Genotypic Variation for Three Physiological Traits Affecting Drought Tolerance in Soybean

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ABSTRACT

Three physiological traits that may affect performance of soybean [*Glycine max* (L.) Merr.] when soil water availability is limiting are (i) water use efficiency (WUE), (ii) regulation of whole plant water use in response to soil water content, and (iii) leaf epidermal conductance (g_e) when stomata are closed. Six soybean plant introductions (PIs), eight breeding lines derived from them, and nine cultivars were compared for variability in these three traits during vegetative growth in two greenhouse studies. In the first experiment, whole plant water use, normalized both to plant size and evaporative demand (the normalized transpiration ratio, NTR), was monitored during a 10-d cycle of gradually increasing drought stress and then for an additional 2 d following rewatering. The critical soil water content at which each plant began to reduce its water use ($FTSW_C$), was determined. The WUE was estimated as the ratio of total plant dry weight to total water used. In the second experiment, g_e was determined for these same 23 genotypes by measuring leaf water vapor exchange after a 36-h dark adaptation. Substantial variation was found among genotypes for WUE, $FTSW_C$, g_e, and also the extent to which NTR recovered on rewatering. Generally, adapted cultivars had greater WUE and lower g_e than did PIs. However, PI 471938 and its progeny N98-7264 were clear exceptions to this trend. An unexpected finding was that WUE was significantly negatively correlated with g_e across genotypes.

D ROUGHT is the leading cause of soybean yield loss in the southeastern USA (Palmer et al., 1996), and so increasing productivity under water deficit stress is an important goal of soybean breeding efforts in this region. Historically, drought tolerance in soybean has been a rather intractable breeding trait for the USA. In the first few decades of modern soybean breeding (1940s through 1970s), breeders were not able to identify any obvious sources of drought tolerance in the adapted breeding pool. Few efforts were undertaken to identify drought tolerance in exotic germplasm because most physiological measures of drought tolerance were time consuming and thus did not lend themselves well to a search for tolerance in the global germplasm collection. In the 1980s and 1990s, several slow-wilting plant introductions (PIs) were discovered (Carter et al., 1999). It quickly became obvious that the slow-wilting trait had

some relation to yield under stress, and a recent quantitative trait loci (QTL) analysis confirmed that two QTL from PI 471938 of Nepal were associated with both slow wilting and improved yield under stress (Lee et al., 2002). Several genetic sources of the slow-wilting trait are now being used in U.S. soybean breeding (Carter et al., 1999). The genetic and physiological bases for this trait are poorly understood at present, and it is unknown whether slow wilting embodies a single mechanism of drought tolerance or perhaps the integration of several.

One mechanism for improving drought tolerance involves developing soybean lines with higher water use efficiency (WUE, the quantity of crop dry matter accumulated per unit of soil water transpired). Genetic variability for WUE has been found in cultivars or lines of several crop species including peanut (*Arachis hypogaea* L.; Hubick et al., 1988; Wright et al., 1994), cowpea [*Vigna unguiculata* (L.) Walp; Ismail and Hall, 1993; Ashok et al., 1999], cotton (*Gossypium* spp.; Quisenberry and McMichael, 1991; Saranga et al., 1998), sorghum [*Sorghum bicolor* (L.) Moench; Donatelli et al., 1992], barley (*Hordeum vulgare* L.; Hubick and Farquhar, 1989), wheat (*Triticum aestivum;* Ehdaie and Waines, 1993; Van Den Boogaard et al., 1997), and soybean (Mian et al., 1996; 1998).

Another physiological trait that may affect drought tolerance is the decline in whole plant water use during a soil water deficit event. As a soil water deficit develops, plants undergo a transition between the water-replete phase where whole plant water use is not dependent on the soil water content and a second phase where water use is directly related to the availability of soil water (Sinclair and Ludlow, 1986). This transition is associated with a reduction in the average stomatal conductance (e.g., Earl, 2003) and can occur at different soil water contents in different species. For example, Sinclair and Ludlow (1986) found that black gram (*Vigna mungo* L.) reduced its whole plant transpiration at higher soil water content than did cowpea as soil water content was depleted. Intraspecific differences in this trait have been studied much less extensively than differences in WUE and only in a very few species. Ray and Sinclair (1997) found significant differences among several maize (*Zea mays* L.) hybrids in the soil water content [expressed as the fraction of transpirable soil water, (FTSW) (Sinclair et al., 1998)] at which whole plant water use began to decline.

Abbreviations: DAS, days after sowing; FTSW, fraction of transpirable soil water; $FTSW_C$, critical FTSW; g_e, minimum epidermal conductance; NTR, normalized transpiration ratio; PPFD, photosynthetic photon flux density; RSWC, relative soil water content; $RSWC_{10}$, relative soil water content when NTR = 0.1; $RSWC_C$, critical relative soil water content; TR, transpiration ratio; W_D, weight of pot + lid + dry soil; W_P, plant fresh weight; W_T, target pot weight; WUE, water use efficiency; W_W, weight of pot + lid + saturated soil.

A third physiological trait that may increase drought tolerance and prolong crop survival during severe water stress is low leaf epidermal conductance (g_e). Total leaf conductance to water vapor is the sum of the stomatal and cuticular diffusive pathways acting in parallel (van Gardingen and Grace, 1992). When stomata are open, cuticular conductance is generally a negligible fraction of total conductance (Lambers et al., 1998). However, in water stressed or dark-adapted leaves, stomata tend to be closed and the cuticular component of leaf epidermal conductance may exceed the stomatal conductance (van Gardingen and Grace, 1992; Boyer et al., 1997). Under severe water deficit when stomatal closure is maximized, g_e (the total of cuticular conductance and any residual stomatal conductance) determines the rate of water loss from leaf tissues and, therefore, the rate of progression toward a critically low (injurious) leaf water content. Species adapted to arid environments tend to have low g_e (Schreiber and Riederer, 1996; Helbsing et al., 2000; Riederer and Schreiber, 2001), and crop species or varieties with low g_e are often those that survive the longest under severe soil water deficits (Hull et al., 1978; Sinclair and Ludlow, 1986; Jovanovi et al., 1996). Intraspecific variability for minimum g_e has been identified in several species including rice (*Oryza sativa* L.) (O'Toole et al., 1979), maize (Dubé et al., 1975), oat (*Avena sativa* L.) (Bengtson et al., 1978), durum wheat (*Triticum turgidum* var. *durum* L.) (Araus et al., 1991), cotton (Quisenberry et al., 1982), sorghum (Muchow and Sinclair, 1989), and soybean (Paje et al., 1988).

