Examining the Effects of Varying Nitrogen and Water Availability on the Quality and Structure of Transgenic and Non-Transgenic *Sorghum bicolor* Grain

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ABSTRACT

Sorghum bicolor L. Moench is a globally important cereal grain that has limited nutritional value because of low grain digestibility. Building upon research of the redox protein, thioredoxin in other cereal grains, the Lemaux laboratory in the University of California's Plant and Microbial Biology Department used transgene-based genetics to create a sorghum line overexpressing a barley thioredoxin gene construct to target the sorghum grain endosperm, improving digestibility. By growing transgenic and non-transgenic sorghum under a combination of nitrogen and drought treatments, this project examined the interaction between growth conditions and an improved digestibility genotype developed with genetic engineering. Both nitrogen availability and drought treatments had significant effects on growth form and reproductive capacity. Growth data suggest that the high-digestibility construct has no observable effect on growth or the plants' responses to different growing conditions. However, an agriculturally favorable growth interaction between low nitrogen availability and late-stage drought may illustrate a stress-induced 'stay green' phenotype from the 296B parental line. Preliminary electron micrographs suggest that adverse water availability during grain fill reduces hard endosperm formation. However, cross-treatment tissue analysis shows the formation of expected endosperm structures regardless of experimental treatment or genetic engineering with the high-digestibility construct.

KEYWORDS

B hordein promoter, Crop science, Genetically modified organism/Genetic engineering, Scanning electron microscopy (SEM), Thioredoxin

INTRODUCTION

Sorghum bicolor L. Moench is a globally important crop for meeting human and animal nutritional requirements with a combination of ecologically desirable and nutritionally unfavorable characteristics. Grain sorghum is well adapted to poor growing conditions, and as a C4 plant from the semi-arid tropics, sorghum has high water use efficiency, resistance to periods of flooding, and tolerates high temperatures (Doggett 1988). But, because of poor nutritive qualities, worldwide interest in sorghum as a food crop has declined since the Green Revolution (Rooney et al. 2007). Cultivated since the third century AD, grain sorghum is currently grown on over 39 million hectares worldwide (Doggett 1988, US FAS and WAOB 2009). Worldwide consumption of sorghum for the last five years exceeded 57 million tons per year (US FAS et al. 2009). The United States is the number one producer of sorghum in the world, responsible for more than 15% of global sorghum production. Domestic sorghum production ranks third among the other cereal grains, behind corn and wheat (US FAS et al. 2009).

In commercial-farming conditions, sorghum yield could meet or exceed other grain crops (Rooney et al. 2007). Yet, sorghum's poor nutritive qualities have limited its use as a worldwide human and animal food source (Doggett 1988). Sorghum grain is characterized by having low available protein during human digestion and protein digestibility decreases sharply after cooking (Table 1) (Hamaker et al. 1987). Relatively high concentrations of phenolic compounds in the grain of some varieties are distasteful to some animals but can impart antifungal or antimicrobial characteristics. These biochemical traits set sorghum aside from the other major grains (O'Kennedy et al. 2006, Duodu et al. 2003, Godwin et al. 2009). With seemingly opposed growth and grain characteristics, many suggest that *S. bicolor* has great potential for improvement through biotechnology by disrupting or bolstering key traits (Rooney et al. 2007, O'Kennedy et al. 2006, Godwin et al. 2009)

Table 1. *Pepsin digestibility of cereals highlighting sorghum's relative indigestibility, especially after cooking*

	% digestibility*		
Cereal	Uncooked	Cooked	Decrease
Sorghum	**80.8**	**56.3**	**24.5**
Maize	83.4	79.3	4.1
Barley	93.2	80.2	13
Rice	91.1	82.1	9.1
Wheat	91.3	85.9	5.4

from Hamaker et al. 1987

The low available protein content of sorghum grain, along with its unfavorable response in digestibility to cooking has been linked to the presence of oxidized proteins which create a resistant matrix of covalently bonded and disulfide-linked proteins around the starch bodies of the grain endosperm. Strong interactions between proteins make it difficult for humans and

animals to use tissues from sorghum grain as a source of amino acids (Duodu et al. 2003, Hoseney et al. 1974, Svihus et al. 2005). Molecular genetics research involving the introduction of transgenes containing desirable proteins has attempted to manipulate the oxidation state of these protein structures through the regulation of the thioredoxin family of enzymes (Wong et al. 2009). Thioredoxin has been shown to play an important role in the oxidation state of proteins found in wheat and barley grain, though the effects of its up-regulation or down-regulation vary across species (Wong et al. 2009, Li et al. 2009). The laboratories of Dr. Peggy Lemaux and Professor Bob B. Buchanan from the University of California, Berkeley are examining the effects of over expression of thioredoxin in the sorghum grain endosperm and its effect on the availability of protein and starch for human and animal digestion.

Through field experiments examining different fertilizer and irrigation methods combined with different crop rotation schedules, it is known that variations in growth conditions alone will have statistically significant effects on many properties of growing plants, including the grain. Several researchers have found that the hardness of the mature grain product is statistically correlated to growing conditions, with wetter or higher nitrogen environments yielding harder kernels (Peterson et al. 1992, Kaye et al. 2007, Bayu et al. 2006, Sweeney and Lamm 1993). Kaye et al. (2007) used electron microscopy of sorghum grain to demonstrate changes in grain structure affected by growth environment and showed that harder kernels produced by more nitrogen-rich, irrigated growth environments have a tighter packing of starch structures than starch structures found in softer kernels produced under nitrogen-limited, dryer growing conditions. It is not surprising that increased fertilization during the growing season leads to a statistically significant increase in total nitrogen content of the grain, with according increases in protein content (Kaye et al. 2007, Bayu et al. 2006, Sweeney and Lamm 1993, Calderón-Chinchilla et al. 2008). However, data for these experiments were collected from field-grown plants whose grain properties can differ between years of cultivation due to variations in temperature and precipitation (Sweeney and Lamm 1993). Furthermore, fertilization with variable sources of nutrients like manure or rotation with a legume also creates variation in the properties of yielded grain, limiting the viable conclusions gleaned from these studies (Kaye et al. 2007, Bayu et al. 2006).

Although previous studies in sorghum have determined the optimal application of nitrogenous fertilizers and different irrigation schedules along with their relationship to grain

quality and protein structure and digestibility, the relationship between growth environment and grain quality for improved sorghum cultivars engineered using transgene technology is currently unknown (Doggett 1988, O'Kennedy et al. 2006, Duodu et al. 2003, Godwin et al. 2009, Svihus et al. 2005, Wong et al. 2009, Kaye et al. 2007, Bayu et al. 2006, Calderón-Chinchilla et al. 2008, Vendemiatti et al. 2008). It is commonly agreed that changes in environment are more significant than genotype in creating observable changes in grain quality, though some differences remain statistically unrelated (Peterson et al. 1992). Even though genetically engineered sorghum lines that express transgenes can boast increased nutrition and increased digestibility when grown under optimal conditions, the nature of the activity of these improved lines in suboptimal growth conditions is unknown.

This project uses controlled growth conditions to approach the question: which is more important for providing high quality sorghum grain, improved genetics or improved environment? Specifically, I examined the relationship between improved genetics generated from transgene biotechnology and soil nitrogen conditions under irrigated and drought scenarios for an increased-digestibility cultivar of sorghum developed in the Lemaux laboratory using a barley thioredoxin gene, *btrxh*. Although the improved cultivar shows increased digestibility under optimal conditions, the same quality has not been examined for grain grown under sub-optimal growth conditions. Though growth conditions are known to affect some grain qualities, *btrxh* is a key redox protein that acts on the formation of inter-protein and intra-protein disulfide structures that are characteristic of the sorghum endosperm and pericarp.

Because changes in environment will affect many genetic pathways during the growth and development of the plant, changes to nutrient and water availability are likely to cause significant observable changes in all parts of the sorghum plant and its grain, both in a plant transformed with the transgene, *btrxh* and its untransformed counterpart. Although the addition of the transgene, *btrxh* will lead to specific changes in grain chemistry and redox state that can be recorded by biochemical assays, any structural changes observable by scanning electron microscopy are likely to be dwarfed by the structural changes that the number and breadth of pathways affected by environment are likely to yield.

