not used to show water stress effects on photosynthetic capacity. Despite difference in water-stress levels, all samples analyzed had acclimated to their environment to provide optimum photosynthetic capacity at dawn. This is exemplified by the 0.8 CFY value obtained for all samples regardless of water stress level analyzed over the 3 years of the study (see Figs. 3 and 5–13). Rather, the fluorescence measurements of the leaf samples before and during exposure to elevated respiratory demands in the dark were used as a surrogate.
for cell viability. The relationship between cell viability determined using TTC and the CFY (Fig. 2) justified our use of this tool to provide a rapid, nondestructive, measure of the viability of the tissue.

Impact of Sampling Time on the Stress Test Determination

Throughout the day carbon assimilated by photosynthesis is stored in the chloroplast as starch and at night, this transitory starch is degraded, the degradation products exported to the cytosol, converted to Suc, and exported from the leaf (Geiger and Batey, 1967). Previous reports that nonstressed plants have only a small amount of starch left at the end of the night (Fondy and Geiger, 1985) led us to initially evaluate samples harvested at dawn. Clear differences were observed within 30 min of heat treatment between irrigated (white circles) and water-deficit stressed (black circles) field-grown cotton using the stress bioassay on samples harvested at sunrise (Fig. 3). Samples harvested at sunset, however, required an additional 2 h exposure to elevated temperatures before treatment differences were apparent. This observation is presumably the result of elevated photosynthate levels in the tissues at sunset that contributed to the overall time that the tissue was able to meet the increased respiratory demands thereby maintaining tissue viability.

Clearly, factors other than carbohydrate reserves (Fig. 4) also contribute to the overall viability of the tissues. Changes in membrane composition (Wilson et al., 1986), organic acids (Timpa et al., 1986), accumulation of osmolytes (Hitz et al., 1982; Timpa et al., 1986), and synthesis of stress protection proteins (hsp 116, Late Embryogenesis Abundant proteins, etc.; Burke et al., 1985b; Rodriguez et al., 2005) all contribute to the rate of tissue decline in the high respiratory environment used in the bioassay. Further study is needed to determine the overall contribution of individual metabolic pathways to the observed water-deficit stress responses of this bioassay.

Comparison of Varietal Water-Deficit Stress Responses

Variatel differences among cotton responses to water availability and water deficits in the field have been reported previously (Quisenberry and McMichael, 1991; Radin et al., 1994). Differences included the environmental and genetic components of stomatal behavior in two genotypes of upland cotton (Roark and Quisenberry, 1977), the genetic relationship between turgor maintenance and growth in cotton germplasm (Quisenberry et al., 1984), root vascular bundle arrangements among cotton strains and cultivars (McMichael et al., 1985), and the genetic variation among cotton germplasm for water-use efficiency (Quisenberry and McMichael, 1991).

The results of this study also identified varietal differences in cotton responses to available soil water. Differences in the rate of CFY decline among six commercial cotton varieties are shown in Figure 5. The observed differences occurred in both the irrigated and water-deficit stressed treatments. The significance of the observed differences was brought into question, however, after we identified the significant impact of field spatial variability on the overall well being of the cotton within a single variety. The spatial variability of...
crop water status results from variability of soil, crop canopy, topography, irrigation level (either inherent in the application method or caused by malfunctions), or from other factors such as salinity, which typically is spatially variable (Cohen et al., 2005). The results presented in Figure 9 demonstrate the magnitude of the spatial variability within a relatively small region of the field. Plants sampled approximately 5 m apart within the irrigated treatment exhibited difference CFY declines indicating different levels of water deficit stress. The rate and level of the decline were as great, if not greater, than the differences observed in the 2003 field study shown in Figure 5. These findings showed the need for greater sampling of the plants throughout a treatment, rather than focusing only on a small region of the field. The 2005 study was sampled more robustly. Clearly, varietal differences were observed in among the four varieties evaluated under irrigated and water-deficit stress conditions (Fig. 10). Although there are significant differences among the three broad leaf cotton varieties (FM989, DP444, and SG215), a more striking difference is apparent when comparing the okra leaf variety (FM800) with the three broad leaf varieties.

