

RESEARCH PAPER

# Independent variation in photosynthetic capacity and stomatal conductance leads to differences in intrinsic water use efficiency in 11 soybean genotypes before and during mild drought

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## Abstract

**Intrinsic water use efficiency ( $WUE_{intr}$ ), the ratio of photosynthesis to stomatal conductance to water, is often used as an index for crop water use in breeding projects. However,  $WUE_{intr}$  conflates variation in these two processes, and thus may be less useful as a selection trait than knowledge of both components. The goal of the present study was to determine whether the contribution of photosynthetic capacity and stomatal conductance to  $WUE_{intr}$  varied independently between soybean genotypes and whether this pattern was interactive with mild drought. Photosynthetic capacity was defined as the variation in  $WUE_{intr}$  that would occur if genotypes of interest had the same stomatal conductance as a reference genotype and only differed in photosynthesis; similarly, the contribution of stomatal conductance to  $WUE_{intr}$  was calculated assuming a constant photosynthetic capacity across genotypes. Genotypic differences in stomatal conductance had the greatest effect on  $WUE_{intr}$  (26% variation when well watered), and was uncorrelated with the effect of photosynthetic capacity on  $WUE_{intr}$ . Thus, photosynthetic advantages of 8.3% were maintained under drought. The maximal rate of Rubisco carboxylation, generally the limiting photosynthetic process for soybeans, was correlated with photosynthetic capacity. As this trait was not interactive with leaf temperature, and photosynthetic capacity differences were maintained under mild drought, the observed patterns of photosynthetic advantage for particular genotypes are likely to be consistent across a range of environmental conditions. This suggests that it is possible to employ a selection strategy of breeding water-saving soybeans with high photosynthetic capacities to compensate for otherwise reduced photosynthesis in genotypes with lower stomatal conductance.**

**Key words:** Breeding strategies, drought stress, intrinsic water use efficiency, photosynthesis, soybean, stomatal conductance.

## Introduction

Every year US soybean [*Glycine max* (L.) Merr.] fields transpire more water than that which evaporates from all the Great Lakes combined (Snyder, 1960; Purcell *et al.*, 2007). Given that irrigation and rainfall limitations on agriculture are expected to grow in the coming decades (Wang, 2005), the development of soybean genotypes with enhanced drought

tolerance, escape or avoidance traits would be highly desirable. To date considerable genetic variation in soybean whole-plant transpiration (Fletcher *et al.*, 2007; Sinclair *et al.*, 2008b; Sadok and Sinclair, 2009a) and whole-plant water use efficiency ( $WUE_{wholeplant}$ ) has been demonstrated (Hufstetler *et al.*, 2007). A particularly promising trait is the break in the

Abbreviations: A, assimilation rate;  $C_a$ , atmospheric  $CO_2$  concentration;  $C_i$ , intercellular  $CO_2$  concentration;  $g_{CO_2}$ , stomatal conductance to  $CO_2$ ;  $g_{H_2O}$ , stomatal conductance to water;  $J_{max}$ , the maximal rate of RuBP regeneration; LMA, leaf mass area; PPFD, photosynthetic photon flux density;  $Q_{10}$ , ratio of a process over a 10°C change in temperature; RIL, recombinant inbred line; SWC, soil water content;  $T_{leaf}$ , leaf temperature; TR, transpiration rate;  $V_{cmax}$ , maximum rate of Rubisco carboxylation; VPD, leaf to air vapour pressure deficit;  $WUE_{g_{H_2O}}$ , water use efficiency estimated from variation in  $g_{H_2O}$ ;  $WUE_{intr}$ , intrinsic water use efficiency ( $A/g_{H_2O}$ );  $WUE_{PC}$ , water use efficiency estimated from variation in A at a constant  $g_{H_2O}$ ;  $WUE_{wholeplant}$ , water use efficiency of whole plant.

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linear relationship between the whole-plant transpiration rate (TR), measured under well-watered and light-saturating conditions, and atmospheric vapour pressure deficit (VPD) that has been demonstrated for some soybean genotypes (Sadok and Sinclair, 2009a). These genotypes show a linear TR to VPD response under mesic conditions but have constant whole-plant TR at higher (drier) VPDs, putatively related to leaf-level hydraulic limitations (Sinclair *et al.*, 2008b), or canopy-level processes such as changes in leaf orientation. Thus these genotypes may conserve soil moisture during the dry periods leading up to short-term water deficits and therefore enable maintenance of productivity (Sinclair *et al.*, 2005, 2010). This is important as a large proportion of US soybeans are grown under water-limiting conditions (Boyer, 1982), and simulations suggest that in 70% of years many soybean-growing areas would benefit from a constant TR at high VPD (Sinclair *et al.*, 2010). Similarly, an early reduction in stomatal conductance upon low soil water contents would be beneficial to productivity in many areas of the USA (Sinclair *et al.*, 2010).

One unstudied effect is whether genotypes with traits such as constant TR at high VPD, or reduced stomatal conductance at low soil water content, also have depressed productivity through low stomatal conductance and thus lower photosynthetic rates. This would seem a severe penalty for breeding water-conserving plants. However, variation in photosynthesis is also dependent on the ability of a leaf to draw down CO<sub>2</sub> within the leaf—defined here as photosynthetic capacity. If photosynthetic capacity is independent of stomatal conductance to water, then breeding for increased photosynthetic capacity offers a way around this trade-off.