The first objective of the present study was to quantify genetic variability for vegetative stage WUE among a selection of soybean cultivars, breeding lines, and plant introductions, specifically chosen for putative differences in responses to soil water deficits, on the basis of field observations and prior greenhouse research. The second objective was to compare these genotypes for their regulation of whole plant water use in response to a slowly developing soil water deficit. To ensure that the water stress developed at the same rate for all entries regardless of any differences in whole plant transpiration, computer automated null balance lysimetry was used to strictly regulate the decline in soil water content in each pot. Recovery of whole plant water use following relief of the water stress was also recorded. The final objective was to quantify variability for g_e among these lines and to determine if this trait was related to other measured traits. It was hypothesized that soybean lines with low g_e would sustain less injury during a severe drought stress and so would recover more quickly on rewatering.

MATERIALS AND METHODS

All experiments were conducted in a greenhouse between August 2001 and January 2003 at the Univ. of Georgia in Athens, GA (33.9°N, 88.3°W). Greenhouse air temperatures were maintained at 27 ± 4°C during the day and 20 ± 2°C during the night with a thermostatically controlled combination of fan–evaporative pad cooling and electric heating. Photoperiod was extended to 16 h with overhead 400-W metal halide lamps that produced a supplemental photosynthetic photon flux density (PPFD) of approximately $230\ \mu\mathrm{mol}\ \mathrm{m}^{-2}\ \mathrm{s}^{-1}$ at the tops of plants. Plant material consisted of 23 soybean genotypes chosen on the basis of the results of prior field screening (T.E. Carter, unpublished data, USDA-ARS, Raleigh, NC) and putative water use characteristics (Table 1).

Table 1. Test Set designation, maturity group, and putative drought response characteristics of the 23 soybean plant introductions, breeding lines, and cultivars used in these experiments. Putative characteristics are based on prior greenhouse and field research (unpublished data, except where reference citations listed).

Set	Genotype	Maturity group	Type of germplasm	Characteristics/comments
1	Fendou 34	IV	plant introduction	Slow wilting cultivar introduced from China; developed near Gobi desert
	H2L16	V	plant introduction	Slow wilting line from Egypt; progeny of U.S. cultivars Celest × Crawford. Also designated as N94-7784
	PI 416937	V	plant introduction	Slow wilting introduction from Japan; prolific rooting; compared previously with Young for WUE (Mian et al., 1996; Earl, 2002)
	PI 471938	V	plant introduction	Slow wilting introduction from Nepal, high yield under stress
	PI 407859-2	V	plant introduction	Slow wilting introduction from Korea
	N98-7265	V	breeding line	High yielding line; progeny of PI 471938 × Hutcheson
	N94-7589	VI	breeding line	Slow wilting line; progeny of Young × PI 416937
	Holladay	V	cultivar	Adapted to high yield environments
	Hutcheson	V	cultivar	Stable yield over environments
	Dillon	VI	cultivar	Adapted to high yield environments
	Young	VI	cultivar	Stable yield over environments; Compared previously with PI 416937 for WUE (Mian et al., 1996; Earl, 2002)
1 and 2	Boggs	VI	cultivar	Stable yield over environments; control cultivar in both Set 1 and 2
2	Tokyo	VII	plant introduction	Ancestor of U.S. soybean cultivars; good N₂ fixation
	G2120	IX	plant introduction	Introduction from Indonesia identified as having low g_e (Paje et al., 1988)
	N95-7424	VII	breeding line	Fast vegetative growth; progeny of Davis × Tokyo
	N96-6809	VII	breeding line	High yielding line; progeny of N90-7202 × N7001. N90-7202 is a slow wilting progeny of adapted breeding line N77-114 × PI416937; grandchild of PI 416937
	N96-7031	VIII	breeding line	Very slow wilting line; progeny of N7001 × N90-7241; N90-7241 is the slow wilting progeny of GASOY × PI 416937; grandchild of PI 416937
	N97-9765	VIII	breeding line	Very slow wilting line; progeny of N7001 × N91-7254; N91-7254 is the slow wilting progeny of Davis × PI 416937; grandchild of PI 416937
	Benning	VII	cultivar	Standard cultivar
	Cook	VIII	cultivar	High yield
	Haskell	VII	cultivar	Standard cultivar
	Jackson	VII	cultivar	Old U.S. cultivar; good N₂ fixation under drought
	N7001	VII	cultivar	High yielding cultivar; progeny of adapted breeding line N77-114 × PI 416937

Plant Culture—Experiment 1

For Exp. 1, soybean plants were grown using the greenhouse culture system described by Mian et al. (1996). The growth medium was a Pacolet sandy loam soil (a member of the clayey, kaolinitic, thermic family of Typic Hapludults) amended with sand to a texture of 800 g kg^{-1} sand, 120 g kg^{-1} silt, and 80 g kg^{-1} clay, and 3.2 kg of this mixture was placed into each 2.5-L pot without drainage holes (actually white plastic food containers from Berry Plastics Corp., Evansville, IN). Previous work has demonstrated that whole plant water use of soybean exhibits a normal response to FTSW in soil mixtures with sand contents similar to those used here (Sinclair et al., 1998).

Before planting, soil water holding capacity was determined. In addition to the pots that were prepared for the soybean plants, two extra pots with mesh-covered drainage holes were filled, then watered to excess, capped with a plastic lid, and allowed to drain until reaching a constant weight. The constant weight was the wet weight of the soil + pot + lid (W_W). A third soil-filled sample pot was emptied into a pan and the soil placed in an 80°C forced air dryer until it had reached constant weight. The oven-dried soil weight + pot weight + lid weight (W_D) was subtracted from W_W to calculate the amount of water held by the soil at 100% pot capacity (maximum amount of water held after free drainage has stopped). This was calculated for each of the two pots, and the mean value was used as the soil water holding capacity estimate.

Seeds were sown four to a pot and fertilized with 50 mL of a 0.8% (w/v) solution of 20–20–20 (%N, P and K as N, P_2O_5, and K_2O equivalents) fertilizer plus micronutrients (Miller Greenhouse Special, Miller Chemical and Fertilizer Co. Corp., Hanover, PA). Cotyledons were expanded and horizontal at 10 to 12 d after sowing (DAS); at this time, plants were thinned to one per pot, an additional 50 mL of fertilizer solution was added, and each pot was capped with a plastic lid to reduce evaporation of water from the soil surface. Each lid had two 1-cm diameter holes—one to accommodate the plant stem and another to permit water additions.

