Selection System for the Stay-Green Drought Tolerance Trait in Sorghum Germplasm

J. J. Burke,* C. D. Franks, G. Burow, and Z. Xin

ABSTRACT

Post-flowering drought tolerance is an essential trait for increasing the production of sorghum [*Sorghum bicolor* (L.) Moench] and other cereals in Mediterranean and semiarid tropical climates. Current methodologies for identifying the nonsenescent (stay-green) trait require the right intensity of drought stress at the right developmental stage to visually evaluate lines in the field. Field-based evaluations of drought tolerance are notoriously difficult to manage, and often require growing lines in multiple locations across several years to acquire a meaningful assessment of the stay-green trait. By means of a 30-min high-temperature challenge to leaf tissue during flowering of well-watered sorghum and a 30-min room temperature recovery, we show that stay-green lines can be readily identified. Using chlorophyll fluorescence to monitor tissue injury, we found that tissue with higher intercellular sucrose concentrations exhibited higher chlorophyll fluorescence yield following the temperature challenge. Stay-green lines evaluated in this study maintained higher dawn leaf sucrose levels than the senescent lines among the five youngest leaf positions. Evaluation of 10 known stay-green and senescent sorghum lines, previously reported in the literature, with this bioassay allowed us to separate the two classes of sorghum from well-watered flowering plants. The stay-green lines can also be separated from senescent lines under well-watered greenhouse conditions from the boot stage onward. This technology will greatly reduce the selection time needed to identify drought tolerant sorghum.

As a food, feed, and biofuel crop, sorghum is a fail-safe crop in the global agroecosystem. Worldwide, sorghum is the fifth most important grain crop (www.fao.org; verified 14 Apr. 2010). Recently, sorghum has become the number 2 crop for grain-based ethanol in the United States after maize. In this regard, sorghum is particularly advantageous, as it can be grown on marginal land without competing for more productive land for food and fiber production. Sorghum is a C4 plant and exhibits excellent resistance to high temperatures and drought, and low input levels (Doggett, 1988), an essential trait for the U.S. Southern Plains that receive too little rainfall for most other grains. Sorghum is often planted in marginal environments with little input of water or fertilizers. As such, sorghum yield is particularly influenced by various abiotic stresses that limit its yield potential. Improving sorghum's tolerance to various abiotic stresses would improve yield and yield stability in these marginal growing areas and thus increase production efficiency and ensure a more reliable and stable crop for farmers growing sorghum under these stressful environmental conditions.

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Three growth stages have been identified in sorghum that are critical in understanding drought tolerance: GS1, seedling establishment (early vegetative stage); GS2, pre-flowering (panicle differentiation to flowering); and GS3, post-flowering (grain fill to physiological maturity of grain). Two distinct types of drought stress responses have been identified and described in sorghum and are related to GS2 and GS3. Plants under stress from panicle differentiation to flowering will exhibit a pre-flowering response. This type of stress directly affects panicle size, grain number, and grain yield. Symptoms include leaf rolling, uncharacteristic leaf erectness, leaf bleaching, leaf tip and margin burn, delayed flowering, saddle effect, poor panicle exertion, panicle blasting and floret abortion, and reduced panicle size (Rosenow et al., 1996). Symptoms of post-flowering drought stress, which occurs from flowering through maturity (GS3), include premature plant death, increased susceptibility to charcoal rot of sorghum, stalk collapse and lodging, and a significant reduction in seed size, all of which result in decreased yield potential (Rosenow et al., 1996). Identification of the genetic factors underlying drought tolerance would provide a solid foundation for improving drought tolerance.