Intrinsic water use efficiency (WUE<sub>intr</sub>), the leaf-level ratio of photosynthesis to stomatal conductance (an indication of leaf transpiration), has been widely used to screen for heritable genotypic variation in water use characteristics of crops, as has the whole-plant equivalent (WUE<sub>wholeplant</sub>). As both the drawdown of CO<sub>2</sub> by Rubisco and diffusion through the stomata result in fractionation of carbon isotopes, a mass spectrometer can measure WUE<sub>intr</sub> instantaneously or integrated for the lifespan of the CO<sub>2</sub> fixed into cellular components in a tissue (Seibt *et al.*, 2008). However, if the measurements of carbon isotope discrimination are not combined with knowledge of the variation in the components of WUE<sub>intr</sub> then it is difficult to draw conclusions about whether WUE<sub>intr</sub> changes might be beneficial to crop water use or productivity, or both (see Theory section). That is, a crop with low photosynthetic capacity and stomatal conductance to water (the components of WUE<sub>intr</sub>) may have the same WUE<sub>intr</sub> as a crop with proportionally higher photosynthetic capacity and stomatal conductance to water. Indeed for soybeans, carbon isotope discrimination, and, by extension WUE<sub>intr</sub>, are not strongly related to WUE<sub>wholeplant</sub> (Specht *et al.*, 2001). In contrast, carbon isotope fractionation in combination with gas exchange measurements has been useful for breeding drought-adapted wheat genotypes (Condon *et al.*, 1990, 2002), and such gas exchange measurements, accounting for the components of WUE<sub>intr</sub>, have been applied to other crops (Pettigrew and Turley, 1998; Centritto *et al.*, 2009).

However, it is felt that a simple experimental approach that would allow for determination of WUE<sub>intr</sub> components is missing.

Here practical formulations that enable the two components of WUE<sub>intr</sub> to be measured are defined. This allows testing of: (i) whether there is a trade-off between reduced transpiration in ‘constant TR’ genotypes and photosynthesis at high VPDs; (ii) whether there are photosynthetic differences between genotypes, independent of variation in  $g_{H_2O}$ , and whether these are maintained under conditions necessary for this trait to be useful in the field such as mild drought or varying temperature conditions; and (iii) which traits determine the photosynthetic differences. The answer to these questions then enables a conceptual framework to be offered by which water-saving soybean genotypes may be bred.

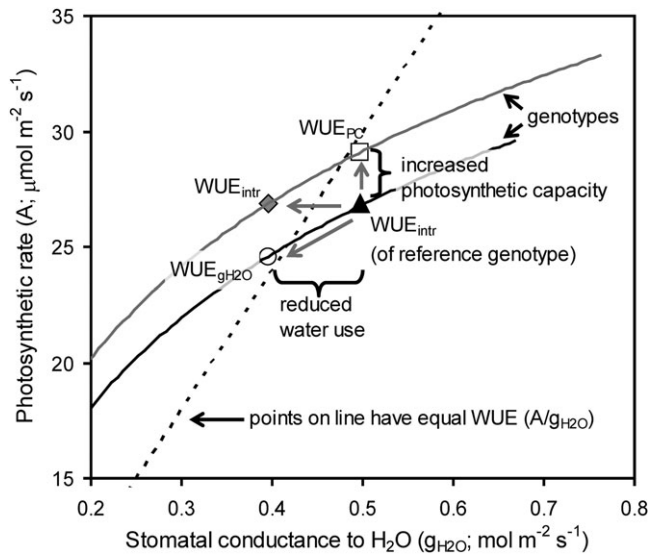
## Theory

Water use efficiency of a crop is defined as a yield, or carbon gain, relative to water used to fix that carbon (or grain). Much of the exchange of water and CO<sub>2</sub> is determined at the leaf level, where stomata co-regulate the influx of CO<sub>2</sub> to photosynthesis and the transpirational loss of water to the atmosphere. As transpiration is determined by both atmospheric dryness (VPD) and stomatal characteristics it does not relate directly to photosynthesis. For this reason, WUE<sub>intr</sub> is defined as the ratio of photosynthesis to stomatal conductance to water, thereby accounting for the effects of VPD on transpiration (Seibt *et al.*, 2008). Similarly, photosynthetic rate ( $A$ ) is determined by two processes:

$$A = (g_{CO_2} \times C_a - C_i) \quad (1)$$

where  $g_{CO_2}$  is the stomatal conductance of CO<sub>2</sub> into the leaf and  $C_a - C_i$  is the CO<sub>2</sub> drawdown, due to photosynthesis, between the atmosphere ( $C_a$ ) and the intercellular ( $C_i$ ) CO<sub>2</sub> concentrations. Stomatal conductance to CO<sub>2</sub> is strictly proportional to stomatal conductance to water ( $g_{CO_2} = g_{H_2O} / 1.6$ ), while the drawdown in CO<sub>2</sub> is proportional to the amount of nitrogen invested in photosynthetic components of the leaf such as Rubisco (Evans, 1989; Wright *et al.*, 2003).

The relationship between  $A$  and  $g_{H_2O}$  is not linear for many species including soybeans, therefore, WUE<sub>intr</sub> is affected by two independent sources of variation: (i) variation in the position along this relationship, relating to  $g_{CO_2}$  and  $g_{H_2O}$ ; and (ii) variation in the height of the relationship, relating to photosynthetic capacity or ability to drawdown CO<sub>2</sub> (Fig. 1). By comparing the typical operating points of two genotypes and knowing the relationship between  $A$  and  $g_{H_2O}$ , the contributions of variation in photosynthetic capacity (PC) and stomatal conductance ( $g_{H_2O}$ ) to WUE<sub>intr</sub>, which are defined as WUE<sub>PC</sub> and WUE <sub>$g_{H_2O}$</sub> , respectively, can be estimated. Thus WUE<sub>PC</sub> is defined as the WUE<sub>intr</sub> that would occur if a genotype of interest had the same  $g_{H_2O}$  as a reference genotype and only differed in photosynthetic capacity. With reference to the