After capping, all water added to the pots was recorded by weight, and pots were maintained between 55 and 85% relative soil water content (RSWC; 100% is equivalent to the soil water content at saturation) before placement on the lysimeter balances. In this culture system, 55% RSWC is equal to approximately 50% FTSW as defined by Ray and Sinclair (1998) and is above the soil water content where soybean plants first begin to reduce their water use (Ray and Sinclair, 1998; Earl, 2003).

Drought Stress Simulation—Experiment 1

Water stress treatments were imposed using a 16-balance gravimetric lysimeter and computer controlled watering system, which is described in detail by Earl (2003). Each pot was positioned on an electronic balance, and its weight was monitored continuously by a computer. Whenever plant transpiration caused the weight of a pot to decline by 15 g below the target weight for that pot at that point in the experiment, the computer activated a solenoid valve, allowing water to flow to that pot via vinyl tubing until the pot weight was 15 g above its target weight. The target weight (W_T) for each balance was calculated by the computer software as:

$$W_T = W_D + W_P + \text{RSWC} (W_W - W_D)$$

where W_P is an estimate of plant fresh weight (roots plus shoots) determined from destructive harvest of two extra plants at the time that pots were placed on the lysimeter, and RSWC is the target relative soil water content expressed as a fraction between 0 and 1. New target RSWC values for each balance were entered manually on a daily basis as required by the experimental protocol. Pot weights were recorded every 10 min by the software, and each time water was added to a pot the amount added was also recorded in the data file.

After pots were placed on the lysimeter at approximately 30 DAS, drought stress was imposed and water use of both drought pots and control pots was monitored. For the first 2 d, all pots were maintained at 80% RSWC (±3%) by the lysimeter to determine initial water use under water replete conditions. Four pots of cultivar Boggs were maintained at 80% RSWC (water-replete controls) throughout the experiment. For drought pots, the system was programmed to allow RSWC to decline by 15% per day until 50% RSWC was reached, and then 10% per day until 40% was reached. Subsequently, the RSWC was allowed to decline by 5% per day until 10% was reached. After one complete day at 10% RSWC, the pots were returned to 80% RSWC, and the whole plant water use was recorded for an additional 2 d (Fig. 1).

Experimental Design—Experiment 1

The availability of only 16 lysimeter units required grouping the 23 soybean genotypes into an early and a late maturing set (Table 1). Boggs was included in both sets. Eighteen pots were planted for each run—16 for the lysimeter (12 lines including Boggs to be exposed to drought stress, plus four pots of Boggs to serve as water-replete controls) and two additional pots of Boggs for the initial fresh weight determination. Each run of the experiment constituted a replication of either Set 1 or Set 2 (Table 1), and sets were randomly ordered with respect to time within each pair of runs. Lines were randomly assigned to the balances within each set. Six replications were completed for Set 1; however, for Set 2, there were six replications of WUE data but only five replications of transpiration data because of a technical failure. In addition, one replication of Set 1 is lacking the transpiration data for Day 13 (second recovery day after rewatering) because of an error in data recording.

Calculation of Normalized Transpiration Ratio and Water Use Efficiency—Experiment 1

The normalized transpiration ratio (NTR) was calculated for each drought pot for every day of the dry-down phase and for the two recovery days following rewatering, as described by Ray and Sinclair (1997). First, daily water use of each plant was divided by that same plant's water use on the initial full day of the experiment, when all pots were at 80% RSWC. This value is the transpiration ratio (TR), and it adjusts for any initial differences in plant size. Second, the daily TR of each drought stressed plant was divided by the mean TR of the control plants (four pots of the cultivar Boggs) on that day. This ratio is the NTR, and it adjusts for day-to-day differences in environmental conditions affecting transpiration such as solar radiation, humidity, and temperature. Mean daily RSWC for each pot (adjusted for actual plant fresh weights determined at the end of the experiment) was also calculated from lysimeter data for each day of the experiment. The NTR by definition has a value of 1.0 under water-replete conditions on the normalization day.

When each run of the lysimeter protocol was finished, plants were harvested, and shoot and root fresh weights were determined. After the shoots were removed and weighed, roots were thoroughly washed with water, blotted dry with paper towels, and weighed. Shoots and roots were dried at 80°C to constant weight. Water use efficiency was estimated as the ratio of total plant dry weight to total water used since pots were capped, including water use recorded by the lysimeter.
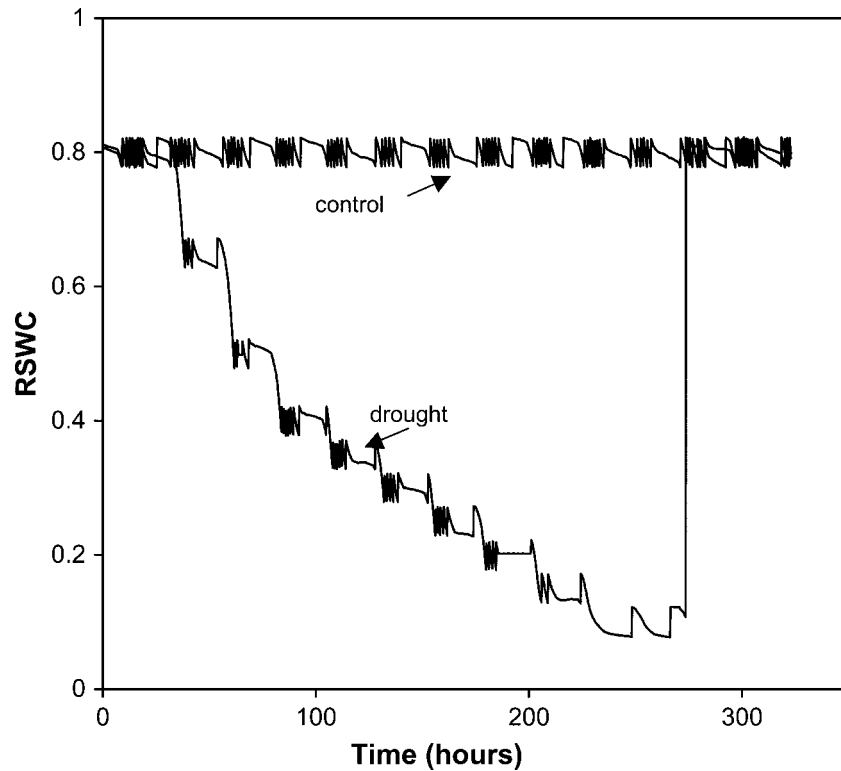
Fig. 1. Water stress protocol, using null balance lysimetry. Relative soil water content (RSWC) vs. time is shown for one drought pot and one control pot in a single replication. After reaching their new target weights on each day of the experiment, drought pots were maintained within ± 3% RSWC of the target weight by frequent water additions. Control pots were maintained between 77 and 83% RSWC at all times. The first 24 h of data were used to normalize subsequent days' water use, to account for differences in plant size.