Retention of green leaf area at maturity (GLAM), known as stay-green, is used as an indicator of postanthesis drought resistance in sorghum programs in the United States and Australia (Borrell et al., 2000). The critical issue is whether maintaining green leaves under postanthesis drought increases grain yield in stay-green compared with senescent hybrids. Borrell et al. reported that under terminal water deficit, grain yield was correlated positively with GLAM ($r = 0.75^{**}$) and negatively

Abbreviations: GS1, seedling establishment (early vegetative stage); GS2, pre-flowering (panicle differentiation to flowering); GS3, post-flowering (grain fill to physiological maturity of grain); GLAM, green leaf area at maturity.

with rate of leaf senescence ($r = -0.74^{**}$) (Borrell et al., 2000). Their results indicated that sorghum hybrids possessing the stay-green trait have a significant yield advantage under postanthesis drought compared with hybrids not possessing this trait. Sorghum containing the stay-green trait retain more photosynthetically active leaves under drought than do hybrids that do not contain this trait (Borrell and Hammer, 2000). Nine hybrids varying in the B35 and KS19 sources of stay-green were grown under a fully irrigated control, post-flowering water deficit, and terminal water deficit. Genotypic differences in delayed onset and reduced rate of leaf senescence were explained by differences in specific leaf N and N uptake during grain filling. Leaf N concentration at anthesis was correlated with onset ($r = 0.751^{**}$, $n = 27$) and rate ($r = -0.783^{**}$, $n = 27$) of leaf senescence under terminal water deficit.

Alleles that contribute to the stay-green trait have been mapped to four major QTL, Stg1 to Stg4, using a population derived from BTx642 and RTx7000 (Harris et al., 2007). Physiological analysis of four RTx7000 near-isogenic lines (NILs) containing only Stg1, Stg2, Stg3, or Stg4 showed that BTx642 alleles in each of these loci could contribute to the stay-green phenotype. RTx7000 NILs containing BTx642 DNA corresponding to Stg2 retained more green leaf area at maturity under terminal drought conditions than RTx7000 or the other RTx7000 NILs. Under postanthesis water deficit, a trend for delayed onset of leaf senescence compared with RTx7000 was also exhibited by the Stg2, Stg3, and Stg4 NILs, whereas significantly lower rates of leaf senescence in relation to RTx7000 were displayed by all of the Stg NILs to varying degrees, but particularly by the Stg2 NIL. Greener leaves at anthesis relative to RTx7000, indicated by higher SPAD values, were exhibited by the Stg1 and Stg4 NILs (Harris et al., 2007).

Stay-green is an important trait for post-flowering drought tolerance in sorghum (Thomas and Howarth, 2000). However, its evaluation requires the right intensity of drought stress at the right developmental stage. Current methodologies consist exclusively of evaluating the lines visually for the stay-green trait under field conditions. Field-based evaluations of drought tolerance are notoriously difficult to manage, and often require growing lines in multiple locations across several years to acquire a meaningful assessment of the stay-green trait. There is a need for an assay that would allow the trait to be measured year-round under controlled and easily reproducible conditions. This report describes the development of an assay for the stay-green trait that meets the requirements of high throughput and reproducibility.

MATERIALS AND METHODS

Cultural Practices and Experiment Design

Five stay-green (B4R, BTx642, B1778, 1790E, and P898012) and five senescent (RTx430, Tx7078, Tx7000, BTx623, and SC1211) sorghum inbred lines previously described in the literature (Rosenow et al., 1983; Sowder et al., 1997; Harris et al., 2007; and D.T. Rosenow, personal communication, 2007) were randomly selected from our collection and used to evaluate the reproducibility of the stay-green assay in the field during the 2007 and 2008 growing seasons. Randomized complete block design was used with three replicate plots: 4.67 m long with 1.02-m row spacing. Sorghum seeds were planted at 100 seeds per plot at a depth of 3 cm using a John Deere MaxEmerge Planter modified for use in small plot research. The plants received 5 mm of water per day from underground drip lines located on 1-m centers. Soil type was an Amarillo fine sandy loam (fine-loamy, mixed, superactive, thermic Aridic Paleustalfs). Plants were evaluated during the 2007 and 2008 growing seasons in Lubbock, TX.