MATERIALS AND METHODS

Study Subject

The Lemaux laboratory in the University of California's Plant and Microbial Biology Department generated a transformed line of sorghum, homozygous for the expression from a barley thioredoxin *h* gene (*btrxh*). Transformation was conducted using biolistic bombardment of tissues cultured from immature embryos in a medium containing 2,4-D (2,4-Dichlorophenoxyacetic acid), 6-benzylaminopurine, and copper sulfate (Cho et al. 1998). The immature embryos for transformation were derived from plants of the inbred sorghum line 296B (National Research Centre for Sorghum, Hyderabad, India), grown under controlled growth conditions (28ºC; 16h light/8h dark).

Two plasmids were used in the transformation of the tissues derived from 296B so that cells containing *btrxh* could be identified through their genetic linkage with a selectable marker gene that allows only those cells expressing the marker gene to grow. The selectable marker plasmid contains a gene coding for hygromycin phosphotransferase (*hpt*) expressed under the contol of the rice actin1 (*act1*) promoter along with its intron (*act1I*) and the nopaline synthase terminator region from Agrobacterium (*nos*). The second plasmid was created with a sequence encoding a barley (*Hordeum vulgare*) thioredoxin *h* (*btrxh*) gene expressed under control of the barley endosperm-specific B1-hordein promoter with its signal peptide sequence to target trxh to the protein body and the nos terminator. Green regenerative tissues were selected as targets for biolistic bombardment and, after a period of nonselection, bombarded tissues were plated on media containing hygromycin B. Tissues that survived multiple rounds of selection were regenerated into plantlets and transferred to soil after sufficient root and shoot formation.

PCR analysis of genomic DNA, Southern blot analysis of genomic DNA digested with restriction enzymes, and western blot analysis of protein extracts confirmed the presence, transcription, and translation of the gene of interest to produce barley thioredoxin *h* (btrxh). Biochemical assays of digestibility, available protein and starch gelatinization suggest the introduction of *btrxh* yielded a sorghum variety with a nutritive advantage for consumption over its parental wild-type line.

Growth and Harvest

I grew two *S. bicolor* lines at the Oxford Tract facility in Berkeley, CA; the homozygous T3 progeny of 296B with the *btrxh* insertion and its companion null segregant that lacks *btrxh*. I

planted 80 plants in 8" individual pots with sterilized Super Soil (The Scotts Company LLC) from the Oxford Tract facility. During planting, I separated the pots into two groups marked with blue labels for nitrogen treatment or white labels for no treatment (suboptimal conditions). I used tap water to saturate the soil of both groups of pots before planting. Greenhouse staff used dissolved Peter's 20-20-20 fertilizer (Group for United Industries Corporation, St. Louis MO) at application rates specified by the manufacturer during normal watering of the marked-blue nitrogen treatment plants. Marked-white plants only received tap water. Greenhouse staff conducted normal watering of designated plants six days a week. Plants were exposed to a mixed sunlight/lamplight 12-hour photoperiod. Plants were treated for insects and fungi according to the Oxford Tract pesticide spray schedule, often requiring weekly spraying.

I planted each pot with two seeds near the center of the pot. Pots were thinned to one plant one week after first seedling emergence and, if at that time the pot contained two seedlings, the extra seedling was either be transferred to another pot without a viable seedling or destroyed.

As the flowering structures of the plant emerged from the main stalk of the plant, I placed a paper bag over the developing panicle to prevent cross fertilization between transgenic and non-transgenic plants.

When plants had approximately 14 leaves and the emerged reproductive structure had become reproductively active throughout the inflorescence (marked by the emergence of the stamen from every individual flower on the primary inflorescence), odd-numbered plants entered into a "drought" watering schedule. I labeled plants entering the drought treatment with a yellow label and subsequently no longer received water or water with dissolved fertilizer. Yellow-labeled plants were placed on elevated growing tables to prevent accidental watering. Plants in the nitrogen fertilizer treatment still received Peter's 20-20-20 and plants in the suboptimal treatment still received tap water only.

Grain was hand-harvested from all individuals five to six months after planting. Grain was only harvested from primary panicles. The presence of tillers was noted and considered part of the biomass of the plant body. Panicles were stored at 25°C until threshed. Panicles were threshed individually and the grain was weighed to produce a kg/1000 figure of grain yield for each individual. After weighing, grain from individual plants was kept separate. Around 20 mg of grain from an individual was used for biochemical analysis. Granules were selected at random for analysis by electron microscopy.

Preharvest Metrics

In order to establish the statistical significance of the different water and nutrient conditions, I took weekly measurements of plant height. Plant height was measured using a ruler or tailor's tape from the soil surface to the top of the highest leaf petiole.

Scanning Electron Microscopy of Grain

Samples of grain taken from the experimental treatments found to have the greatest biochemical differences were imaged using scanning electron microscopy of the endosperm and pericarp according to previously established tissue preparation techniques to examine physical reflection of growth or genetic differences (Hoseney et al. 1974, Wong et al. 2009, Kaye et al. 2007). All scanning electron microscopy was done on a Hitachi TM-1000 environmental SEM (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) according to the protocols available at the University of California, Berkeley's Robert D. Ogg Electron Microscope Laboratory.

Statistical Analyses

I collected data in Excel databases and imported them into R (R Development Core Team 2009) to create graphics and calculate analyses of variances, according to the comparisons of genetics and environment outlined in Peterson et al. 1992.

Repeated Measures ANOVA

Plant heights were analyzed with a multivariate Repeated Measurements ANOVA (α=.05) comparing height to different genetic and growth conditions to establish which growth and genetic conditions were statistically different from one another.

RESULTS

Growth

Data and plant materials were gathered from the experimental groups for 181 days after sowing (DAS). All 80 individual plants grew to reproductive maturity during this study period. Growth data collected in weekly intervals until 134 DAS showed different growth and vigor between combinations of fertilizer, tap water only, continuous daily watering, and late-stage drought.

After the first few weeks of growth, distinct visual differences distinguished the fertilized treatment group compared to the non-fertilized treatment group (Fig. 1). Generally, plants that

received fertilizer accumulated qualities associated with high vigor and good health. Plants receiving fertilizer grew to be dark green with many (between 15-18 total) broad, thin leaves while the plants which only received tap water were light green with few (between 10-14 total) narrow, spongy leaves. Plants from the fertilized treatment group had thicker stalks than the non-fertilized treatment group. Plants that received fertilizer flowered earlier and produced larger reproductive structures than plants that only received tap water. Plants that received fertilizer were additionally more likely to produce additional reproductive tillers, or secondary reproductive stalks, and none of the plants that received only tap water formed reproductive tillers. There were no visible cues that distinguished plants with the transgene *btrxh* from plants without the transgene.

Figure 1. Images of early growth (A, ~50DAS) and late growth (B, ~120DAS) showing divergent growth and vigor between fertilized (left) and unfertilized plants (right). Rows are made up of both transgenic and non-transgenic plants arranged randomly. Transgene presence has no observable affect on growth form.

A multiple variant repeated measures ANOVA of plant height across 134 DAS divided the study into two divergent groups (Fig. 2). One group of plants grew faster and achieved a greater total height than the other, more slowly growing group. A repeated measures ANOVA revealed a statistically significant effect in height when compared to fertilizer treatment only (Table 2). Highly significant p-values for combinations of fertilizer treatment and presence of the transgene *btrxh* in explaining height suggest that the presence of the transgene *btrxh* did not have a significant affect on the overall growth of the studied plants.