Analysis of the Normalized Transpiration Ratio/Relative Soil Water Content Relationship—Experiment 1

Plateau regression was applied to the response of NTR to RSWC for each drought pot to find the threshold for the decline in evapotranspiration (Fig. 2). NTR was assumed to be unaffected by soil drying until the RSWC reached some critical value (usually between 0.2 and 0.3) and to decline in a linear fashion below this threshold. The intersection of the unaffected plateau region where NTR = 1 and the linear phase of the curve, as fit by PROC NLIN in SAS (SAS Institute, Cary, NC), was defined as the critical relative soil water content ($RSWC_c$; that soil water content where the plant begins to reduce its evapotranspiration rate). A third phase of the NTR-RSWC curve was revealed as a nonlinear tail once NTR declined below about 0.1. Consistent with previous practice (Sinclair and Ludlow, 1986; Ray and Sinclair, 1998), all data for which NTR < 0.1 were removed so that these nonlinear data would not unduly affect the plateau regression. As expected, removal of the "tail" significantly increased the average x-intercept for the regression (data not shown).

To allow for convenient comparisons between the current and previous work, soil water content was also expressed as a fraction of transpirable soil water (FTSW) for each pot (Sinclair and Ludlow, 1986; Ray and Sinclair, 1997–1998). From the regression of NTR response to RSWC for each pot, the RSWC at which NTR is predicted to be 0.1 ($RSWC_{10}$) was determined. Then, estimates of RSWCc were converted to FTSWc as FTSWc = $(RSWCc − RSWC_{10})/(1 − RSWC_{10})$ (Fig. 2).

Plant Culture—Experiment 2

Culture conditions for Exp. 2 were identical to Exp. 1, except (i) the growth medium was Brown Earth potting mix (Craven, Inc., Commerce, GA), (ii) pots had drainage holes and were watered daily without recording plant water use, and (iii) fertilizer was added weekly. Plants were maintained in well-watered and fertilized conditions for 30 to 35 d before g_e measurements. Six replications were planted sequentially. Within each replication, genotypes were randomized in terms of their location on the greenhouse bench.

Minimum Epidermal Conductance (g_e) Measurements—Experiment 2

Epidermal conductance measurements were taken at approximately 30 d after sowing (DAS). Plants were placed in a dark room for ≥36 h before the start of gas exchange measurements. This dark adaptation period was sufficient to maximize stomatal closure and prevent circadian rhythms such as stomatal opening in response to time of day (i.e., daybreak) according to preliminary experiments (H. Earl, unpublished data). To facilitate data collection during the measurements, a dim source of green light was added to the room. Two low output (25 W) bulbs (Philips Lighting Company, Somerset, NJ) were used. Measured PAR from these bulbs in the room ranged from 0 to 1 μmol m^{-2} s^{-1}, which was considered negligible in terms of inducing stomatal opening (Frechilla et al., 2000).

Two LI-6400 gas exchange systems (LICOR Inc., Lincoln, NE) were used to measure g_e after the dark adaptation period. On each measurement day, the water and CO_2 infra-red gas analyzers of the LI-6400s were calibrated using a verified CO_2 standard and a dew point generator. Measurements were made with the instruments' light sources and temperature controllers deactivated. Ambient air was drawn from within the laboratory and passed through a 4-L buffer volume.
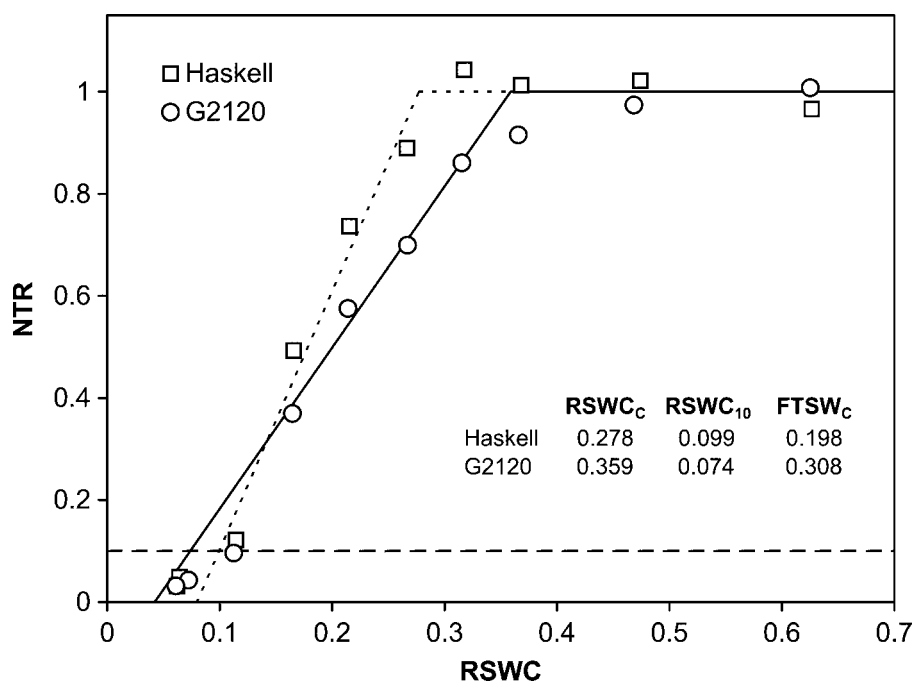
Fig. 2. Plateau regression applied to the relationship between the normalized transpiration ratio (NTR) and relative soil water content (RSWC) for two entries (Haskell and G2120) in a single replication. Data for which NTR < 0.1 (horizontal dashed line) were not included in the regression. The critical relative soil water content ($RSWC_c$) is the RSWC where NTR is estimated to begin to decline; above $RSWC_c$, NTR is assumed to be 1.0. The RSWC at which NTR = 0.1 ($RSWC_{10}$) was estimated by interpolation from the regression. This value was used to calculate the critical fraction of transpirable soil water ($FTSW_C$) from $RSWC_C$ as described in the text.

Flow rate of air through the sample chamber was maintained at 200 μmol s^{-1}. After a leaf was installed in the chamber, gas exchange was allowed to equilibrate until a graph of the water vapor concentration differential between the sample and reference paths stabilized. Then, data points were logged at 10-s intervals for 120 s, providing 12 sequential measurements of g_e for each leaf, which were then averaged to get the g_e estimate for that leaf. For each plant, these measurements were made on both the youngest fully expanded main stem leaf and an older leaf three mainstem nodes lower. The middle leaflet of the trifoliolate was measured in each case.