**Fig. 1** Schematic of components of intrinsic water use efficiency, where  $WUE_{intr}$  is the ratio between  $A$  and  $g_{H_2O}$  for a genotype of interest. A soybean genotype of interest has an increased  $WUE_{intr}$  (diamond;  $WUE_{intr}=26.9/0.395=68.1\mu\text{mol CO}_2\text{ mol}^{-1}\text{ H}_2\text{O}$ ) relative to the average value measured for a reference genotype (triangle). This difference is due to a combination of two shifts: (i) a decrease in  $g_{H_2O}$  resulting in an increase in  $WUE_{intr}$ , which has been termed  $WUE_{gH_2O}$  (circle;  $WUE_{gH_2O}=24.6/0.395=62.3\mu\text{mol CO}_2\text{ mol}^{-1}\text{ H}_2\text{O}$ ) and (ii) the genotypes have different  $A$  to  $g_{H_2O}$  relationships, resulting from an increase in photosynthetic capacity of the genotype of interest relative to the reference genotype, which has been termed  $WUE_{PC}$  (square;  $WUE_{PC}=29.1/0.498=58.4\mu\text{mol CO}_2\text{ mol}^{-1}\text{ H}_2\text{O}$ ). As the high  $WUE_{PC}$  of the genotype of interest is maintained under lower  $g_{H_2O}$ , photosynthetic capacity differences can compensate for variation in water use. All points along a line passing through the origin (e.g. the dashed line) have the same  $WUE_{intr}$ . The  $A$  to  $g_{H_2O}$  relationship and variation in  $WUE_{PC}$  and  $WUE_{gH_2O}$  represent realistic values for soybean genotypes.

theoretical example shown in Fig. 1, the  $WUE_{PC}$  of the genotype of interest is:

$$WUE_{PC} = \frac{A(\text{of genotype of interest at } g_{H_2O} \text{ of reference genotype})}{g_{H_2O}(\text{of reference genotype})}$$

Similarly,  $WUE_{gH_2O}$  is the  $WUE_{intr}$  that would occur if the genotype of interest had the same photosynthetic capacity ( $A$  to  $g_{H_2O}$  relationship) as the reference genotype and only differed in  $g_{H_2O}$ . In the case of Fig. 1, the  $WUE_{gH_2O}$  is:

$$WUE_{gH_2O} = \frac{A(\text{of reference genotype at } g_{H_2O} \text{ of genotype of interest})}{g_{H_2O}(\text{of genotype of interest})}$$

The resultant  $WUE_{intr}$  for a soybean genotype of interest is then the combination of the change in  $WUE_{gH_2O}$  and  $WUE_{PC}$  from the reference genotype ( $WUE_{intr}=A/g_{H_2O}$  of the genotype of interest).

To evaluate these two components of  $WUE_{intr}$  considerable data are required, specifically, gas exchange measurements of a genotype of interest are needed in combination with an established  $A$  to  $g_{H_2O}$  relationship for both the genotype of interest and a reference genotype. As VPD changes may not result in variation in  $g_{H_2O}$  for the genotypes with a linear TR response to VPD, variation in soil water content may be used instead to cause the variation in  $g_{H_2O}$  needed to establish the  $A$  to  $g_{H_2O}$  relationships. This approach is based upon the empirically testable assumption that the  $A$  to  $g_{H_2O}$  relationship differs significantly between genotypes, and that it is relatively invariable within a genotype, as may be the case for sun-leaves in the uniform canopies of crops, particularly nitrogen fixers such as soybean. Virgona and Farquhar (1996) propose a similar approach, review its limitations, and use it to determine the reasons for differences in water use efficiency amongst sunflower genotypes in response to nitrogen treatments.

Two chloroplast processes may limit photosynthesis, individually or jointly: the  $\text{CO}_2$ -limited rate of Rubisco carboxylation, indicated by  $V_{cmax}$ , and the rate of RuBP regeneration ( $J_{max}$ ), estimated from the  $\text{CO}_2$ -saturated photosynthetic rate. These processes are differentially affected by leaf temperature, and a leaf may transition between these two limitations due to changes in stomatal conductance (Hikosaka, 1997; Hikosaka *et al.*, 1999). Thus, it is possible that photosynthetic advantages under humid mesic conditions will not translate to conditions of mild drought, high temperatures associated with increasing VPD, or where stomata are limiting to photosynthesis and other stresses are yet to influence productivity. There is also genetic variability in the TR to VPD relationship (Bunce, 1984; Sadok and Sinclair, 2009a). If there are interactions between genotype and mild drought for both  $WUE_{gH_2O}$  and  $WUE_{PC}$ , it would complicate breeding efforts to improve soybean water use. Therefore,  $WUE_{PC}$  and  $WUE_{gH_2O}$  have been measured over a range of conditions for 11 soybean genotypes, with the aim to elucidate whether genotype by environment interactions are present.

## Materials and methods

Experiments were conducted in the Harvard Greenhouse Facility between August and November 2009 at Harvard University, Cambridge, MA, USA (42°N, 71°W). The first two experiments and the drought experiment were used to screen as many genotypes as possible for classic gas exchange traits, while the third experiment involved imposition of a mild drought (Table 1). For all experiments, air temperatures were maintained between a daily maximum of 27°C and minimum of 17°C measured within a screened box. The natural light environment was supplemented with a combination of 500W metal halide and high-pressure sodium lamps, enhancing the natural photosynthetic photon flux density (PPFD) by 500–600  $\mu\text{mol m}^{-2}\text{ s}^{-1}$  for 16h  $\text{d}^{-1}$ .