The impact of leaf shape has been evaluated previously for CO2 exchange rates of whole plants of four cultivars (FiberMax 832, Stoneville 474, DeltaPine 5690, and Paymaster 1220) measured at eight different temperatures (6°C–34°C) in 4°C steps (Bednarz and Van Iersel, 2001). Net photosynthesis of the plants decreased at temperatures over 20°C, while dark respiration increased exponentially with increasing temperature. Cotton cultivar did not influence the response of net photosynthesis or dark respiration to
high temperature stress; however, the initial temperature response of carbon use efficiency and net assimilation rate differed significantly between the okra leaf variety FiberMax 832 and the other cultivars. Carbon use efficiency and net assimilation rate of FiberMax 832 decreased at a greater rate with increasing temperature.

In this study the FM800 showed a greater level of stress for the irrigated treatment exemplified by the CFY decline to a value of 0.3 while the broad leaf varieties showed declines between 0.1 and 0.2. It is possible that the daily exposure to air temperatures between 30°C and 36°C may have had a greater impact on the FM800 than the broad leaf varieties similar to the findings of Bednarz and Van Iersel (2001). The response to the water-deficit stress treatment, however, was the reverse of the irrigated treatments. The FM800 showed the least water-deficit stress with CFY declines falling to 0.45 while the broad leaf varieties ranged between 0.53 and 0.61. Canopy architecture clearly impacts the microenvironment around the plant, thereby placing unique demands on the okra leaf variety compared with the broad leaf varieties.

Early studies of okra leaf cotton showed enhanced light penetration into the canopy (Wells and Meredith, 1986) and more recent studies showed enhanced water-use efficiency (carbon exchange rate/g) in okra leaf isolines compared with normal leaf isolines in the absence of moisture stress (Pettigrew et al., 1993). It is possible that the improved water-use efficiency, combined with the reduced leaf area index (Wells and Meredith, 1986), allow the okra leaf variety to avoid the water-deficit stress levels experienced by the broad leaf varieties.

The Dynamics of the Recovery from and Onset of Water-Deficit Stress

This study evaluated the time course of physiological responses to water-deficit stress in the field using the newly developed stress bioassay. The time course graphs in Figures 6, 7, and 8 show the progression of the onset of field-grown cotton water-deficit stresses and the recovery from these stresses in response to either irrigation or rain events.

The source leaves of greenhouse-grown cotton also showed the changes in the rate of the CFY decline in response to reduced water availability (Fig. 11). The response of the plants grown in a restricted soil volume to a sudden, severe water stress showed only a slight change in the rate of fluorescence yield decline during the stress process and suggests a continued mobilization from the source to the sinks (Fig. 12, A–B). The slow rate of fluorescence yield decline, characteristic of a water-deficit stress, was not observed until 24 h after the pots had been rewatered (Fig. 12C). It is interesting to speculate that the delay in the stress phenotype, and recovery from the stress may relate to the time required for the stress-induced signals to be synthesized and transported in sufficient concentrations.

Figure 12. Graph of the time course of fluorescence yield decline of well-watered greenhouse-grown Suregrow 215 cotton (A), 24 h after the withholding of water (B), 24 h after rewatering (C), 72 h after rewatering (D), 96 h after rewatering (E), and 120 h after rewatering (F).
to reduce sink growth (Sharp and LeNoble, 2002; Verslues and Zhu, 2005).