Data Analysis

The PROC MIXED procedure in SAS was used to detect significant ($P < 0.05$) genotype effects on all measured parameters. The experimental design required Set 1 and Set 2 lines to be considered separately in Exp. 1, but all 23 lines could be compared directly in Exp. 2. Because there were missing data in some replications (see Results), genotype means were expressed as LSMEANS. $LSD_{0.05}$ for comparing lines were calculated from the standard errors for LSMEANS differences provided by the DIFF option.

RESULTS

Water Use Efficiency

In both Sets 1 and 2, genotypes differed significantly ($P < 0.05$) for WUE (Tables 2 and 3). In Set 1, the percent difference from the lowest WUE line (PI416937) to the highest WUE line (Dillon) (calculated as [(highest WUE − lowest WUE)/lowest WUE] × 100) was 21.4%. In Set 2, Boggs had the highest WUE value, 3.24 g DM kg^{-1} H$_2$O, while Tokyo had the lowest WUE, 2.72 g DM kg^{-1} H$_2$O, which amounted to a variation of

19.1%. Boggs, a common control in both sets, had a high WUE in Set 1 as well and was not significantly different ($P > 0.05$) from the most water use efficient line in that set, Dillon. Because the WUE value for Boggs was so similar in both sets (Set 1, 3.23; Set 2, 3.24), a comparison of genotypes across sets could be considered. Total variation across sets from lowest to highest WUE was 25.4%. In Fig. 3, dry weight is graphed against water used for all genotypes in the study, and the WUE of any genotype is the slope of the line through the origin to that data point on the graph. The average WUE across

Table 2. Water use efficiency (WUE) and critical values for the fraction of transpirable soil water at which transpiration is first reduced during a drying cycle ($FTSW_C$) for soybean genotypes in Set 1 (Maturity Groups IV-VI). Sample size is shown in parentheses.

Genotype	WUE	$FTSW_C$
	g DM kg^{-1} H$_2$O	
Dillon	3.41 (6)	0.271 (6)
PI 471938	3.38 (6)	0.244 (6)
N98-7265	3.30 (6)	0.226 (6)
Boggs	3.23 (6)	0.256 (6)
Young	3.22 (6)	0.217 (6)
Hutcheson	3.19 (6)	0.259 (6)
Holladay	3.17 (6)	0.239 (6)
Fendou 34	3.10 (5)	0.252 (5)
N94-7589	3.02 (5)	0.252 (5)
H2L16	2.94 (6)	0.221 (6)
PI 407859-2	2.94 (6)	0.224 (6)
PI 416937	2.81 (6)	0.287 (6)
$LSD_{0.05}$†	0.209	0.049
$LSD_{0.05}$‡	0.219	0.051
$LSD_{0.05}$§	0.229	0.053

† Comparisons between genotypes with $n = 6$.
‡ Comparisons between genotypes with $n = 5$ and $n = 6$.
§ Comparisons between genotypes with $n = 5$.

Table 3. Water use efficiency (WUE) and critical values for the fraction of transpirable soil water at which transpiration is first reduced during a drying cycle (FTSW$_C$) for soybean genotypes in Set 2 (Maturity Groups VI–IX). Sample size is shown in parentheses.

Genotype	WUE	FTSW$_C$
	g DM kg^{-1} H$_2$O	
Boggs	3.24 (6)	0.246 (5)
Benning	3.14 (5)	0.269 (4)
N95-7424	3.04 (6)	0.215 (5)
N96-6809	3.01 (6)	0.243 (5)
Cook	2.98 (6)	0.212 (5)
Haskell	2.96 (6)	0.186 (5)
Jackson	2.94 (6)	0.229 (5)
N7001	2.91 (6)	0.234 (5)
N97-9765	2.90 (6)	0.251 (5)
G2120	2.87 (6)	0.270 (5)
N96-7031	2.82 (6)	0.255 (5)
Tokyo	2.72 (6)	0.241 (5)
LSD$_{0.05}$†	0.268	0.079
LSD$_{0.05}$‡	0.281	0.084

† For pairwise comparisons not including Benning.
‡ For pairwise comparisons including Benning.

all lines was 3.0 g DM kg^{-1} H$_2$O. Several lines with unusual properties emerged from this regression analysis. Boggs, PI 407859-2, Dillon, PI 416937, PI 471938, and Tokyo were most notable. Boggs, present in both sets, showed low water using and low dry matter producing properties and near average WUE. PI 407859-2 also had a WUE near the average, but in contrast to Boggs, it was a very high water using and very high dry matter producing line. Dillon used a moderate amount of water but produced higher than average dry matter. It was identified by Cook's distance measure (Neter et al., 1996) in SAS as an influential point in the regression, which could skew the regression when it is allowed to remain in the data set. PI 416937 also was a moderate water user but produced unusually low dry matter. PI 471938 displayed moderate water use but high dry matter production. It was a leveraged point (extreme observation) because it had a studentized deleted residual or externally studentized residual (R-student value in SAS) of >|2.0| at 2.02 (Belsley et al., 1980). Tokyo was a very high water user but moderate dry matter producer. It was an influential point like Dillon.

Critical Fraction of Transpirable Soil Water and Normalized Transpiration Ratio

Genotypes differed significantly for FTSWc in both Set 1 and Set 2, but differences were greater in Set 1. In Set 1, Dillon and PI 416937 reduced whole plant water use earlier in response to drying soil than did the cultivar Young, Egyptian breeding line H2L16, or PI 407859-2. In Set 2, the cultivar Benning and Indonesian land race G2120 reduced whole plant water use earlier than did the cultivar Haskell (Tables 2 and 3).