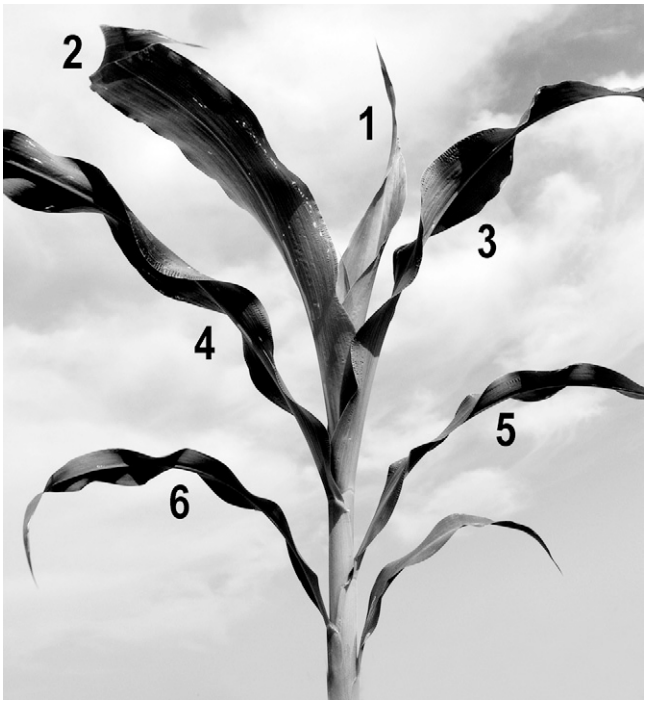

Fig. 1. Photograph of a sorghum plant illustrating the leaf numbering system used in this study. Leaf position numbers increased from the youngest to the oldest leaves.

Sucrose Analysis on HPLC

The dawn leaf sucrose concentrations were determined for BTx642, 1790E, Tx7000, and BTx623 lines during the 2007 and 2008 growing seasons. A leaf punch from each of the upper six leaf positions on the plant was obtained on three consecutive dates. Ten plants were sampled on each date, for a total sampling of 30 random plants per plot. The youngest leaf was considered Position 1, and leaf positions increased as you move basipetally (Fig. 1). Soluble sugars from 10 1-cm punches per set were extracted in 1 mL 80% ethanol at 60°C for 1 h followed by a 5-min incubation at room temperature. The extract was centrifuged at 10,000 rpm for 10 min and 400 μL supernatant was transferred into a clean Eppendorf tube and dried on a Speed Vac. Soluble sugars were redissolved in 0.2 mL deionized water overnight at 4°C. Dried samples were redissolved in 200 μL deionized water overnight at 4°C. Precipitate was removed by centrifugation at 10,000 rpm, and one-fourth volume of acetonitrile was added, vortexed, and centrifuged for 5 min at 10,000 rpm. Supernatant (100 μL) was transferred to HPLC vials for analysis.

The amount of sucrose was analyzed using a VP Series HPLC system fitted with a SIL-10AD auto-injector and an evaporative light scattering detector-LT (Shimadzu, Columbia, MD). Samples equivalent to 50 to 200 μg were separated on a 4.6 by 250 mm YMC Polyamine II column (Waters, Milford,

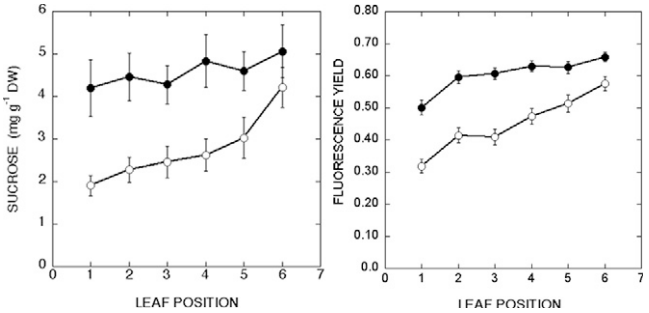

Fig. 2. Graphs of (A) leaf sucrose concentrations from Leaf Positions 1 to 6 of senescent (open circles) and stay-green (closed circle) lines; and (B) yield of quantum efficiency values from Leaf Positions 1 to 6 of senescent (open circles) and stay-green (closed circle) lines following a 30-min 40°C challenge and a 30-min 25°C recovery. The data for the senescent lines Tx7000 and BTx623 were combined, as was the data for the stay-green lines BTx642 and 1790E. Sixty leaf samples were evaluated for each yield of quantum efficiency data point. Error bars represent standard error values.