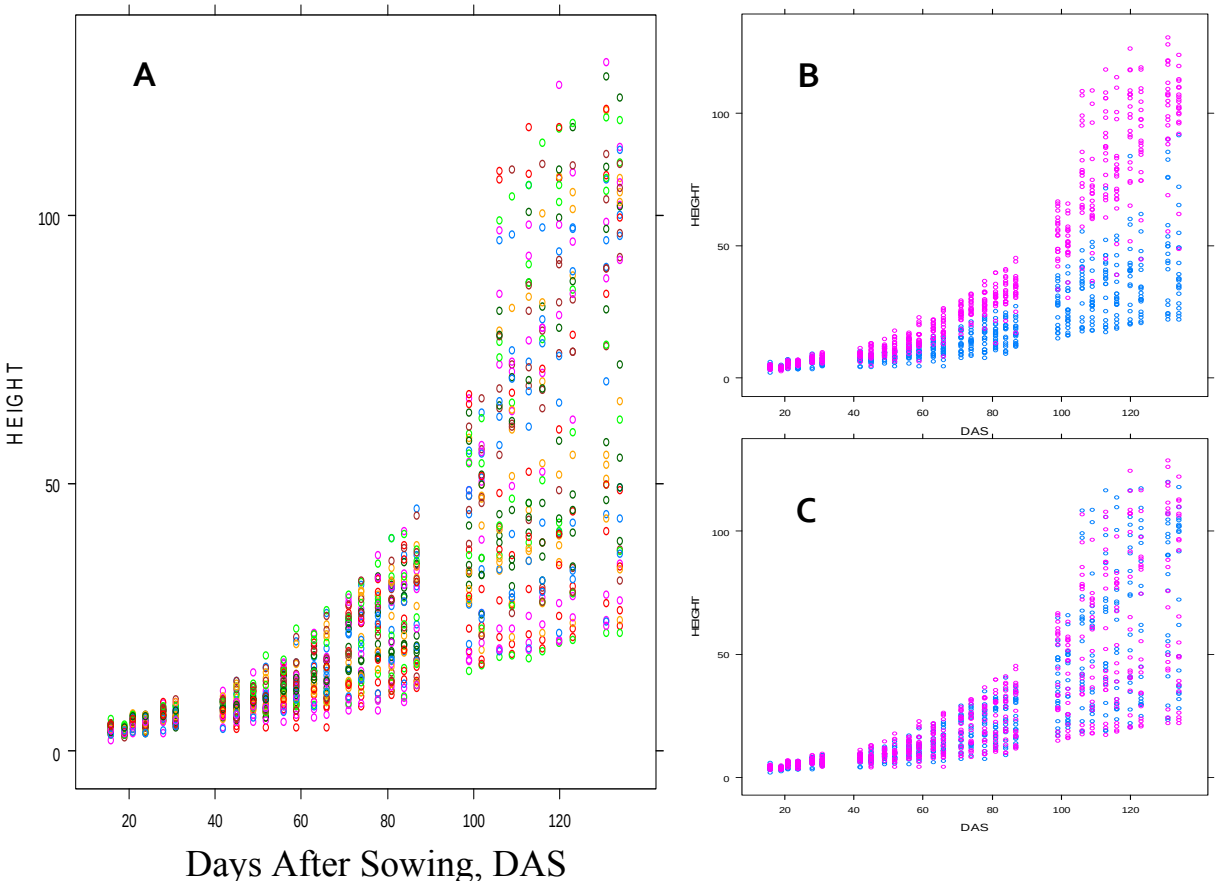

Figure 2. Plant height against number of days after sowing. The overall trend shows two divergent groups (**A**). A visual trend can be seen when data are grouped into fertilized (pink) and unfertilized (blue) experimental groups (**B**), but not when data are grouped into transgenic (blue) and non-transgenic (pink) genotypic groups (**C**).

Table 2. Repeated Measures ANOVA of Height against Days After Sowing (DAS), Genotype (TRANS), Fertilization (FERT). '***' indicated a highly significant relationship (p<0.0001).

	Df	SumSQ	MeanSQ	F-value	Pr(>F)	
Error:factor(PLANT_ID)						
DAS	2	516	258	0.50	0.61	
TRANS	1	125	125	0.24	0.62	
FERT	1	108796	108796	210.85	<2e-16	***
DAS:TRANS	2	517	258	0.50	0.61	
DAS:FERT	1	22	22	0.04	0.84	
TRANS:FERT	1	673	673	1.30	0.26	
DAS:TRANS:FERT	1	240	240	0.47	0.50	
Residuals	70	36119	516			
Error:Within						
DAS	28	632540	22590.7	440.39	<2e-16	***
DAS:TRANS	28	878	31.4	0.61	0.94	
DAS:FERT	28	122326	4368.8	85.17	<2e-16	***
DAS:TRANS:FERT	28	1623	57.9	1.13	0.29	
Residuals	1006	51605	51.3			

Striking visual differences in response to the late-drought condition distinguished plants grown with fertilizer from plants that were grown without fertilizer (Fig. 3). Upon entering into the drought treatment, plants which has previously been receiving fertilizer-enriched water quickly wilted and browned. In most plants, the leaves wilted to the point of being brittle after 1 week of drought treatment. By this time, the reproductive structures had begun to yellow. Plants which were grown with tap water only retained their pigment and did not wilt until well after 3 weeks of drought treatment. During this transition, the reproductive structures of droughted, unfertilized plants continued to develop similarly to plants of similar physiological development that continued to receive tap water only. After more than 3 weeks in drought conditions, the reproductive structures of droughted, unfertilized plants began yellowing, (similar to the changes that droughted, fertilized plants underwent within the 1st week of drought treatment). As with response to fertilizer, the late-stage drought treatment did not have a distinct visual effect on transgenic plants when compared to non-transgenic plants. In both experimental groups, the soil growth medium felt dry to the touch and began to shrink and compact within days of entering the drought treatment.