No significant ($P < 0.05$) genotype effect was found among Set 1 genotypes for NTR following rewatering
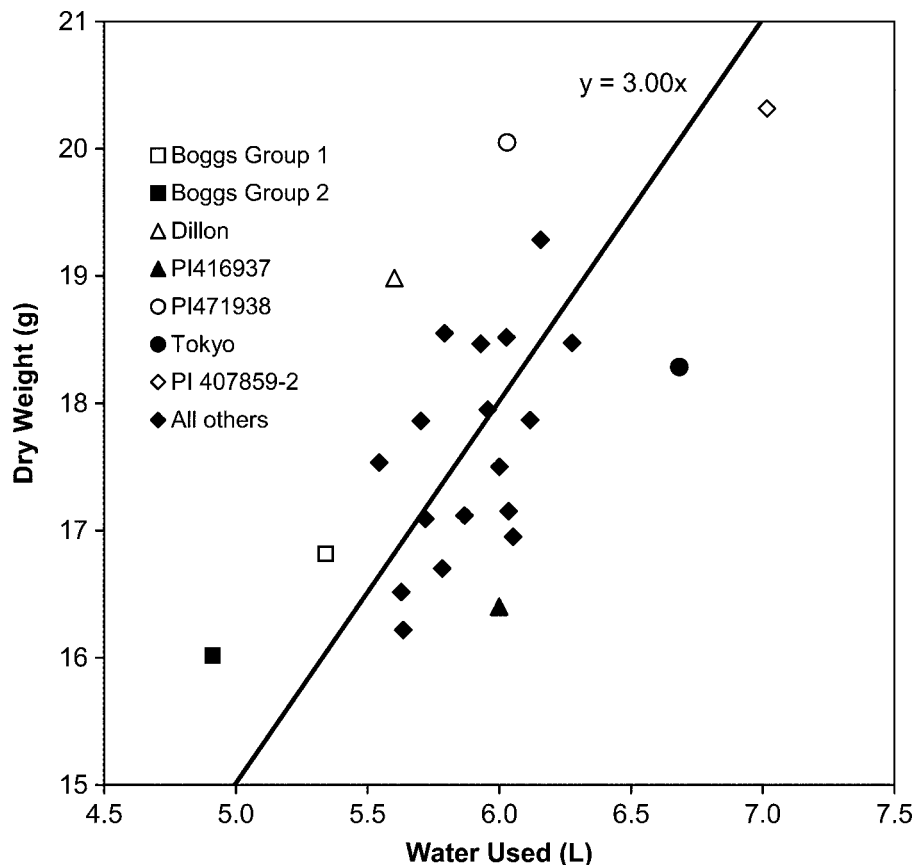
Fig. 3. Average dry matter production vs. average water used for 23 soybean lines during vegetative growth in a greenhouse. Each data point is the mean of five or six observations depending on genotype, as shown in Tables 2 and 3. The slope of the regression line through the origin (3.00 g L^{-1}) closely estimates mean water use efficiency (3.04 g L^{-1}).

Table 4. Normalized transpiration ratio (NTR) for Set 2 (late maturing) soybean genotypes on the first and second recovery days after rewatering following a water stress. Sample size for each genotype is as shown in Table 3.

Line	NTR, first recovery day	NTR, second recovery day
Boggs	0.270	0.515
N96-6809	0.229	0.542
N7001	0.216	0.513
Cook	0.190	0.448
Jackson	0.184	0.398
Tokyo	0.175	0.366
Benning	0.165	0.388
N97-9765	0.163	0.452
G2120	0.161	0.402
N95-7424	0.156	0.338
Haskell	0.136	0.374
N96-7031	0.126	0.335
LSD$_{0.05}$†	0.073	0.125
LSD$_{0.05}$‡	0.077	0.132

† For pairwise comparisons not including Benning.
‡ For pairwise comparisons including Benning.

(data not shown). Recovery of NTR following rewatering for Set 2 is shown in Table 4. On Recovery Day 1, the range in NTR between the highest and lowest entries in Set 2 was 0.144. Boggs had the highest NTR value which was more than double that of the lowest entry, N96-7031. On Day 2, the range between highest and lowest entries was 0.207. N96-7031 still had the lowest NTR value, while N96-6809 had the highest with an NTR of 0.542.

Minimum Epidermal Conductance (g_e)

Significant differences were found among genotypes for minimum g_e values ($P < .001$) across leaf positions, but there was no difference in g_e on the basis of leaf position (upper vs. lower), and no genotype × leaf position interactions were present. Chinese cultivar Fendou 34 had the highest average g_e value (18.5 mmol m^{-2} s^{-1}) while USDA breeding line N98-7265 had the lowest

Table 5. Mean minimum leaf epidermal conductance (g_e) for 23 soybean lines. Measurements averaged across two leaf positions per plant. $n = 6$.

Number	Genotype	g_e
		mmol m^{-2} s^{-1}
1	Fendou 34	18.5
2	PI 416937	17.0
3	PI 407859-2	14.9
4	Tokyo	14.6
5	N97-9765	13.7
6	N7001	13.2
7	N96-7031	13.0
8	N95-7424	12.9
9	Haskell	12.3
10	G2120	12.3
11	PI 471938	11.5
12	Cook	11.4
13	Holladay	11.3
14	Jackson	11.2
15	N94-7589	11.2
16	Young	10.8
17	Boggs	10.5
18	H2L16	10.4
19	N96-6809	10.3
20	Dillon	10.3
21	Benning	10.0
22	Hutcheson	9.90
23	N98-7265	8.60
	LSD $_{(0.05)}$	5.67

(8.60 mmol m^{-2} s^{-1}) (Table 5). N98-7265 was also significantly ($P < 0.05$) lower than PI 416937, PI407859-2 and US soybean ancestor Tokyo.

Few significant ($P < 0.05$) correlations were found between any of the measured traits (WUE, FTSW$_C$, NTR on Recovery Day 1 and Recovery Day 2, and g_e). One trivial exception was a significant correlation between NTR on Recovery Day 1 and NTR on Recovery Day 2 ($r = 0.86$, $P < 0.0001$ across all 23 genotypes). The other exception was the strong negative correlation between g_e and WUE, shown in Fig. 4 ($r = -0.56$, $P < 0.01$). Fendou 34 was found to be a statistical outlier from this relationship, with a studentized residual $> |2|$. When Fendou 34 was removed, the correlation was much stronger ($r = -0.74$, $P < 0.0001$). This correlation between g_e and WUE was also statistically significant when considered within individual sets ($r = -0.70$ and $P < 0.05$ in Set 1 with Fendou 34 excluded; $r = -0.57$ and $P = 0.052$ in Set 1 with Fendou 34 included; $r = -0.79$ and $P < 0.01$ in Set 2).

Although we hypothesized that genotypes with lower g_e might recovery more quickly from water stress, no correlation was found between g_e and NTR on either Recovery Day 1 ($P = 0.92$) or Recovery Day 2 ($P = 0.63$).