### Plant culture

Seeds were germinated in seedling trays, and transplanted to 2.2l pots once the first leaves had started to develop. Pots were filled with Farfard 3B potting soil including Osmocote Plus slow release fertilizer as per the manufacturer's instructions (15/9/12/1 N/P/K/Mg+trace elements: S, B, Cu, Fe, Mn, Mo, Zn; Scotts Company). The pots

**Table 1.** Summary of the experiments

Experiment	Measurements	Sowing date	Measurement period	Conditions
Experiment 1	$V_{\text{cmax}}$ and $J_{\text{max}}$ gas exchange measurements	22 August	16–23 September	Well watered
Experiment 2	$V_{\text{cmax}}$ and $J_{\text{max}}$ gas exchange measurements	8 September	28 September–6 October	Well watered
Drought experiment	$A$ , $g_{\text{H}_2\text{O}}$ , and $V_{\text{cmax}}$ gas exchange measurements, leaf water potentials, and soil water contents	7 October	1–12 November	Drought imposed on half of the plants

were watered daily and fertilized weekly with a water-soluble fertilizer (21/5/20 N/P/K+trace elements: B, Cu, Fe, Mn, Mo, Zn; Peters Excel All Purpose fertilizer, Scotts Company).

Seeds of 11 soybean genotypes were provided by T.E. Carter (USDA-ARS, Raleigh, NC, USA), and selected to represent genotypes with a variety of TRs at high VPDs and a variety of TR to VPD responses (Table 2). Four and seven genotypes were chosen to represent genotypes with increasing TR at high VPD and constant TR at high VPD, respectively. Of the 11 genotypes, the genotypes with increasing TR at high VPD were reported to have the highest TR at a VPD of 3.5kPa similar to that which occurred in these experiments (Table 2). PI416937 has been demonstrated to be slow wilting (King *et al.*, 2009), corresponding to a constant TR at high VPD (Fletcher *et al.*, 2007), and consequently was crossed with the standard cultivar Benning. The progeny, ‘G00BP’-labelled recombinant inbred lines (RILs), displayed a range of TR to VPD responses in excess of that shown by the parents, which both had constant TR at high VPD (Sadok and Sinclair, 2009a). Five of these RILs, and the parents, were selected to represent the range of these responses, including one genotype with increasing TR at high VPD, G00BP-147. In addition, a standard reference cultivar (Biloxi), two recent commercial cultivars (N96-7031 and N01-11136), and a second plant introduction (PI471938) were also included. PI471938 was chosen as it had a high  $\text{WUE}_{\text{wholeplant}}$  relative to PI418937 (Hufstetler *et al.*, 2007), suggesting that variation in  $\text{WUE}_{\text{intr}}$  may be present, and was slow wilting (King *et al.*, 2009); however, this is likely to be due to a mechanism different from that in PI416937 as this genotype has increasing TR at high VPD (Fletcher *et al.*, 2007). N96-7031 has similar  $\text{WUE}_{\text{wholeplant}}$  to PI416937 (Hufstetler *et al.*, 2007), but an increasing TR to VPD response (Sadok and Sinclair, 2009a). N01-11136 represents the most extreme genotype with increasing TR at high VPD, with the highest whole-plant TRs (Sadok and Sinclair, 2009a). As the majority of the selected genotypes were descended from PI416937, this was selected as the reference genotype.

#### Drought experiment

Once the seedlings reached the V3 stage of growth, ~20–30cm tall and with between two and three fully expanded trifoliolate leaves, the pots were randomly assigned to a control (well-watered) or drought treatment. Sample sizes were five control and six drought treatment plants per genotype. Initial measurements were made on 1 and 2 November on all plants prior to imposition of drought. Pots were submerged in water to achieve saturation and, after allowing 2h for excess water to drain, the pot water-holding capacity was determined. Subsequently pot weights were measured every day, within a 2h period, after sunset. Upon imposition of the drought treatment on 3 November, all pots were watered to a pre-determined weight, calculated to result in an 85% soil water content (SWC) for control plants and a standard SWC for drought-stressed plants. In this way, drought could be imposed in a consistent and gradual manner for all plants, regardless of leaf

**Table 2.** VPD response characteristics and origin of the 11 soybean genotypes for which the drought experiment was undertaken

Genotype categorization and TR predictions were made from data in Fletcher *et al.* (2007), Sinclair *et al.* (2008b), and Sadok and Sinclair (2009a, b)

Genotype	Origin	$\text{WUE}_{\text{wholeplant}}$ (g DM kg <sup>-1</sup> H <sub>2</sub> O) <sup>a</sup>	TR predicted for 3.5 kPa (mg H <sub>2</sub> O m <sup>-2</sup> s <sup>-1</sup> )
Genotypes with increasing TR at high VPD			
N01-11136 <sup>b</sup>	NTCPR94-5157×N96-7031		76.7
PI471938	Plant introduction from Nepal	3.38	75.3
G00BP-147 <sup>b</sup>	PI416937×Benning		63.7
N96-7031 <sup>b</sup>	N7001×N90-7241	2.82	62.0
Genotypes with constant TR at high VPD			
PI416937	Plant introduction from Japan	2.81	62.0
Biloxi	Historical cultivar		59.6
G00BP-60 <sup>b</sup>	PI416937×Benning		52.6
G00BP-160 <sup>b</sup>	PI416937×Benning		48.5
G00BP-59 <sup>b</sup>	PI416937×Benning		39.8
G00BP-78 <sup>b</sup>	PI416937×Benning		39.3
Benning	Hutcheson×Coker 6738	3.14	34.6

<sup>a</sup> Hufstetler *et al.* (2007)

<sup>b</sup> Descendant of PI416937.

area. Drought was imposed over 7d, during which the SWC of the drought-treated plants was returned to 85, 75, 65, 50, or 45% SWC each day at the time of weighing. At the end of the experiment, the leaves and stems were removed, and pots were dried for 2 weeks in a forced draught oven at 85°C, and SWC was determined as (fresh weight–dry weight)/(field capacity weight–dry weight)×100.