MA) with a mobile phase of 75% acetonitrile in water (vol/vol) at a flow rate of 1.5 mL/min. Sucrose was identified by its retention time in comparison with the corresponding sucrose standard. Quantification of sucrose concentration was calculated using peak area.

Stay-Green Bioassay

At sunrise during the 2007 and 2008 seasons, a leaf punch was harvested from Leaf Positions 1 to 6 (Fig. 1) from BTx642, 1790E, Tx7000, and BTx623 using a number 6 cork borer and rubber stopper. This was repeated on 30 random plants per line. Leaf samples from each of the six leaf positions were simultaneously harvested either for sucrose determination or for yield of quantum efficiency determination. The punches were transferred to a well in a Costar 3524 24-well cell culture cluster (Corning Inc., Corning, NY) that had been half filled with water. The lid was returned to the cell culture plate immediately following the addition of more leaf punches. This process was repeated until samples from all treatments had been harvested.

Upon returning to the lab, the leaf punches were placed on moistened Model 583 Gel Dryer Filter Paper (Bio-Rad Laboratories, Hercules, CA) in a Pyrex baking dish. The leaf punches and filter paper were covered with CO_2 permeable Glad Cling Wrap (The Glad Products Company, Oakland, CA) and pressed flat with a speedball roller for Microseal film (MJ Research, Inc., Waltham, MA) to remove air bubbles and ensure good contact between the tissue and filter paper. The yield of quantum efficiency (Fv/Fm) was determined at the start of the experiment using an Opti-Science OS1-FL Modulated Fluorometer. Samples were then placed in the dark in a VWR Model 2005 incubator (Sheldon Manufacturing, Inc., Cornelius, OR) set to 40°C. The samples were challenged for 30 min in the 40°C incubator. Following the temperature challenge, the Pyrex baking dish was removed from the incubator, placed on the laboratory bench (25°C) for 30 min, and then the yield of quantum efficiency was determined a second time. The data for the yield of quantum efficiency following the heat treatment for BTx642 and 1790E were combined as stay-green lines, and the Tx7000 and BTx623 data was combined as senescent lines to emphasize the characteristic responses of the stay-green and senescent lines evaluated.

Five known stay-green sorghum lines (B4RB4R, BTx642, B1778, 1790E, and P898012) and five senescent lines (RTx430, Tx7078, Tx7000, BTx623, and SC1211) were evaluated in 2008 using the stay-green bioassay on Leaf Position 1. Leaf Position 1 was selected because it showed the maximum difference in cellular sucrose and in the yield of quantum efficiency in the preliminary experiments. Seven plants were sampled per line. The yield of quantum efficiency was determined following a 30-min 40°C treatment and 30-min 25°C recovery.

Greenhouse Evaluation of Stay-Green

BTx642, Tx7000, and BTx623 seeds were planted into 18.9-L pots containing Sunshine 3-Mix soil (Sun Gro Horticulture Canada Ltd, Bellevue, WA). Three seeds per line were planted into every pot. Five replicate pots were prepared for evaluation. Following germination and emergence, seedlings were thinned to one plant per line per pot. The pots were well watered using an automated drip irrigation system. Nutrients were maintained by daily application with Peters Excel fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH) through the automated watering system. When the plants reached boot stage, three leaf punches were harvested from Leaf Position 1 (Fig. 1) using a number 6 cork borer and rubber stopper from each of the three lines in each pot. The punches were transferred to a well in a Costar 3524 24-well cell culture cluster (Corning Inc., Corning, NY) that had been half filled with water. The lid was returned to the cell culture plate immediately following the addition of the leaf punches. Upon returning to the lab, the punches were analyzed using the stay-green bioassay described above.

Significant differences between stay-green and senescent lines were determined using a least square mean differences student's *t* test using JMP Version 5.0 statistical discovery software (SAS Institute, Cary, NC).