DISCUSSION

A few of these lines have been compared for WUE in previous studies, and in general, the present findings are consistent with these prior reports. For example, Mian et al. (1996) and Earl (2002) reported that Young had 18.9 and 9.1% (respectively) higher WUE than PI 416937, as compared with 14.6% in the present work. These previous authors used a similar culture system as in the present study, but grew the plants under cyclic drought stress by repeatedly allowing soil moisture to drop to 20 to 25% RSWC before rewatering. It is not clear whether constant water-replete conditions or some sort of managed water stress is most relevant for comparing lines for WUE. Earl (2002) reported that the WUE difference between Young and PI 416937 was constitutive in nature; that is, the difference was observed whether the plants were grown under cyclic drought stress or under continuous water-replete conditions. In the present work, the genotypes were well watered for 30 d and then exposed to dry down (to 10% RSWC) and recovery (at 80% RSWC) periods. On the basis of comparisons between water-replete control pots of Boggs and droughted pots of Boggs, this water stress protocol significantly reduced whole plant dry matter accumulation (from 21.5–16.4 g plant^{-1}, $P < 0.0001$), but it did not significantly affect whole plant WUE ($p = 0.24$, 3.23 vs. 3.18 g/L for control and drought pots respectively). Mian et al. (1998) identified Tokyo as a low-WUE line. In the present work, Tokyo had the lowest WUE of any genotype studied. The range of WUE found in the present work (3.4 g DM kg^{-1} H$_2$O for Dillon vs. 2.7 for Tokyo, a 25% difference) is substantial but not unusually large compared with other experiments of this type [compare for example Van den Boogaard et al. (1997), wheat, WUE range of 20.6%;
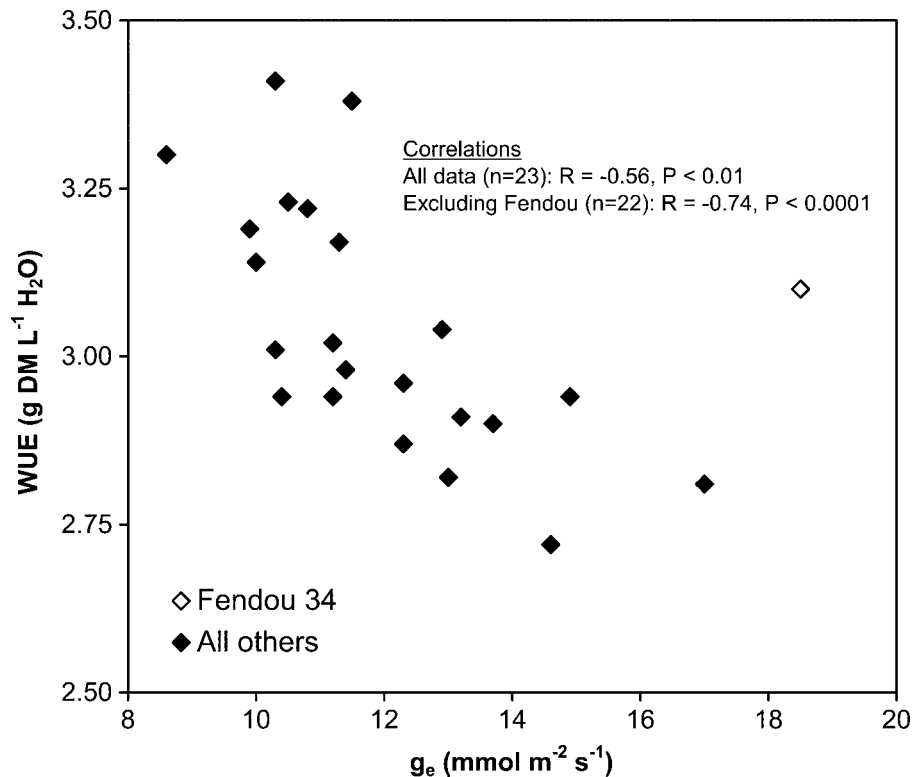
Fig. 4. Linear correlation between water use efficiency (WUE) and minimum epidermal conductance (g_e) for 23 soybean genotypes. Data are the same as shown in Tables 2 and 3 (WUE) and Table 4 (g_e). WUE and g_e data were collected in separate experiments using different greenhouse culture systems. n = 5 or 6 for WUE data, and n = 6 for g_e data. Fendou 34 was identified as an outlier (see text).

Donatelli et al. (1992), cowpea, WUE range of 35%; Ismail and Hall, 1993, soybean, WUE range of 25%], despite the fact that in the present work entries were chosen specifically on the basis of putative differences in water use characteristics.

There have been no previously published reports of experiments to compare soybean genotypes for their regulation of whole plant water use under drought stress. Indeed, these responses have not been compared across such a broad range of germplasm in any crop species. In general, the critical soil water content (expressed as fraction of transpirable soil water) at which NTR began to decline was in the range of 0.2 and 0.3, as previously reported for the soybean genotype CPI 26671 by Sinclair and Ludlow (1986). The reduction in NTR as FTSW declines is assumed to be due primarily to stomatal closure (Ray and Sinclair, 1997); indeed, Earl (2003) reported a very strong correlation between whole plant water use and single leaf stomatal conductance in soybean in drying soil.

Results of the present work indicate that there is variability among soybean genotypes for regulation of water use under drought stress. For example in Set 1, slow wilting PI 416937 from Japan had the highest FTSW$_C$ and reduced whole plant water use earlier than did breeding line N98-7265, slow wilting Korean PI 407859-2, Egyptian breeding line H2L16, and Young. In Set 2, Benning and Indonesian PI G2120 had the highest FTSW$_C$ and reduced whole plant water use sooner than Haskell. The relative advantage or disadvantage of early stomatal closure in the field might depend on the duration

of water stress or drought. If these greenhouse results are indicative of how these lines might respond under field conditions, then under a long-term drought, PI 416937 would be at an advantage over N98-7265, PI 407859-2, H2L16, and Young because it could conserve water and increase its chances for survival. However, under short-term stress, PI 416937 could be at a disadvantage by prematurely closing stomata and thereby sacrificing potential carbon assimilation.

One potential disadvantage of expressing NTR in terms of FTSW instead of RSWC is that by calculating FTSW independently for every pot, any real differences that may exist between genotypes in their abilities to extract water from very dry soil are obscured; this is because, in the calculation of FTSW, the point at which "transpirable" soil water is considered to be exhausted is determined by the measured water use of each plant. If such genetic differences in the ability to extract soil water at very low RSWC were to exist, then FTSW would be a biased measure of soil water content that is affected by the genotype under consideration. Such a result would imply that FTSW$_C$ is not a suitable measure of sensitivity of whole plant water use to soil drying when comparing different genotypes. In this study, analysis of the x-intercepts of the regression of NTR on RSWC revealed few significant differences among genotypes, indicating that genotypes generally dried pots to the same degree. The only significant ($P < 0.05$) difference found was in Set 2, between Tokyo (intercept = 0.057) and Haskell (intercept = 0.091) (data not shown).