The average canopy air temperatures and humidity measured simultaneously with the gas exchange measurements for the drought experiment, using a screened Vaisala probe, ranged from 29.5°C to 31.6°C and 14.2% to 28.5% relative humidity, corresponding to a range in mid-day average VPD of 3.0–4.0kPa. All drought-stressed plants (SWC <50%) had leaf temperatures well below the reported >33–35°C leaf temperature threshold for thermal inhibition of soybean photosynthesis (Harley *et al.*, 1985), eliminating the possibility of artefacts due to the reduced evaporative cooling of drought-stressed leaves. The SWCs chosen to represent mesic and mild drought conditions corresponded to average leaf water potentials of –0.62MPa and –1.05MPa, respectively (leaf water potentials

were measured on six genotypes on the final day of drought for both well-watered and drought-stressed plants;  $n=4-5$  per treatment). The most severe leaf water potentials were between  $-1.2$  MPa and  $-1.4$  MPa, close to the minimum extractable soil water potential for soybean of approximately  $-1.2$  MPa (Brisson *et al.*, 1993; Sinclair *et al.*, 2008a). Leaf water potentials were measured with a Scholander-type pressure chamber after transpiring leaves were bagged, excised, and allowed to equilibrate for 10 min in a dark cool container.

#### Gas exchange measurements

Gas exchange for the drought experiment was measured using a LI-6400 photosynthesis system with a red-blue light source and  $6\text{ cm}^2$  cuvette. At the start of the drought experiment the youngest fully expanded primary leaflet (mostly second trifoliolate) was marked and subsequently used for all gas exchange measurements. To standardize the environmental conditions, an hour before measurement each plant was placed on the edge of the greenhouse bench, with the marked leaf oriented to receive full sunlight. Gas exchange measurements were made on the majority of plants on 1, 4, 6, and 8 November from 10:00 to 15:00 h. As not all plants could be measured on a single day, the remaining third of the plants were measured on 2 and 7 November. Cuvette conditions were: PPF,  $2000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ; flow rate,  $500\ \mu\text{mol s}^{-1}$ ; sample  $\text{CO}_2$ ,  $390\ \mu\text{mol mol}^{-1}$ ; VPD,  $2.3 \pm 0.5\ \text{kPa}$ ; leaf temperature,  $31.3 \pm 1.6^\circ\text{C}$  (mean  $\pm$  SD); and the cuvette fan was set to fast. Leaf temperature was allowed to track ambient conditions; however, the cuvette was shaded, and block temperature cooled to  $32^\circ\text{C}$  when leaf temperatures were  $>33^\circ\text{C}$ .

Well-watered plants from the drought experiment and the two earlier experiments were used to determine the response of photosynthetic traits to leaf temperature. A screening approach was used to allow many plants to be measured and  $\text{CO}_2$  response curve traits related to  $\text{WUE}_{\text{PC}}$ . Namely, the  $\text{CO}_2$  and light-saturated rate of photosynthesis was used to estimate the maximal rate of RuBP regeneration ( $J_{\text{max}}$ ), and the initial slope of the  $\text{CO}_2$  response curve was used to provide an estimate of Rubisco carboxylation capacity ( $V_{\text{cmax}}$ ) (see Supplementary Fig S1, available at *JXB* online). Leaf temperature was allowed to vary with ambient conditions, which resulted in a  $>10^\circ\text{C}$  range across days.  $\text{CO}_2$ -saturated photosynthetic rates were measured on the second trifoliolate, or marked leaf of on average 16 plants per genotype. The  $2\text{ cm}^2$  LI-6400 fluorescence chamber was used for this experiment. Cuvette conditions were: PPF,  $2000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ; flow rate,  $250\ \mu\text{mol s}^{-1}$ ; sample  $\text{CO}_2$ ,  $1990\ \mu\text{mol mol}^{-1}$ ; VPD,  $1.6 \pm 0.3\ \text{kPa}$  (mean  $\pm$  SD); leaf temperature,  $22.5-32.9^\circ\text{C}$ . Additional full  $\text{CO}_2$  response curves demonstrated that photosynthetic rates at high  $\text{CO}_2$  were not significantly triose phosphate limited, thus photosynthetic rates at very high  $\text{CO}_2$ , where photorespiration is competitively inhibited, are approximately equal to a quarter of the maximal rate of RuBP regeneration (von Caemmerer, 2000) ( $J_{\text{max}} \approx 4 \times A$  at  $1990\ \mu\text{mol mol}^{-1}\ \text{CO}_2$ ).  $V_{\text{cmax}}$ —the initial slope of the  $\text{CO}_2$  response curve—was measured for a subset of the 11 genotypes by varying sample  $\text{CO}_2$  from 225, 175, 125, 75, to  $50\ \mu\text{mol mol}^{-1}$ . The initial slope value was estimated from linear regression of points with  $C_i$ s  $<200\ \mu\text{mol mol}^{-1}$ , and was equated to the derivative of the Rubisco-limited photosynthesis function (von Caemmerer, 2000) at the photocompensation point, which was then solved for  $V_{\text{cmax}}$ , using the temperature corrections and values of  $K_c$ ,  $K_o$ , and  $\Gamma^*$  of von Caemmerer (2000). The genotypes used represented three with constant TR at high VPD (PI416937, G00BP-160, and Benning) and three with increasing TR at high VPD (N96-7031, PI471938, and N01-11136). The LI-6400  $6\text{ cm}^2$  cuvette was used for these experiments. Cuvette conditions were: PPF,  $2000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ; flow rate,  $400\ \mu\text{mol s}^{-1}$ ; VPD,  $1.6 \pm 0.6\ \text{kPa}$  (mean  $\pm$  SD); leaf temperature,  $21.9-34.4^\circ\text{C}$ . This data set was augmented with measurements of  $V_{\text{cmax}}$  made on 3–5 leaves of three other genotypes at leaf temperatures within  $1^\circ\text{C}$  of  $30^\circ\text{C}$ . Both sets of measurements were corrected for  $\text{CO}_2$  leaks using an

empirical relationship, where  $\text{CO}_2$  leaks were measured during  $\text{CO}_2$  response curves as the  $\text{CO}_2$  concentration difference between the previously matched reference and sample infrared gas analysers (IRGAs) in an empty chamber with the above conditions.