Evaluation of HSP101 Overexpression in Cotton on the Stress Bioassay

The stress bioassay uses the enhanced respiratory demand associated with an elevation in ambient temperature as the engine behind this stress treadmill. A question that arises from the technique is what role do hsp50 play in the observed slowing in the rate of CFY decline associated with water-deficit stress. Association of hsp accumulation with water-stressed field-grown cotton was first observed by Burke et al. (1985b) and the contribution of the heat shock response to tissue heat tolerance has been clearly established (Queitsch et al., 2000). To test the possible involvement of hsp50 in shifting the tissue death rate in this bioassay, we developed a hsp101 overexpressing transgenic cotton. Hsp101 overexpression in Arabidopsis has been shown to enhance tissue heat tolerance in the absence of the prior heat exposure (Queitsch et al., 2000). No change in the rate of CFY decline in response to the exposure to elevated temperatures was observed in this study. Although this does not rule out the involvement of hsp50 in protecting stressed tissues in the bioassay, it does suggest that the large difference between CFY decline of irrigated and water-deficit stressed are more likely a result of changes in source leaf export of photosynthate. Additionally, all samples are exposed to the prolonged exposure to 39°C, therefore hsp accumulation is likely to occur in all samples during the test as long as sufficient energy supplies are available within the cells.

MATERIALS AND METHODS

Crop Management: 2003

Eight 61-m rows of Suregrow 215 RR/BG, Fibermax 989 BR, DP 555 BG/RR, Beltwide-24R, PM 2326 BG/RR, and PM 2280 BG/RR were planted in a North-South orientation on DOY 149 (May 29, 2003) using a John Deere 7300 MaxEmerge 2 VacuMeter Planter. The plants received 5 mm of water per day per acre from underground drip lines located on 1-m centers. Roundup (Monsanto) was sprayed over the top on DOY 168 for additional weed control. Pix (BASF Corporation) was sprayed over the irrigated crop according to manufacturer’s instructions on DOY 217. The plots were sprayed with Ginstar (Bayer CropScience, RTP) according to manufacturer’s instructions on DOY 273, and the plots were harvested on DOY 304.

Crop Management: 2004

Sixteen 61-m rows of Suregrow 215 RR/BG were planted in a North-South orientation on DOY 194 (May 12, 2004) using a John Deere 7300 MaxEmerge 2 VacuMeter Planter. The plants received 5 mm of water per day per acre from underground drip lines located on 1-m centers. Roundup (Monsanto) was sprayed over the top on DOY 145 for additional weed control. Pix was sprayed over the irrigated crop according to manufacturer’s instructions on DOY 217. The plots were sprayed with Ginstar on DOY 289, and the plots were harvested on DOY 320.

Crop Management: 2005

Four 61-m rows of FM800, DP444, Suregrow 215 RR/BG, and FM089 were planted in a North-South orientation per replication on DOY 158 (June 7, 2005) using a John Deere 7300 MaxEmerge 2 VacuMeter Planter. Six replications were plants with three replications only receiving rain after planting and three replications fully irrigated. The plants received 5 mm of water per day per acre from underground drip lines located on 1-m centers. Pix was sprayed over the irrigated crop on DOY 206. The plots were sprayed with Ginstar on DOY 280, and the plots were harvested on DOY 306.

Meteorological Measurements

The U.S. Department of Agriculture-Plant Stress and Water Conservation Meteorological Tower is located immediately adjacent to the experimental...
plots. Five-minute measurements of wind speed (m/s), rainfall (mm), and temperature (°C) were collected and hourly averages calculated.