Genotypes with more rapid recovery of whole plant water use (NTR) after rewatering following a drought stress could potentially regain full productivity more quickly than lines with slower recovery. Significant variation for this trait was found among Set 2 genotypes (Table 4). If the rapidity of recovery were somehow related to the extent of desiccation injury experienced by leaves during the stress, one might expect recovery to be related to other traits that could protect plants from such injury, such as a high $FTSW_C$ or a low g_e. No such correlations were found in the present work, so the physiological basis of the genotypic differences shown in Table 4 remains uncertain.

The finding that soybean genotypes varied for g_e by a factor of about 2 is consistent with a previous report by Paje et al. (1988). They found a range in g_e of 17.1 to 31.5 mmol m^{-2} s^{-1} in the first 40 soybean accessions they tested and a range of 10.3 to 23.9 mmol m^{-2} s^{-1} in the next 34 accessions they screened [data converted from mm s^{-1} according to Jones (1992, p. 56), assuming pressure was 100 kPa]. A "low g_e" genotype that was identified in that previous work, Indonesian plant introduction G2120, was also evaluated in the present study. While we found a g_e value of 12.3 mmol m^{-2} s^{-1} for G2120, Paje et al. (1988) found a value of 18.2 mmol m^{-2} s^{-1} in their first experiment and 11.4 mmol m^{-2} s^{-1} in their second. In our study, G2120 was not significantly different from the genotype with lowest g_e, USDA breeding line N98-7265. Thus G2120 would not appear to be priority breeding material for the USA because it is much less adapted than N98-7265 and the other low g_e genotypes in Table 5.

An unexpected result of the present work was the significant negative correlation between WUE and g_e ($r = -0.74$, with Fendou 34 removed). While several authors have established that variation for g_e and WUE exists in various crop species (see Introduction), we are not aware of any studies that demonstrate a correlation between these two traits. While this relationship between g_e and WUE does not necessarily indicate causality (lower g_e values do not necessarily cause higher WUE values), it is interesting to speculate as to any mechanistic link that might exist. Several theories have been proposed to explain variation in g_e, including differences in the quantity of cuticular wax (Clark and Levitt, 1956; Blum, 1975), quality of wax such as the structure and chemical composition (Rama Das et al., 1979), and stomatal density (Muchow and Sinclair, 1989). In sorghum, Muchow and Sinclair (1989) found that g_e was highly correlated to stomatal density ($R^2 = 0.82$). They hypothesized that at minimum stomatal apertures water loss from the cuticle above guard cell teichodes (holes in external cell walls of guard cells) becomes a significant source of leaf water loss. Greater stomatal density, if linked to higher average stomatal conductance and leaf internal CO_2 concentrations, would be consistent with lower WUE according to existing theory about how WUE is determined at the leaf level (Farquhar et al., 1989). However, Araus et al. (1991) found conflicting results in durum wheat. They found no significant correlation between g_e and total stomatal density or between g_e and either adaxial or abaxial stomatal density in the fourth leaf, first node leaf, or flag leaf. Also, Paje et al. (1988) conducted an experiment to test whether responses in stomatal density to environmental change were associated with environmentally induced changes in g_e. In the six soybean accessions tested, they found that differences in g_e in response to differences in environmental conditions were not associated with stomatal density.

The relationship between WUE and g_e may not be unique to soybean; indeed, we have recently observed an even stronger correlation of this type among a selection of cotton genotypes (H.J. Earl and D.A. Fish, unpublished data). Attention should be focused on the physiological nature of this relationship since it may provide new insights into how WUE is actually determined. Other future work might include using g_e measurements as a screening tool to predict drought resistance among genotypes. A necessary requirement for this approach is that the environmental, leaf-age induced, or random variability within a test be much smaller than the inherited differences between genotypes (Kersteins, 1996)— that is, that the trait has sufficiently high heritability. If individual alleles controlling g_e have sufficiently large effects, then a QTL analysis of g_e could be employed to identify and track alleles in breeding, and could also lead to development of near isogenic lines for physiological and agronomic study (Richards, 1988). Given the strong correlation that was found between WUE and g_e, there is a possibility they will share QTL, so that study of one trait will lead to QTL identification for the other. Of all of the traits studied in the present work, g_e is by far the most convenient to measure and so may be best suited to future work such as QTL mapping. The correlation between g_e and WUE identified in these experiments also argues for this trait's potential importance.

Implications to Breeding

The PIs and their progeny breeding lines in the present study were selected primarily because they are slower wilting in the field than the cultivar controls. Although the physiological basis for slow wilting is not yet known, it is likely to involve WUE, FTSWc, g_e, and/or deep rooting. The present study examined genetic variation in three of the four traits. Genetic differences in the fourth, deep rooting, could not be examined because of the nature of the pot-based culture system. Although the evaluations reported in the present study were made only on vegetative plants and need to be validated in older reproductive plants, the results nevertheless suggest that the slow wilting trait in the field may not be consistently associated with WUE, FTSWc, or g_e. Generally, adapted cultivars had more favorable WUE and g_e values than did PIs. Slow wilting PI 471938 and its progeny N98-7264 were clear exceptions to this trend in that they were comparable to the cultivars for both traits. However, these two genotypes were no more extreme than the cultivar controls for these traits, while exhibiting considerably slower wilting in the field (T.E. Carter, Jr., personal communication, 2005). Distinctions among cultivars, PIs, and breeding lines were less clear

for FTSWc, with both PIs and cultivars at either extreme of the rating scale. These results leave open the possibility that slow wilting is associated primarily with deep rooting or deep rooting in combination with the above traits.

The fact that almost all genotypes with highest WUE and lowest g_e were released cultivars and not PIs suggests that breeders who are engaged in drought-tolerance breeding efforts need to be cautious as they introduce the slow-wilting trait into their populations. In many cases, they may also introduce inferior WUE and g_e (and perhaps inferior yield under stress) at the same time. In that regard, some PIs were as much as 25% less water use efficient than cultivars under stress, a magnitude of loss on a scale comparable to the potential benefits associated with slow wilting (Carter et al., 1999). To prevent this unwanted occurrence, breeders should monitor and maintain the favorable WUE and g_e that may already exist in their program. That PI 471938 was better in terms of WUE and g_e than most other slow wilting PIs suggests that, as drought-tolerance breeding programs mature, it may be a breeding priority to use progeny of this PI in schemes which pyramid alleles from multiple slow-wilting sources into an adapted background. A priority use of alleles from PI 471938 will certainly improve the ease with which favorable WUE and g_e can be maintained in breeding.

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