#### Data analysis

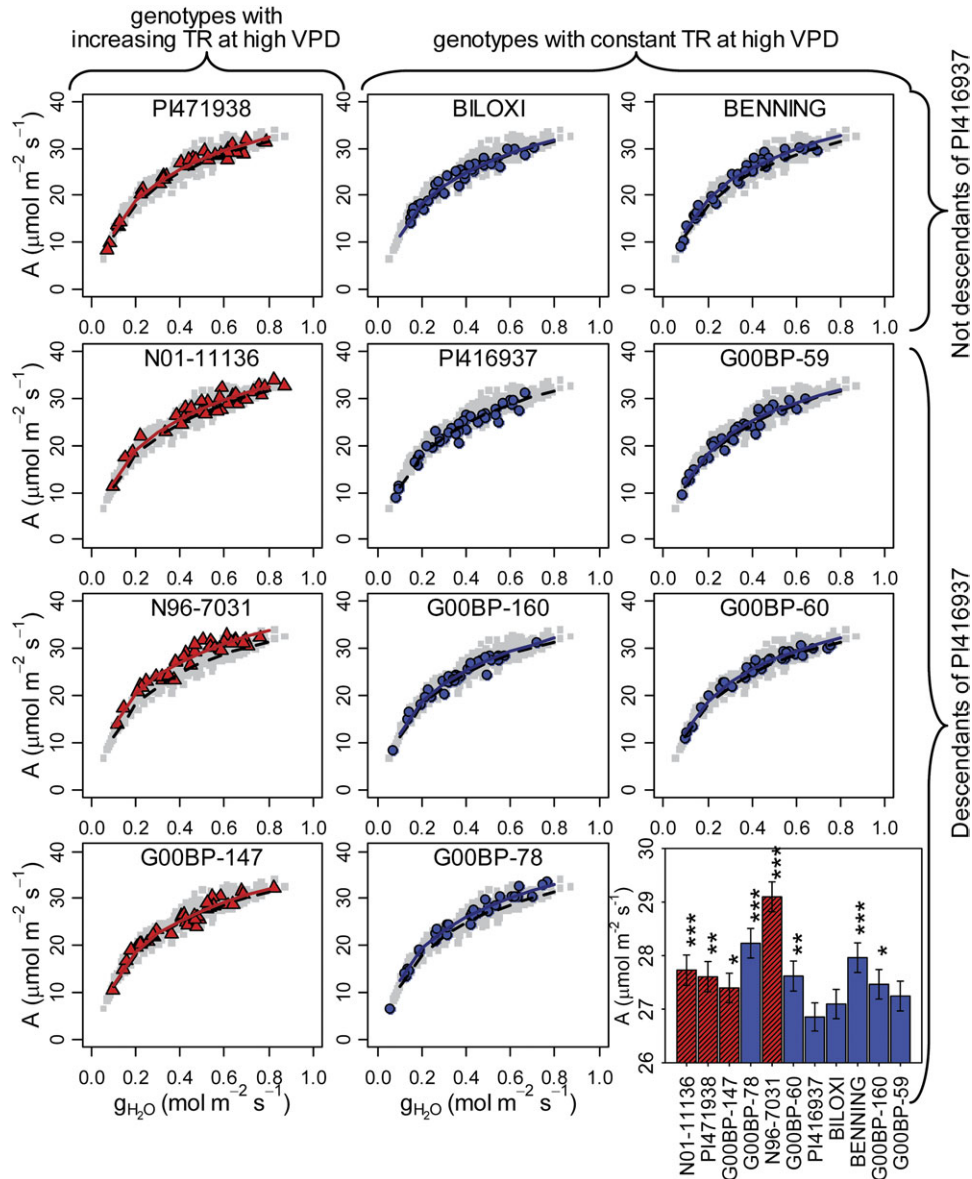
The R statistical and graphical package (R\_Development\_Core\_Team, 2010) was used to perform general linear model (GLM) analyses as follows. As the photosynthesis versus stomatal conductance relationship scaled non-linearly, it was not appropriate to analyse  $\text{WUE}_{\text{intr}}$  as a ratio (Jasienski and Bazzaz, 1999), therefore a standard GLM approach to data analysis was adopted (Müller *et al.*, 2000; Crawley, 2005). Hence, photosynthesis was analysed through a GLM of the natural logarithm of stomatal conductance to  $\text{H}_2\text{O}$ ; similarly  $J_{\text{max}}$  was predicted by leaf temperature, and the natural logarithm of  $V_{\text{cmax}}$  predicted by the natural logarithm of leaf temperature (a power function). In these analyses, genotype and the interaction with the linear predictor were initially included—testing for differences in slope. However, the presented results represent minimum adequate models where unnecessary (non-significant) higher order interactions have been removed using the *step()* function of the R statistical package (Crawley, 2005). That is, if there was no significant genotype with linear predictor interaction, the slopes were assumed to be the same and this term was removed from the analysis, as per a standard analysis of covariance (ANCOVA). PI416937, an ancestral genotype of the majority of the genotypes measured in this study, was used as the reference genotype with which changes in most variables including  $\text{WUE}_{\text{intr}}$ ,  $\text{WUE}_{\text{PC}}$ , and  $\text{WUE}_{g_{\text{H}_2\text{O}}}$  were compared, and relative to which the significance of linear model coefficients were compared. This avoided the use of many *post hoc* comparisons, whilst still providing information on significant differences between genotypes.