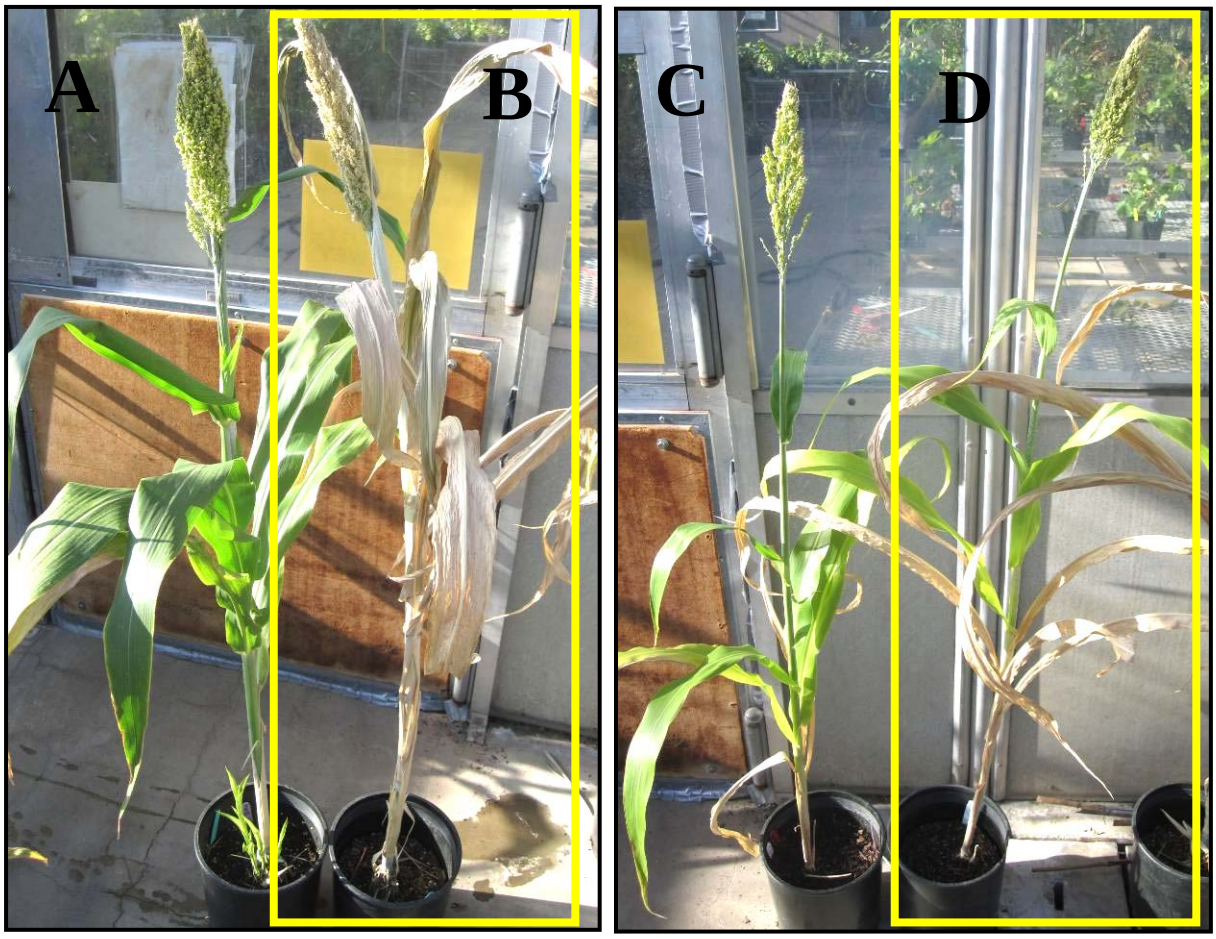

Figure 3. Images showing differential response to 3 weeks of drought treatment (no water added, no nutrient input). Plants **A** and **B** were grown with Peter's 20-20-20 fertilizer. Plants **C** and **D** were grown with tap water only. All four were at similar stages of development before **B** and **D** (outlined in yellow) entered began a drought treatment. After three weeks, **B** (grown with fertilizer) dried completely and grain development stopped; under the same treatment, **D** (growth without fertilizer) remained green and grain development continued. None of the plants grew vertically during the 3 week period.

Harvest and Yield

Although all plants reached reproductive maturity during the study period, only 13 of the total 80 plants reached a harvestable level of grain development. The majority of these (12 plants/13 harvested) were droughted plants. 4 plants reached harvestable maturity, but yielded no grain. Among non-zero fertilized, droughted individuals, the average harvest yielded 5.96 ± 3.02g of grain (n=6), with a 1000-grain weight of 0.00675 ± 0.00161 kg (n=6). The only recorded unfertilized, droughted individual yielded 3.96g of grain, with a 1000-grain weight of 0.015kg. The only recorded fertilized, non-droughted individual yielded 48.86g of grain, with a 1000-grain weight of 0.3485kg.

Grain Quality and Appearance

As fertilized and unfertilized plants responded to drought treatment in visually distinguishable ways, the grain of fertilized, droughted plants and unfertilized, droughted plants also appeared distinctly different from one another (Fig. 4). Although the grain from fertilized, droughted plants (Fig. 4, A) is uniformly small, shriveled, and largely green, the grain from unfertilized, droughted plants (Fig. 4, B) appear more substantial, less wrinkled, possess a rounder shape and have yellowed more than grain from fertilized, droughted plants. Differences in size/roundness match the trend seen in kg/1000 grain figures.

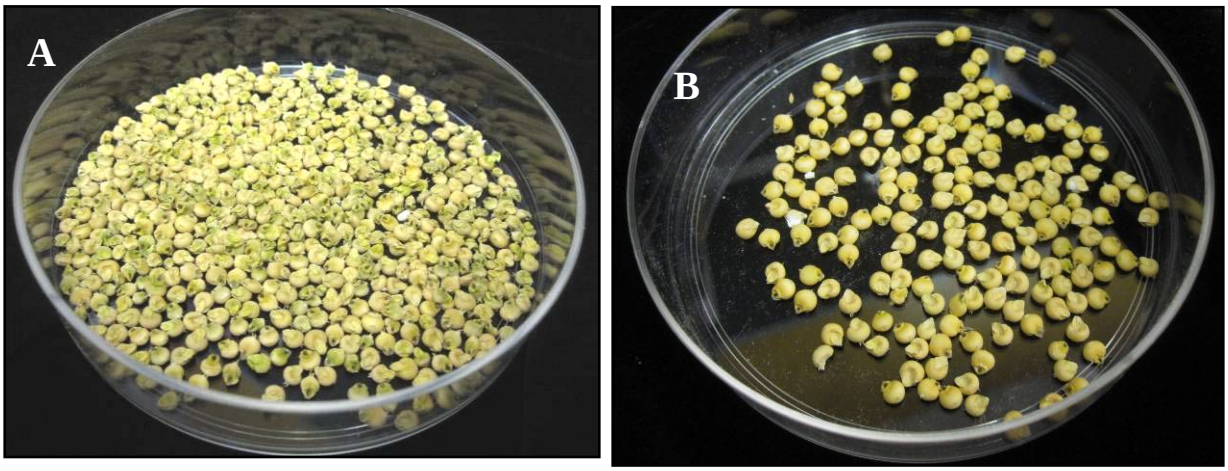

Figure 4. Images comparing the grain yielded from fertilized, droughted (A) and unfertilized, droughted plants (B).

Grain Analysis

Because of the unavailability of grain from key experimental groups, grain analysis was supplemented with fertilized, non-droughted grain from plants transformed with the transgene, *btrxh* and untransformed segregate plants. The plants were grown alongside the experimental groups in the Oxford Tract facility at the University of Cailifornia, Berkeley as part of another study in the Lemaux laboratory interested in generating additional transgenic and non-transgenic seed.

Scanning electron microscopy (SEM) generated images that illustrate the relative distribution of grain tissues and allow the comparison of the internal structures of different tissue layers. Comparison of the available grain samples using SEM revealed reduced hard endosperm

development in the samples yielded from droughted plants relative to images of non-droughted, fertilized samples (Fig. 5). Although fertilized, non-droughted samples showed the highest proportion of hard endosperm, the droughted, unfertilized sample had a greater proportion of highly compacted hard endosperm than the droughted, fertilized samples. Fertilized, non-droughted samples had highly rounded granules (Fig. 5, A and B). Unfertilized, droughted samples displayed more domed granules (Fig. 5, E). Meanwhile, fertilized, droughted samples had a variety of grain shapes ranging from domed to small, tear drop-shaped granules (Fig. 5, C and D).

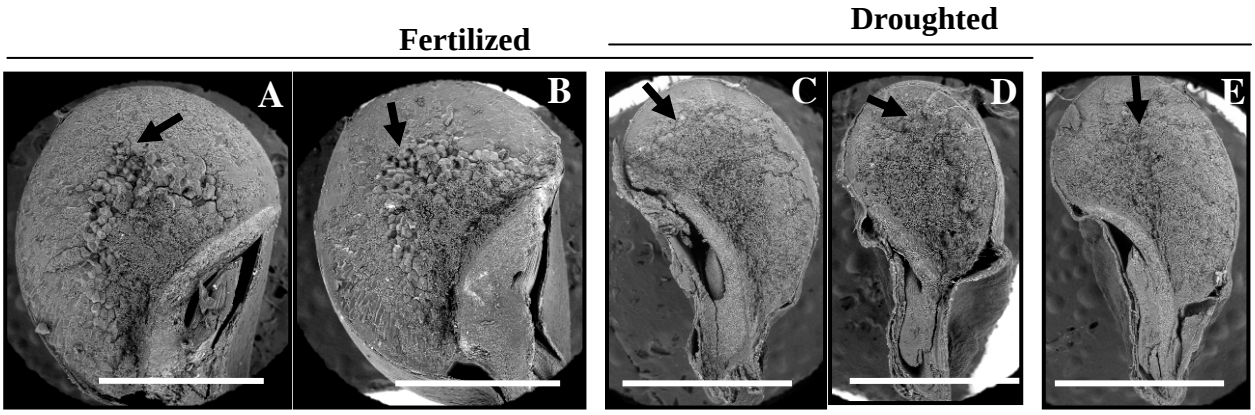

Figure 5. Images of grain bisections showing differential endosperm growth between combinations of fertilization and drought treatments. Arrows mark approximate transition between hard and soft endosperm tissue layers. Whereas the grain from fertilized, non-droughted plants (**A** and **B**) is relatively large and have well developed hard endosperm tissue layers, the grain produced by droughted plants (**C**, **D**, and **E**) is smaller and has proportionally less hard endosperm than the fertilized, non-droughted plants (**A** and **B**). Additionally, the endosperm of grain harvested from non-fertilized, droughted plants (**E**) is somewhat larger and has a somewhat greater proportion of hard endosperm than grain from the fertilized, droughted plants (**C** and **D**). Grain from transgenic plants with *btrxh* (**B** and **D**) do not have distinct visual characteristics that set them apart from grain yielded from non-transgenic plants (**A**, **C**, and **E**). Images taken at 40x. Scale bars represent 2mm.