RESULTS

Sucrose levels in leaves harvested at dawn were determined for the upper six leaf positions on the plant. The data for two stay-green lines (BTx642 and 1790E) and the data for two senescent lines (Tx7000 and BTx623) were combined for each leaf position to emphasize the characteristic responses of the stay-green and senescent lines evaluated. Figure 2A shows that sucrose levels during 2007 were lowest in Leaf Position 1 of the senescent lines (open circle) with values of 1.6 g kg^{-1} DW. Sucrose levels increased with increasing leaf maturity in the senescent lines, reaching a maximum level of 3.2 g kg^{-1} DW in Leaf Position 6. Sucrose levels in the leaves of the stay-green lines (closed circle) were between 3.2 and 3.7 g kg^{-1} DW across all leaf positions. The results of the student's *t* test showed significant differences ($P < 0.05$) between the stay-green and the senescent lines. The stay-green lines had a least square mean of 4.568, and the senescent lines had a least square mean of 2.842.

The yield of quantum efficiency data for two stay-green lines (BTx642 and 1790E) and for two senescent lines (Tx7000 and BTx623), following a 30-min 40°C challenge and a 30-min 25°C recovery period, were combined for each of the first six leaf positions on the plant to emphasize the characteristic responses of the stay-green and senescent lines evaluated. The yield of quantum efficiency was lowest in Leaf Position 1 of the senescent lines (Fig. 2B, open circle) with values of 0.32.

The yield of quantum efficiency increased with increasing leaf maturity in the senescent lines, reaching a maximum level of 0.58 in Leaf Position 6. The yield of quantum efficiency was 0.50 in Leaf Position 1 of the stay-green lines (Fig. 2B, closed circle), 0.60 in Leaf Position 2, and gradually increased to 0.66 by Leaf Position 6. The results of the student's *t* test showed significant differences ($P < 0.05$) between the stay-green and senescent lines. The stay-green lines had a least square mean of 0.601, and the senescent lines had a least square mean of 0.440.

The relationship between yield of quantum efficiency values obtained during the 2007 and 2008 field study for two stay-green lines (BTx642 [closed circle] and 1790E [closed square]) and for two senescent lines (Tx7000 [open circle] and BTx623 [open square]) compared with sucrose values obtained from these lines is shown in Fig. 3. Figure 3A shows the combined relationship across all leaf positions. The senescent lines (open symbols) had lower yield of quantum efficiency and sucrose levels compared with the stay-green lines (closed symbols). An R^2 value of 0.54 was observed across all leaf positions. Figure 3B shows the relationship between yield of quantum efficiency and leaf sucrose concentrations for Leaf Position 1. An R^2 value of 0.67 was observed when only Leaf Position 1 samples were evaluated.

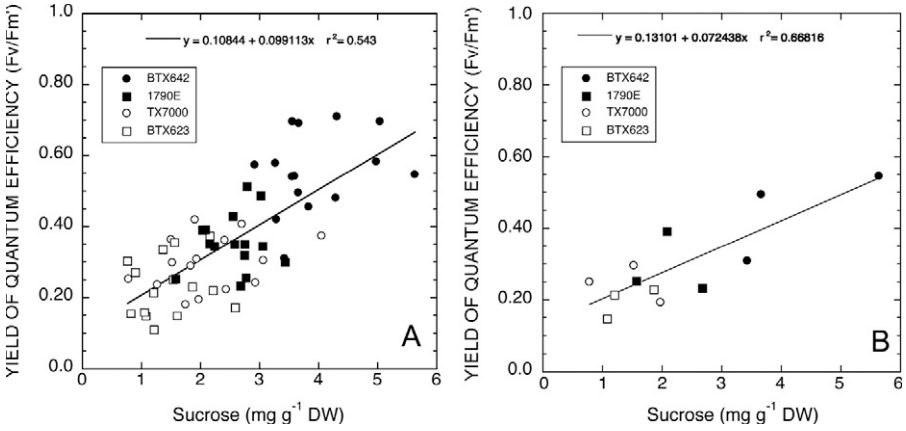

Fig. 3. Graphs of (A) the yield of quantum efficiency values from Leaf Positions 1 to 6 of senescent (open symbols) and stay-green (closed symbols) lines following a 30-min 40°C challenge and a 30-min 25°C recovery vs. sucrose concentrations; and (B) the yield of quantum efficiency values vs. sucrose concentrations for only Leaf Position 1. The senescent lines evaluated were Tx7000 (open circle) and BTx623 (open square), and the stay-green lines were BTx642 (closed circle) and 1790E (closed square). Three plants were evaluated for each leaf position.