Stomatal conductance to  $\text{H}_2\text{O}$  was non-linearly related to SWC, with  $g_{\text{H}_2\text{O}}$  values at high and low SWC reduced relative to SWCs at  $\sim 85\%$ . A quadratic equation best accounted for this variation, and allowed a linear model analysis to be performed testing for effects of genotype, SWC,  $\text{SWC}^2$ , and interactions. To summarize these data, the SWCs used to regulate pot water content were selected to represent mesic (85% SWC) and drought conditions (50% SWC). Using the linear model for  $g_{\text{H}_2\text{O}}$  to SWC, average values of  $g_{\text{H}_2\text{O}}$  were estimated for each genotype and SWC. In turn, the average  $A$  value for these  $g_{\text{H}_2\text{O}}$ s were estimated from the linear model for  $A$  to  $g_{\text{H}_2\text{O}}$ . Using these averages and regression equations, values of  $\text{WUE}_{\text{intr}}$ ,  $\text{WUE}_{\text{PC}}$ , and  $\text{WUE}_{g_{\text{H}_2\text{O}}}$  for each genotype could be estimated relative to PI416937.

## Results

### *Intrinsic water use efficiency and variation in components*

The relationship between  $A$  and  $g_{\text{H}_2\text{O}}$  measured over the course of the mild drought had significant curvature, and the shape was highly conserved across the 11 soybean genotypes (Fig. 2; Supplementary Table S1, at *JXB* online), although there were differences between genotypes in the elevation of the curve. The genotype  $\times g_{\text{H}_2\text{O}}$  interaction was not significant ( $F_{10,358}=0.703$ ,  $P=0.721$ ), although genotype had a significant effect (Supplementary Table S1.). As this analysis used the natural logarithm of  $g_{\text{H}_2\text{O}}$  to predict  $A$ , this signifies that the between-genotype differences in  $A$  for a given  $g_{\text{H}_2\text{O}}$  were consistent across the range of  $g_{\text{H}_2\text{O}}$  induced by drought. Thus, all genotypes apart from Biloxi and G00BP-59 had significantly higher  $A$  than PI416937 across the entire range of  $g_{\text{H}_2\text{O}}$  (Supplementary Table S1.). As Biloxi, a historical



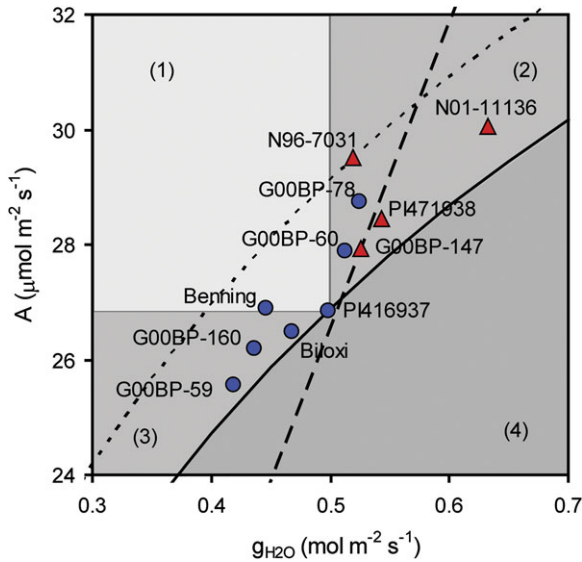
**Fig. 2** Differences between genotypes in photosynthesis ( $A$ ) and intrinsic water use efficiency ( $WUE_{intr}=A/g_{H_2O}$ ) during drought-induced variation in stomatal conductance to  $H_2O$  ( $g_{H_2O}$ ). Each panel shows measurements made for a soybean genotype (dark symbols) and points for all genotypes combined (light squares). The fitted relationships (using the natural logarithm of  $g_{H_2O}$ ) are shown for each genotype (solid line) and relative to the relationship for PI416937 (dashed line). The final panel shows variation among genotypes in photosynthetic rate at the average  $g_{H_2O}$  of PI416937 at a SWC of 85% ( $0.498 \text{ mol m}^{-2} \text{ s}^{-1}$ ). Statistical differences and standard errors of the mean are derived from Supplementary Table S1. at *JXB* online. Genotypes were ranked in order of greatest (N01-11136) to lowest  $g_{H_2O}$  (G00BP-59). Soybean genotypes with a constant TR at high VPD relationship (circles and solid bars) and genotypes with increasing TR at high VPD (triangles and hatched bars) are shown.

reference cultivar, was similar to PI416937 in both  $g_{H_2O}$  and  $A$ , it appears that the choice of a plant introduction as reference genotype affects these results little.

$WUE_{intr}$  varied greatly between genotypes (Figs 3, 4), largely due to the curvature of the relationship between  $A$  and  $g_{H_2O}$ , and variation in  $g_{H_2O}$  between genotypes (Fig. 3; Supplementary Table S1.). The average genotype  $g_{H_2O}$  under well-watered conditions varied by 43%, between a high of  $0.632 \text{ mol m}^{-2} \text{ s}^{-1}$  for N01-11136 and a low of  $0.42 \text{ mol m}^{-2} \text{ s}^{-1}$  for G00BP-59, relative to the reference genotype, leading to 15% variation in  $A$ , given no differences in photosynthetic

capacity (Fig. 3). However, enhanced photosynthetic capacity resulted in up to 8.3% increases in  $A$  for N96-7031, 5.1% increases in G00BP-78, and 4.1% in Benning relative to PI416937. This resulted in increased  $WUE_{intr}$  for genotypes with low  $g_{H_2O}$  [e.g. region (3)] or intermediate to high  $g_{H_2O}$  [e.g. region (2)], and in some genotypes the increased photosynthetic capacity entirely compensated for reductions in  $A$  due to low  $g_{H_2O}$  (e.g. Benning) (Fig. 3).