There were no discernable differences in structures imaged from grain gathered from plants with the transgene, *btrxh* and grain from plants without a transgene at any magnification level captured by the scanning electron microscope.

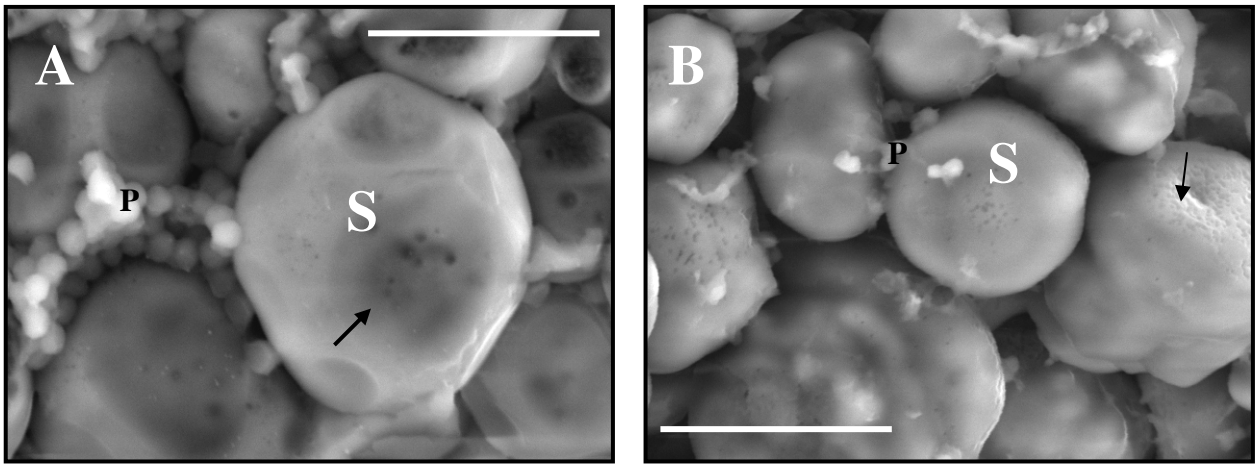

Figure 6. **Close-up images of the architecture of the sorghum endosperm.** Hard endosperm of the non-transgenic, unfertilized, droughted experimental group (**A**) and soft endosperm of the non-transgenic, fertilized, non-droughted experimental group (**B**). In both images, '*S*' denotes a starch granule, '*P*' denotes a protein body, and arrows mark an example of starch body pores. Images are representative of structures found in all experimental groups. 8000x magnification. Scale bars represent 10μm.

Despite differences in proportion of hard and soft endosperm, when images of hard endosperm were compared together and images of soft endosperm were compared together, images of available grain show similar structure regardless of environmental treatment or genetic engineering with the transgene, *btrxh* (Fig. 7). In the hard endosperm, starch granules show a tight-packed arrangement surrounded by a geometric matrix of protein bodies (Fig. 6,A). At times, the compaction of the starch granules can give the endosperm a continuous, glassy appearance. The soft endosperm, on the other hand, is composed of loosely aggregated, rounded starch bodies with intermittent blotches of protein adhered to starch body surfaces (Fig. 6,B). Loose aggregation of starch granules in the soft endosperm can provide even, spongy layers of tissue or, in some areas, less dense tissue regions with irregular, empty hollows. Tissues of every type and from every environmental and genetic experimental group showed substantial pittation on the surface of starch bodies. Although every sample had regions of similar structure, observation at many different locations in the sample revealed a high degree of heterogeneity in tissue appearance. Images taken represent the variety of tissue appearances available within the endosperm and do not reflect the relative abundance of each architecture type.

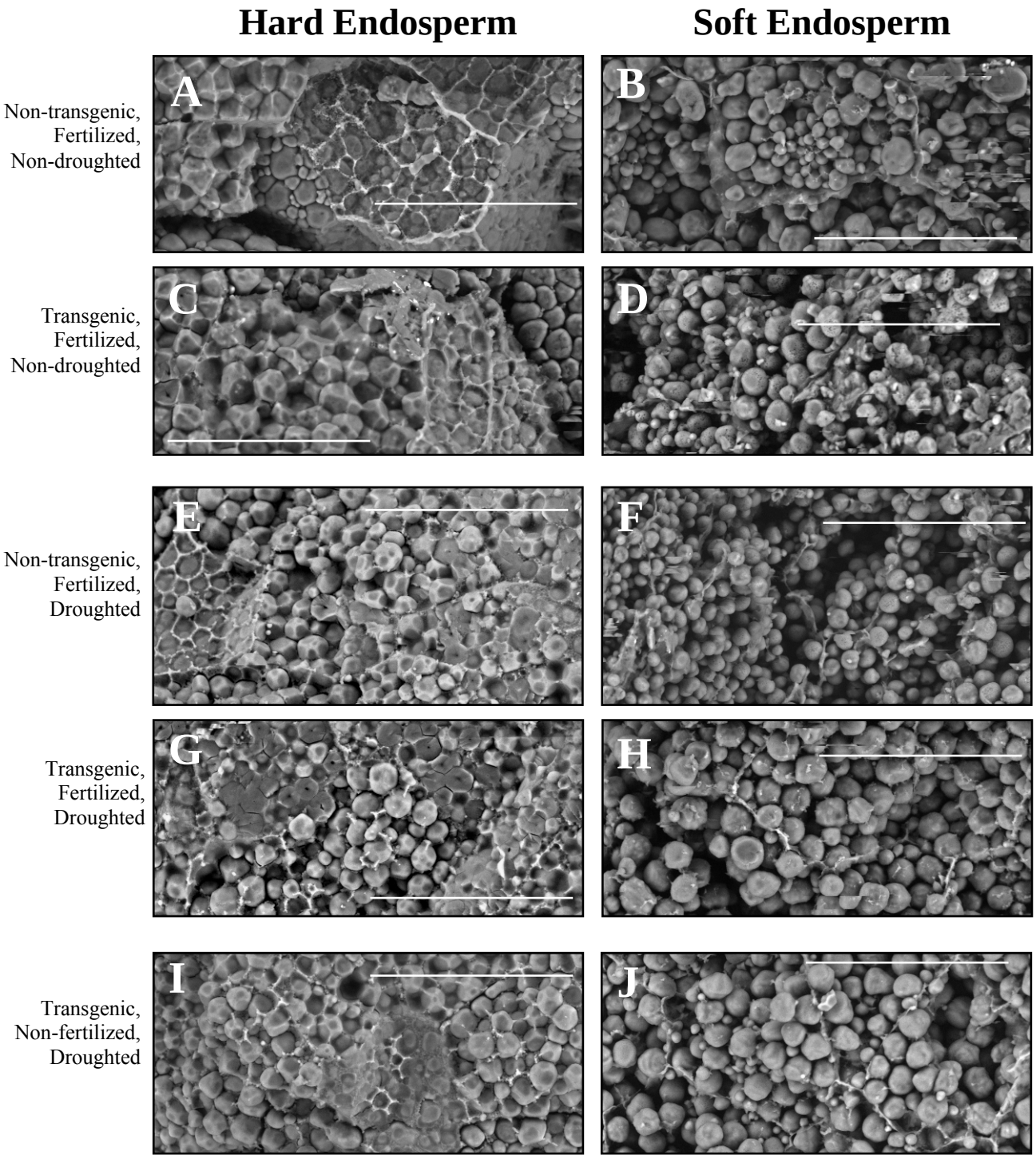

Figure 7. Electron micrographs comparing hard (left) and soft (right) endosperm tissue layers between grain of different treatment groups. The characteristics of either tissue layer remain constant throughout different environmental and genetic experimental groups, although whole tissue layers have high heterogeneity. Images generated from samples of each experimental group match previously documented descriptions of the general characteristics of sorghum endosperm layers. Images collected at 3000x magnification. Scale bars represent 100μm.