Greenhouse Cultural Practices
Suregrow 215 RR/BG seeds were planted into 16-cm diameter pots containing 900 g of Sunshine Mix #1 soil (Sun Gro Horticulture Distributors). Three seeds were planted per pot and five pots were placed on benches in a greenhouse set to provide a 31°C/27°C day/night cycle. The plants were watered daily with a daily application of Peters Excel fertilizer (Scotts-Sierra Horticultural Products Company) through the automated watering system. Water-stress experiments on 2- to 3-week-old seedlings were performed by first thinning seedlings 1 week following planting to one plant per pot. Pots were saturated with water, excess water was allowed to drain from the pot overnight, and the pots were sealed from both ends with Uline 2Mil poly bags (S-3478; permeable to air but impermeable to water). A small hole was cut in the top bag through which the cotton (Gossypium hirsutum) plant was allowed to grow. The hole was further sealed with Scotch packing tape and covered with a layer of dry potting mix to reduce heat load within the pot. The poly bags were fixed on the pots with a rubber band and strengthened with Scotch packing tape. The plants were allowed to grow for 18 d, at which time severe leaf wilting was observed at midday. Following analysis of the plant response leaf punches and filter paper were covered with Glad ClingWrap (CO2 Gel Dryer Filter Paper (Bio-Rad Laboratories) in a Pyrex baking dish. The leaf punches were excised from the fifth main stem leaves when the plants reached the eight-leaf stage. Five replicate punches obtained from well-watered and water-deficit stressed plants were evaluated following incubation at 39°C using an OSi-FL Pulse Modulated Fluorometer (Opti-Science Corporation). Following analysis of the PSII CFY(\(F_v/F_m\)), leaf punches immediately were transferred to 6 mL of 0.1 M phosphate buffer, pH 7.0, containing 0.8% TTC and incubated in the dark at 32°C for 24 h. Following the incubation, the TTC was extracted from the leaf punches with 95% ethanol and TTC reduction determined according to the procedure described by Burke (1998).

Carbohydrate Analyses
Leaves were taken from the youngest fully expanded leaf (fifth mainstem leaf from the top) of irrigated and dryland grown cotton in 2003. Immediately upon removal, the leaves were wrapped in aluminum foil and frozen in liquid nitrogen \(N_2\) within 10 s. The frozen tissue was stored in a freezer at −80°C. Leaf discs were taken from the youngest fully expanded leaf (fifth mainstem leaf from the top) of greenhouse-grown cotton that had been well watered and cotton that had water withheld for 24 h. Samples from well-watered and water-stressed greenhouse-grown cotton were analyzed following 0, 3, and 6 h of dark incubation at 39°C. Starch was assayed according to Ruffy and Huber (1982) with the Sigma Glc kit 115-A. Suc was assayed according to Warner et al. (1995).

Stress Test Bioassay
At sunrise, a leaf punch was harvested from a source leaf (in cotton this is the fifth main stem leaf from the top) using a number 6 cork borer and rubber stopper. This was repeated on five separate plants. The punches were transferred to a well in a Costar 3524 24-well cell culture cluster (Corning) that had been half filled with water. The lid was returned to the cell culture plate immediately following additional of the leaf punches. This process was repeated until samples from all treatments had been harvested.

Upon returning to the lab, the punches were placed on modified model 583 Gel Dryer Filter Paper (Bio-Rad Laboratories) in a Pyrex baking dish. The leaf punches and filter paper were covered with Glad ClingWrap (CO2 permeable; Glad Products Company) and pressed flat with a speedball roller for Microseal film (MF Research) to remove air bubbles and ensure good contact between the tissue and filter paper. Initial \(F_v/F_m\) levels were determined using an Opti-Science OSi-FL Modulated Fluorometer and then samples were placed in the dark in a VWR model 2005 incubator (Sheldon Manufacturing) set to 39°C. The samples were evaluated every 30 min after being placed in the 39°C incubator. The decline in \(F_v/F_m\) over time was used as a relative measure of the stress level of the plant (a slow decline occurring in tissue from stressed plants, and a more rapid decline occurring in tissue from less stressed plants).

Cotton Transformation and Selection
A gene reported to play a crucial role in thermotolerance (Queitsch et al., 2000) encoding the Arabidopsis (Arabidopsis thaliana) hsp101 was placed under the control of the constitutive ocs/mas superpromoter, incorporated into an expression vector, and transferred into cotton hypocotyls cells via Agrobacterium. The callus that developed was a mixture of transformed and nontransformed cells as there was no selection pressure during callus development. The callus was moved to cell suspension medium and the cell suspension was allowed to grow for 9 d. The cells and embryos were transferred to a solid medium, followed by exposure to elevated temperatures in a 50°C incubator, for selection of transgenic cells and embryos according to the procedure described by Burke et al. (2005).