Figure 4 shows the yield of quantum efficiency values of five stay-green and five senescent sorghum lines following a 30-min 40°C challenge and a 30-min 25°C recovery period. A range of yield values was observed from a high of 0.63 for B4R to a low of 0.13 for SC1211. In general, stay-green lines (solid bars) exhibited higher fluorescence yield values than the senescent sorghum lines (open bars). Significant differences ($P < 0.05$) were observed between the B4R, B1778, and BTx642 stay-green lines and the Tx7000, BTx623, and SC1211 senescent lines. The mean yield of quantum efficiency value for the five stay-green lines was 0.48 ± 0.030, and the mean value for the senescent lines was 0.22 ± 0.022. Statistical analysis showed no interaction between line and year, and the block design had no affect.

The yield of quantum efficiency for the greenhouse-grown stay-green line (BTx642) and for two senescent lines (Tx7000, BTx623), following a 30-min 40°C challenge and a 30-min 25°C recovery period, showed a similar trend as in Fig. 4. There was clear separation between the BTx642 and the two senescent lines (Tx7000 and BTx623) at the boot stage. However, no difference was observed between Tx7000 and BTx623 based on the least square means student's *t* test (data not shown).

DISCUSSION

This study describes a novel procedure for identification of stay-green (post-flowering drought tolerant) and senescent lines from well-watered pre-flowering sorghum. We hypothesize that the stay-green trait in sorghum occurs in plants that are inducing low levels of natural drought protection systems (osmoregulation, changes in leaf morphology, etc.) under nonstress conditions. The procedure described in this study is a modification of a stress bioassay used to dissect responses of field and greenhouse-grown cotton (*Gossypium hirsutum* L.) source leaves to water-deficit stresses (Burke, 2007). Burke reported that sucrose levels were lower in nonstressed cotton at sunrise compared to water-deficit–stressed cotton, potentially predisposing the nonstressed tissue to succumb more rapidly when subjected to a prolonged elevated respiratory demand in the dark. In the present study, the modification of the challenge to a 30-min 40°C treatment followed by a 30-min 25°C recovery period before measurement was essential for the successful evaluation of the stay-green trait. Evaluation of the leaf samples immediately following the heat challenge resulted in erroneous fluorescence yield values that were elevated at high temperatures

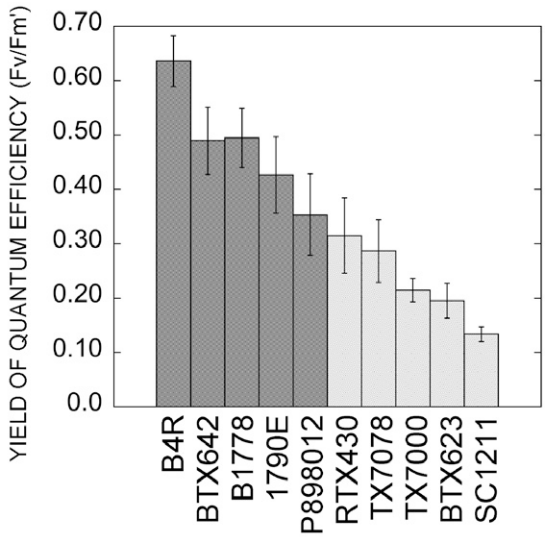

Fig. 4. Graph of the yield of quantum efficiency values for five stay-green and five senescent sorghum lines following a 30-min 40°C challenge and a 30-min 25°C recovery. Sorghum lines evaluated included five known stay-green lines (B4R, BTx642, B1778, 1790E, and P898012) and five senescent lines (RTx430, Tx7078, Tx7000, BTx623, and SC1211). Seven plants were evaluated for each line. Error bars represent standard error values.

and declined as the Pyrex dish cooled to room temperature during the measurements. Although the metabolic cause of this observation is not known, it may be related to high temperature uncoupling of photosynthetic electron transport and re-coupling as the temperature declines following the treatment.