DISCUSSION

As a global food crop, grain sorghum, *S. bicolor* provides an interesting opportunity for the development of strategies for agricultural improvement by genetic engineering. With natural resistance to pests and high drought tolerance but poor nutritive value, the Lemaux and Buchanan Laboratories of the University of California, Berkeley employed transgene-based genetic engineering with a barley thioredoxin gene construct to remedy grain sorghum's low human and animal digestibility. Investigation of the sorghum endosperm has suggested that redox-sensitive, intra- and intermolecular bonds are responsible for grain sorghum's low digestibility. Meanwhile, field growth studies have shown that fertilization and irrigation strategies can affect sorghum grain chemistry and endosperm structure, also affecting grain digestibility.

Because the *btrxh* construct used by the Lemaux laboratory to engineer the high digestibility cultivar of sorghum is designed to express a barley thioredoxin within the sorghum grain endosperm, leaving other regions of the plant unaffected. I expected that the growth and yield of plants would not be significantly affected by transformation with *btrxh*. Knowing that extremes in environment have been shown to affect many qualities of growing sorghum and its grain, I expected that growth environment would have significant affects on the growth, yield and endosperm architecture of both transformed and non-transformed sorghum. At the same time, because the high digestibility modification changes the chemical environment of the grain, I expected that the sorghum grain endosperm architecture would be noticeably affected by the addition of *btrxh*, though this affect would appear minimal next to the changes that modified growth environment could provide.

Varying Environment

Overview

Given the biological need for nutrients and water for the proper growth and development of a mature sorghum plant, it is not surprising that manipulation of the availability of nutrients had significant affects on the growth rate and form of the experimental groups. The imposition of a late-drought alone caused also drastic, predictable visual changes to maturing sorghum plants. The ability of non-fertilized plants to remain green under low water availability, however, may point to an undescribed 'stay green' phenotype activated by nutrient stress (Thomas and Smart 1993). Although the literature cites many different metrics as critical physiological indicators of

yield in sorghum, reproductive activity and grain maturation in this study appeared to be closely linked to the length of time plants remained green after anthesis, the activation of the flower's male reproductive organs (Zhao et al. 2005, Rosenow et al. 1983, Craufurd and Peacock 1993, Kassahun et al. 2010). But neither nutrient nor water regime had a detectable visual affect on the endosperm structures in the grain harvested from the experimental groups as imaged by SEM, suggesting that characteristic endosperm morphological structures form within a broad range of environmental conditions.

Effects of Nutrient Stress on Growth

The high vigor and rapid growth of the fertilized treatment is a result of the availability of nitrogen for the growing plants. Nitrogen compounds are intercepted by roots and converted into photosynthetic machinery that allows the quicker accumulation of biomass, supporting a greater number of leaves and leading to increased production and deposition of sugars (Bayu et al. 2006, Zhao et al. 2005, Muchow and Sinclair 1994). When compared to the narrow, light green leaves of the non-fertilized experimental treatment, the broad, deep green, numerous leaves of the fertilized experimental treatment suggest that the fertilized plants contain a greater concentration of chlorophyll per unit of leaf area while a greater total leaf area (the product of number of leaves and leaf blade width) would allow the fertilized plants to photosynthesize at a greater rate then the non-fertilized plants (Fig. 2) (Zhao et al. 2005, Muchow and Sinclair 1994). Plants receiving daily fertilization benefited from this additional nitrogen, growing statistically faster and to greater heights than their non-fertilized counterparts (Table 2). These data match results comparing the biomass accumulation and photosynthetic activity of diverse sorghum cultivars grown under different nitrogen availabilities (Zhao et al. 2005, Muchow and Sinclair 1994). Non-fertilized plants, on the other hand, unable to match the photosynthetic capacity allowed by abundant nitrogen fertilizer, were not able to grow as quickly or as heartily as the fertilized experimental group.

Effects of Water Stress on Growth

The eventual browning and drying of plants under drought treatment is a result of the lack of water during plant maturation. Drought has been shown to affect the photosynthetic electron transport system, allowing oxidation from exposure to light to disrupt the green-colored biochemical machinery of the plant, causing part of the observed browning (Fig. 3) (Masojidek et al. 1991). At the same time, continual stomatal conductance in the absence of newly

intercepted water led to a decrease in tugor pressure causing a wilted or papery appearance, triggering the plant to further mobilize photosynthetic components and export them from the leaf, known as senescence (Thomas and Smart 1993). Plants that were not droughted did not undergo any browning or wilting because they were not put under the same stress.

Nutrient and Water Stress Interacted in a 'Stay Green' Phenotype

The ability of the unfertilized plants to remain green under drought for several weeks longer than their fertilized counterparts may be the result of a 'stay green' interaction undescribed in the current body of literature. Although reduced leaf area and lower photosynthetic capacity allows for a lower rate of potential transpiration from the unfertilized plants, it is unlikely that the reduced rate of transpiration would allow several additional weeks of photosynthetic activity since the soil available in the 8" pots dried quickly and equally among experimental groups subjected to drought (Zhao et al. 2005). Because plants were elevated off the greenhouse floor, there was no chance for accidental seepage of water between experimental groups.

Although the droughted, unfertilized group was under post-anthesis drought for several weeks, the plants retained green, vigorous leaves, matching the description of a perennial 'stay green' phenotype (Thomas and Smart 1993, Rosenow et al. 1983, van Oosterom et al. 2010a, van Oosterom et al. 2010b, Borrell et al. 2001, Borrell et al. 2000a, Borrell et al. 2000b, Mahalakshmi and Bidinger 2002). Germ lines with 'stay green' activity retain photosynthetically active leaf area while germ lines without 'stay green' undergo rapid leaf senescence during post-anthesis water stress (Thomas and Smart 1993, Borrell et al. 2001). Relatively few cultivars have been identified as potential sources of 'stay green' phenotype for breeding (Mahalakshmi and Bidinger 2002). Among the common parent and hybrid cultivars utilized by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), the 296B cultivar is not recognized as source of 'stay green' and has been shown to fail to mature under post-anthesis drought in fertilized field studies when grown alongside successfully yielding 'stay green' cultivars (Mahalakshmi and Bidinger 2002, Rao et al. 1999). Genetic analysis of the allelic basis for 'stay green' have revealed multiple, dissimilar multi-gene strategies for surviving post-anthesis drought (Thomas and Smart 1993, Kassahun et al. 2010, Borrell et al. 2001, Borrell et al. 2000a, Borrell et al. 2000b).

Increased leaf thickness and spongy mesophyll leaf development are among the associated traits linked to one form of 'stay green' phenotype (Borrell et al. 2001). Borrell et al. (2001) related leaf thickness to an increased demand for nitrogen in the leaves and suggested that relatively high nitrogen demand in leaves prevents the mobilization and export of nitrogen-rich compounds from the leaf, ultimately preventing leaf senescence. Zhao et al. (2005) have shown that although nitrogen availability is linearly related to chlorophyll density in sorghum, photosynthesis rates remain relatively high in plants grown under severe nitrogen stress, suggesting that photosynthetic area in nitrogen stressed plants must operate with high nitrogen use efficiency. Therefore, the spongy and relatively sparse leaves of the unfertilized group match the description of highly nitrogen efficient, highly nitrogen demanding leaves of a potential 'stay green' allele.