The binary vector pE1801-ocs/mas superpromoter-HSP101 was introduced into the EHA 105 strain of Agrobacterium tumefaciens by direct transformation as described by Walkerpeach and Velen (1994). The constructs were subsequently introduced by Agrobacterium transfection into hypocotyl explants, by cutting submerged hypocotyls in a 24-h-old culture of EHA 105, containing the appropriate construct, grown at 28°C. The hsp101A sections were blotted dry on sterile filter paper to remove excess EHA 105, and transferred onto T2 Media [4.4 g/L Murashige and Skoog medium with Gamborg’s vitamins + 0.1 mg/L 2,4-dichlorophenoxyacetic acid and 0.5 mg/L kinetin + 30 g/L D(+)-Glc + 2 g/L phytagel]. The infected hypocotyl tissue was incubated on T2 medium at 28°C for 2 d prior to transfer to MS2NK CL medium [4.4 g/L Murashige and Skoog medium with Gamborg’s vitamins + 2 g/L phytagel + 30 g/L D(+)-Glc + 2 mg/L-naphthaleneacetic acid + 0.1 mg/L kinetin + 266 mg/L cefotaxime]. Hypocotyls were transferred to fresh MS2NK CL medium 3 weeks following Agrobacterium infection. Four weeks after the transfer, calli were cut from the hypocotyl ends and moved onto MS2NK 1/4CL medium [4.4 g/L Murashige and Skoog medium with Gamborg’s vitamins + 2 g/L phytagel + 30 g/L D(+)-Glc + 2 mg/L-naphthaleneacetic acid + 0.1 mg/L kinetin + 67 mg/L cefotaxime]. Six to 7 weeks following the transfer to MS2NK 1/4CL medium the calli were moved into MSNH cell suspension medium [4.4 g/L Murashige and Skoog medium with Gamborg’s vitamins + 30 g/L D(+)-Glc] and placed on a rotary shaker at 110 rpm for 9 d. Nontransformed embryogenic cell suspensions were transferred to MSK plates and placed in a model E-30B incubator (Percival Scientific) for 150 min at 48°C. Following the heat treatment, the petri dishes were moved to a 28°C tissue culture room, the surviving embryos allowed to develop, and seedlings recovered.

PCR Analysis of Putative Transgenic Cotton Lines
Leaf samples from the putative transformants were evaluated for the presence of the superpromoter-HSP101 coding region. PCR analyses were performed according to the procedure of Xin et al. (2003). DNA quality was determined using cotton cellulose synthase (celA1) specific primers. Transgenic lines were taken to a homozygous state prior to use in this study.

Immunoblot Analyses
Cotton leaves were ground into fine powders in liquid nitrogen and then extracted in 400 μL 2X SDS page sample buffer (Laemmli, 1970) containing 1% Tween 20. Homogenates were boiled for 10 min, and then centrifuged at 12,000g for 5 min at 4°C. Supernatants that contain total cellular proteins were stored at −80°C for later use. Protein concentration in the extraction buffer was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. About 75 μg of cotton leaf total proteins per lane was resolved by SDS-PAGE (4.5% stacking gel and 8% separation gel) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad; transfer buffer contains 25 mM Tris pH 7.5, 0.192 mM Gly, and 20% methanol). The PVDF membrane was blocked in Tris-buffered saline (TBS; 20 mM Tris pH 7.5 and 0.5% NaCl) containing 3% gelatin for 1 h, washed in TBS with 0.05%
Twist 20 (TTBS) for 5 min once, and then incubated with anti-HPSP101 antibodies in TTBS containing 1% gelatin for 3 h. The PVDF membrane was then washed three times in TTBS with each wash for 5 min, and then incubated with goat anti-rabbit IgG-alanine phosphatase conjugate in TBS for 1 h. Finally, the membrane was washed three times in TTBS and developed in the color development solution (Bio-Rad).

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LITERATURE CITED