The content of nonstructural carbohydrates in branches was shown to increase twofold more for stay-green than for senescent plants between the time of labeling and 14 d after black layer (Vietor et al., 1990). The concentration and content of nonstructural carbohydrates in main stems of senescent plants declined 40% or more during the same period. Leaf CO_2 exchange and sucrose synthesis rates, carbohydrate concentrations, and ^{14}C-assimilate partitioning in field-grown plants that were exposed to $^{14}CO_2$ for 3 h under steady state labeling conditions were evaluated (Sowder et al., 1997). They reported that leaf CO_2 exchange and sucrose synthesis rates were slower, and concentrations and radioactivity in blade starch were greater, in B35 (stay-green) than in Tx430 (senescent) under well-watered conditions. In addition, the postflowering-tolerant cultivar B35 retained 70% more ^{14}C-assimilate in the labeled blade starch than did Tx430. Leaf sucrose concentrations were not different among Tx430, B35, or their hybrid at preboot, anthesis, or grain filling.

In the present study, we examined leaf sucrose levels at dawn in senescent and stay-green lines. Our findings showed significant differences in the level of sucrose maintenance in developing leaves. The stay-green lines evaluated retained higher dawn sucrose levels and, therefore, had the potential to show differences in the stress assay when subjected to a prolonged elevated respiratory demand in the dark. We observed significant differences between stay-green and senescent lines in the yield of quantum efficiency that mimicked the differences in dawn sucrose concentrations in the leaves (Fig. 2). Evaluation of additional stay-green and senescent lines showed a range of sensitivities to the temperature challenge (Fig. 4). Significant differences were observed between the B4R, B1778, and BTx642 stay-green lines and the Tx7000, BTx623, and SC1211 senescent lines. The sensitivity of the leaf tissue to the temperature challenge was directly related to the sucrose concentration within the tissue (Fig. 3). Greenhouse studies showed that plants sampled at the boot stage exhibited yield of quantum efficiencies similar to those observed from field studies. Clear separation of the genotypes was observed when plants reached the boot stage. Measurements at earlier developmental stages either failed to separate the genotypes or exhibited a reversal in the phenotypes compared with the field and boot-stage plants. The current bioassay requires well-watered plants to function properly. The reduced soil volume for root development in greenhouse pots, and the potential for water channeling in the pots, makes it difficult to ensure nonstressed conditions for young seedlings. With many of the stay-green lines exhibiting elevated internal sucrose contents, it is possible that the stay-green lines may not sense the onset of soil drying as well as the senescent lines. Senescent lines may sense pot drying and respond using osmoregulation mechanisms sooner that the stay-green lines. Should this occur, the bioassay could show higher values in the senescent lines because of stress-induced osmoregulation. The larger the plant, the more soil volume the root system will have explored in the pot, reducing the chance of differential stresses because of uneven watering. The results of this study suggest that the stay-green bioassay provides reproducible readings from the boot stage forward.

In the field, there is a range of stay-green among the stay-green lines. The present study showed a range of Fv/Fm values among the lines evaluated (Fig. 3). It is interesting to speculate that the degree of stay-green expressed in the plant may be identified by the bioassay described in this study. Additional studies are needed to test this hypothesis.

In summary, this study showed that the stay-green trait could be identified in the well-watered sorghum lines evaluated. Using this assay, which involves a 30-min 40°C challenge and a 30-min recovery at 25°C, large numbers of samples can be evaluated in a very short time period. The ability to detect these differences in well-watered sorghum removes a major roadblock in breeding for the stay-green trait. Based on the field and greenhouse findings of this study, we recommend using this bioassay during the grain-fill period of well-watered sorghum to identify the stay-green response.

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