Given that all droughted plants, regardless of experimental group, were subjected to the same water stress, but only the nutrient stressed plants were able to respond with 'stay green' behavior there may be a synergistic relationship between nutrient and drought stresses. Stress has been shown to regulate genetic pathways in maize and other relatives of sorghum (Wang et al. 2008). Perhaps the 'stay green' activity of the unfertilized, droughted experimental group represents the insensitivity of nutrient stressed sorghum to nutrient mobilization cues. Acting as a sink for a largely unavailable resource, establishing the photosynthetic machinery of the unfertilized plants' leaves requires a high leaf demand for nitrogen (Zhao et al. 2005, van Oosterom et al. 2010a). As a result, leaves of the unfertilized plants in this study may be predisposed to resist drought-related nitrogen export signals. Although the molecular explanation for this phenomenon is outside of the scope of this study, it provides an interesting opportunity to study an unlikely resilience, created by an interaction between environment and genetics

Links Between Reproductive Activity and Retention of Green Area

Although different studies have used various physiological factors, fluxes, and rates to predict sorghum yield, maintaining green leaf area during the grain-filling period was closely linked to reproductive activity across this project's experimental groups (Craufurd and Peacock 1993, van Oosterom et al. 2010b, Borrell et al. 2001, Beheshti and Fard 2010, Buffo et al. 1998, Gerik et al. 2004, Eppendorfer et al. 1985). During grain-fill, the plant mobilizes nitrogen-rich compounds and sugars from stalk tissues to provide material for the development of growing granules (van Oosterom et al. 2010b, Borrell et al. 2000b, Beheshti and Fard 2010). As the

developing panicle draws material from storage material in the stalk, new photosynthate is required from leaves to support continued plant development and grain maturation (Borrell et al. 2001).

With a highly productive photosynthetic base providing material during grain-fill, the fertilized experimental group produced large reproductive structures and yielded grain, over one magnitude greater in total mass than yield figures from droughted samples. Using the planting density reported in field studies, the potential yield from the fertilized, non-droughted experimental group is over 7 Mg ha^{-1}, an order of magnitude higher than reports from field studies in a variety of sorghum cultivars, but close to figures seen in field experiments involving generous soil amendment with fertilizers (Kaye et al. 2007, Bayu et al. 2006, Rao et al. 1999). Deposition of material during grain fill in the fertilized experimental group produced grain with an order of magnitude greater 1000-grain mass than reported in field studies for 296B (Rao et al. 1999). These figures together suggest that the daily fertilizer received by fertilized experimental groups provided a greater photosynthetic base for reproductive development than normally achievable in field situations except when rich fertilizers are applied.

On the other hand, unfertilized plants with fewer leaves and a smaller photosynthetic base were unable to match the magnitude of reproductive activity that characterized the fertilized plants and therefore produced reproductive structures that carried much less grain than the fertilized experimental group (Fig. 3).

Under post-anthesis drought conditions, the fertilized plants were unable to maintain green, photosynthetically active leaves during grain fill. Because of the collapse of photosynthetically productive tissue, 4/10 harvested, fertilized, droughted plants failed to yield any grain. The failed harvest of the fertilized, droughted samples matches results from field studies where sorghum line 296B yielded no grain after a post-anthesis drought (Mahalakshmi and Bidinger 2002, Rao et al. 1999).() Wrinkled or green granules harvested from the fertilized, droughted plants were evidence that material drawn from the parent plant was insufficient to allow complete grain maturation. Using the planting density reported in field studies, the potential yield from the fertilized, droughted experimental group is nearly 0.6 Mg ha^{-1}, which is an order of magnitude less than the production achieved by common field studies, but greater than the 0 figure reported for 296B (Kaye et al. 2007, Bayu et al. 2006, Rao et al. 1999). Although the mass of grain yielded in the fertilized, droughted experimental group is non-zero,

the wrinkled, green grain may not be of usable quality, reducing the effective yield to almost nothing.

On the other hand, because the droughted, unfertilized plants had several extra weeks of photosynthetic activity before the eventual collapse of photosynthetic activity, the reproductive structures of the droughted, unfertilized remained green and developed for longer than the droughted, fertilized plants. As a result of additional green-tissue support, harvested grain appeared rounded and more similar to the grain harvested from non-droughted plants. Using the planting density reported in field studies, the potential yield from the unfertilized, droughted experimental group is around .6 Mg ha^{-1}, the same as expected from the droughted, fertilized experimental group (Kaye et al. 2007, Bayu et al. 2006, Rao et al. 1999).

While the calculated yield figures provide interesting information about the potential interaction of environmental stresses and the reproductive potential of 296B, the lack of biological replicates prevent the establishment of statistically sound conclusions that link fertilization and irrigation regime to grain yield.

Characteristic Endosperm Structures form under Diverse Conditions

Whereas Kaye et al. (2007) have reported that grain from well-fertilized treatments is composed of tightly packed hard endosperm and grain from low-fertilized treatments is composed of loosely arranged soft endosperm, grain from every experimental group in this study possessed areas of both hard and soft endosperm (see Fig. 6 for an overview of the difference between hard and soft endosperm, Fig. 5 and Fig. 7 for comparison of experimental grain). Other studies which did not consider environment as a variable agree that the endosperm of sorghum grain is made up of both a dense, compact hard tissue layer and a loosely aggregated soft tissue layer, although precise characteristics vary between cultivars (Hoseney et al. 1974, Wong et al. 2009, Hoang 2008, Chandrashekar and Mazhar 1999). Because the formation of the recognized structures of the two endosperm tissue layers has been linked to physiological reactions to drying during grain maturation, the unanimous presence of both layers across all experimental groups suggests that changes to the nutrient and water regimes in this experiment were insufficient to disrupt the underlying physiological processes of grain maturation in sorghum line 296B (Hoseney et al. 1974).

Disparate ratios of hard to soft endosperm between experimental groups reflect plants' ability to mobilize photosynthate and stored materials from the leaves and stalk into the

developing grain. Plants that were able to sustain reproductive activity during grain maturation yielded more hard endosperm than plants that suffered under drought stress.

Improved Genetics

Genetic engineering with the barley thioredoxin-containing *btrxh* transgene construct did not significantly affect the growth or development of the maturing sorghum plants, or the grain endosperm structures yielded from mature plants. Comparative visual assessments (Fig. 1) and quantitative comparisons of height (Fig.2) could not reveal differences in the overall growth patterns of transgenic plants when compared to an untransformed null segregant. A multiple variant repeated measures ANOVA further suggests that there is no meaningful statistical difference between the genetically engineered plants and untransformed plants (Table 2).

The *btrxh* construct was designed with an endosperm-specific promoter so that only the grain endosperm would express the barley thioredoxin gene. The *b-hordein* promoter's endosperm-specific pattern of expression has not affected the overall growth pattern of the maturing plants, supporting the results of previous literature (Choi et al. 2009, Furtado et al. 2009).

Whereas, other sorghum cultivars with increased digestibility have shown unique endosperm structures, images gathered by scanning electron microscopy were unable to differentiate the increased digestibility line from its non-transgenic null segregant (Oria et al. 2000). Although Hoang (2008) had suggested that the increased digestibility trait provided by transformation with *btrxh* increased the occurrence of pores in the soft endosperm, I was unable to replicate these observations. At high magnification, pores and protein body-related pittation were equally visible in the hard and soft endosperm tissues of all experimental and genetic groups. Because the major protein participants in disulfide-crosslinking that contribute to sorghum's poor digestibility may be localized at the protein body periphery, potential changes in the structure created by the expression of *btrxh* within the endosperm may be limited to the protein bodies that occur throughout the endosperm (see Fig. 6 for an example of protein bodies) (Duodu et al. 2003, Oria et al. 2000).

Study Limitations

This study provided a specific look at interactions between extreme fertilization and irrigation regiments for two closely-related genotypes. Although many of the limitations to this

study are related to the specificity of the material examined, problems with plant harvest, genotyping, and grain analysis limit the breadth of strong conclusions that can be made.

By design, this study aimed to create extremes in environmental conditions for plant growth. Although the results from growth environment established by this study do not directly reflect any real field condition, many of the conclusions relate closely to known information about field-grown sorghum. The plants of this study received daily watering and daily fertilizer (depending on the experimental group). The total invested care for these plants cost more than $1 per plant per month. According to known field densities of sorghum, the same investment into field-grown sorghum would require over 150 thousand dollars per hectare per month and more than 5.85 trillion dollars per month to support US sorghum production (US FAS and WAOB 2009, Rao et al. 1999). Because of this investment, fertilized and non-droughted plants from this study are likely to exhibit growth and development that is uncharacteristically vigorous when compared to field studies. At the same time, small planting pots prevented the droughted or non-fertilized plants from retaining moisture or nutrients. Whereas natural soil can have complicated moisture and mineral patterns and availabilities that can mitigate suboptimal inputs during the growing season, droughted and non-fertilized plants were forced into suboptimal conditions. Droughted and non-fertilized plants from this study are likely to exhibit growth and development that is uncharacteristically poor when compared to field studies. Comparing the performance of the droughted and non-fertilized plants to the fertilized and non-droughted plants, therefore, reveals disparities that are greater in magnitude than those likely found in field studies, where soil resilience in arable land may mitigate deficiencies or abundances in water or fertilizer.

Plants grown in this study were both products of the genetic engineering process. To attempt to isolate the effect of the transgene *btrxh*, a transformed line was grown alongside a null-segregant. Plants grown in this project represent the fourth generation of plants generated from a single biolistic bombardment event. After going through tissue culture and regeneration into a plantlet (see Cho et al. (1998) for a description) subsequent generations of sorghum may exhibit somaclonal variation from the 296B line (Brown and Thorpe 1995). Although both lines used in the study would possess equal genetic variation, these plants may possess patterns of expression that are not found in 296B or any other known line of sorghum. So although the null-segregant isolates the effect of the *btrxh* construct encoding for barley thioredoxin, additional

experiments to a wider variety of lines would be necessary to compare the transformed plants to currently used, untransformed or the progeny of other transformation events.

Even though all the plants from each experimental group reached reproductive maturity during the study period, only one plant from the non-droughted experimental group reached a harvestable level of grain development by 181 DAS. Common cultivars take less than 150 DAS to yield mature grain (Craufurd and Peacock 1993). Although 296B has been known to deviate from typical sorghum phenology when grown under stress, plants from all experimental groups underwent anthesis according to expected phenology (Rao et al. 1999). Mite infestation or greenhouse lamp outages may have slowed grain-fill in all plants, regardless of experimental treatment. Leaves and reproductive structures were infested with mites during most of the study period. Resistant to normal insecticides, the mites were removed by rubbing at 4 times during the study period, although it became more difficult to remove the mites from older fertilized plants with many leaves.

Because most of the study period spanned across short, winter days, supplemental light was critical for the development of the growing sorghum plants. However, for several weeks throughout the study period, the plants were grown without supplemental light. By affecting circadian rhythms, changes to the light regiment could easily disrupt plant maturation. Of course, by shortening the effective photosynthesis per day, the lack of supplemental light would slow the growth of the plants and their ability to provide photosynthate for grain-fill.

Although the original grain stock was carefully labeled and genotyped by southern blotting in separate experiments (Lemaux, unpublished data), I was unable to confirm the presence of the *btrxh* construct in the transformed genetic group or deny the presence of the *btrxh* construct in the non-transformed genetic group. Although I was able to amplify other genes from the sorghum genome, *btrxh* could not be confirmed by polymerase chain reaction and gel electrophoresis. Alternatively, digestion analysis of grain would reveal the increased digestibility phenotype which is linked to the *btrxh* genotype, definitively genotyping the plants.

Biochemical analysis and SEM imagining of grain structures was limited because little grain was available at the end of the project. The slow maturation of experimental grain prevented the analysis of potential interactions of varying environment and a modified digestibility through genetic engineering.

Future Directions

The results of this experiment have provided interesting insight into the interaction of environment and genetics. Novel results and experimental limitations have illuminated new directions for future research that could better explore issues in the interaction between environment and genetics in sorghum.

The 'stay green' behavior observed in the unfertilized, droughted treatment provides a broad range of future related research. If this phenomenon proves to be a repeatable, agriculturally valuable method for the 296B germplasm to escape post-anthesis drought, then it may be a previously unreported source of 'stay green.' Because 'stay green' has been studied using quantitative trait locus (QTL) technology, there are some genetic sequences that are associated with the 'stay green' allele (Kassahun et al. 2010). Likewise, widespread analysis of potential sources of 'stay green' behavior have examined diverse plant physiological characteristics (Thomas and Smart 1993, Rosenow et al. 1983, van Oosterom et al. 2010a, Borrell et al. 2001, Borrell et al. 2000a, Borrell et al. 2000b). Future studies utilizing microarrays of known QTL's and physiological examination of plant characteristics could reveal if or how this 'stay green' behavior fits into the framework of known phenotypes. If the observed 'stay green' phenotype is the result of a change in genetic signaling in response to nutrient stress, then research into the signaling pathway may reveal new transcription factors or signal receptors. The predisposition of plants from the 296B line of sorghum to water stress in response to nutrient stress could provide very interesting information about plant adaptability.

Biochemical analysis of grain, comparing grain digestibility would help reveal the critical environmental and genetic factors in providing high-digestibility grain. Unfortunately, because of slowly maturing grain and time limitations, the biochemical digestibility of grain produced by the experimental groups could not be compared in this project. Comparing the relationship between digestibility and other biochemical qualities could more closely examine the interaction between environment and improved genetics with transformation by *btrxh* and grain digestibility. Although the *btrxh* construct was designed to improve sorghum grain digestibility by affecting grain biochemistry, growth environment is also known to have dramatic affects on the grain biochemistry (Kaye et al. 2007, Eppendorfer et al. 1985). Further experimentation could make use of grain generated outside the study period by the experimental groups and would provide a

more comprehensive image of grain quality variations created by environmental and genetic variations established in this project.

Although scanning electron microscopy provided visually-pleasing, 3D images of the greater endospermic tissues and structures, transmission electron microscopy may capture more information about individual protein bodies. Transmission electron microscopy of protein bodies has been instrumental in exploring another high-digestibility cultivar or sorghum (Oria et al. 2000). And because the proteins involved with sorghum's low human and animal digestibility have been localized within the periphery of protein bodies, transmission electron microscopy's cross-sectional view could better examine the effects of transformation with *btrxh* on potential targets for interaction with the barley thioredoxin encoded by *btrxh* (Duodu et al. 2003).

Given access to the resources, increasing the scale of experimentation would answer larger questions about providing high quality sorghum grain. Comparisons between different high-digestibility cultivars, diverse parent cultivars, or sorghum cultivars transformed with diverse genetic constructs under varying environmental conditions would provide additional information about the interaction between environmental conditions and genetics in sorghum. The strong statistical significance of growth data, however, suggests that additional biological replicates may not be necessary to provide persuasive data in order to compare different experimental and genetic groups.

Producing High Quality Sorghum Grain

The production of high quality sorghum grain involves the interaction of genetic factors and environmental inputs. Even with genetic engineering, drought or nutrient deficiencies can limit sorghum growth and production. Regardless of potential genetic engineering, the fertilized, droughted plants of this study were unable to yield quality grain after experiencing a late-stage drought. Without the capacity to satisfy the basic chemical, nutritional requirements of growing plants, it will be impossible to create a high quality product. At the same time, genetic pathways and developmental strategies may regulate growth and behavior across a variety of environments, potentially mitigating or overcoming deficiencies in environmental inputs. Scanning electron micrographs of grain endosperm structures from a variety of environmental conditions showed the same architectural structures independent of drought or fertilizer during growth. And despite adverse growth conditions, plants grown without fertilizer were able to stay green for more than 3 weeks without water. Some physiological pathways and developmental cornerstones shape

plant growth and maturation despite varying and sometimes antagonistic environmental cues. Targeted genetic engineering strategies can introduce desirable traits from one crop into a specific part of the sorghum plant without causing a cascade of unexpected effects outside the target region. The *b-hordein* promoter's strong endosperm specific pattern of expression allowed a barley thioredoxin to be expressed in the sorghum endosperm without causing any significant changes to plant growth or development, even under stress.

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