When shown as the percentage difference in  $WUE$  between a genotype of interest and the reference PI416937,  $WUE_{intr}$  changed largely due to variation in  $WUE_{g_{H_2O}}$ , and to a lesser



**Fig. 3** Variation in the average photosynthesis ( $A$ ) and stomatal conductance to  $H_2O$  ( $g_{H_2O}$ ) operating points between well-watered soybean genotypes relative to the fitted  $A$  to  $g_{H_2O}$  relationship for PI416937 (solid line) and N96-7031 data (dotted line). Soybean genotypes with a constant TR at high VPD (circles) and genotypes with increasing TR at high VPD (triangles) are shown. The diagonal dashed line represents points of equal intrinsic water use efficiency to PI416937. The numbers and shaded regions represent four categories of soybean genotypes having: (1) increased photosynthetic rates and reduced  $g_{H_2O}$  relative to PI416937; (2) higher photosynthesis, but increased  $g_{H_2O}$  relative to PI416937—genotypes have a range of  $WUE_{intr}$ , some greater or less than PI416937; (3) reduced  $A$  and  $g_{H_2O}$  relative to PI416937; and (4) generally less  $WUE_{intr}$  than PI416937. These categories are described in detail in the Discussion.

degree with  $WUE_{PC}$  (Fig. 4A). A quadratic function was used to conduct a linear model analysis of the relationship of  $g_{H_2O}$  to SWC (Supplementary Table S1. at *JXB* online). The genotype by SWC interaction was significant, as was the  $SWC^2$  term. Due to the significant genotype by SWC interaction, the average  $g_{H_2O}$  measured for genotypes under mesic conditions was uncorrelated to those measured under mild drought ( $R^2=0.254$ ,  $n=11$ ,  $P=0.114$ , for average  $g_{H_2O}$  of the genotypes at 50% and 85% SWC). Genotype  $g_{H_2O}$  response interacted with SWC (Supplementary Table S1.), and therefore  $WUE_{intr}$  advantages under mesic conditions were not maintained for all genotypes under drought conditions, although the  $WUE_{PC}$  advantages were constant (Fig. 4B). Exceptions were Benning, which had high  $WUE_{intr}$  due to favourable  $WUE_{g_{H_2O}}$  and  $WUE_{PC}$  under both mesic and drought conditions, and N01-11136 which had low  $WUE_{intr}$  due to extremely high  $g_{H_2O}$  under all conditions.

#### Mechanisms of variation in $WUE_{PC}$

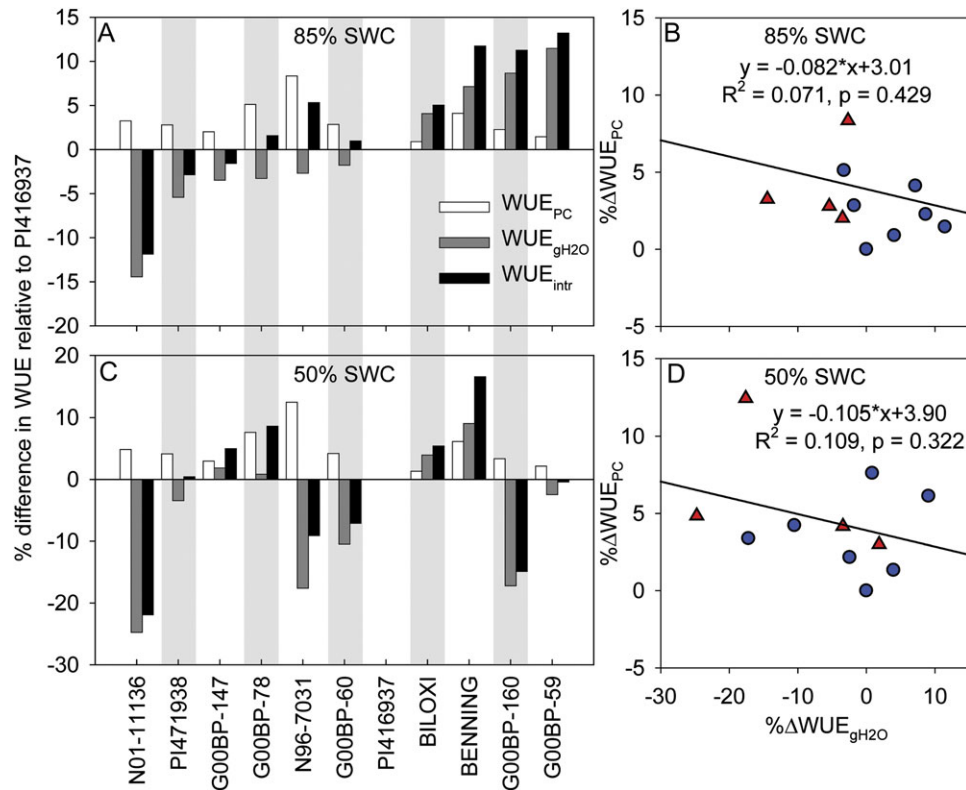
Knowledge of  $WUE_{PC}$  seems particularly useful for breeders, because variation in photosynthetic capacity was

not correlated to  $g_{H_2O}$  (Figs 3, 4), and  $WUE_{PC}$  did not interact with drought. It was thus sought to establish the mechanisms underlying variation in  $WUE_{PC}$ , and to establish a link with a trait that would allow easy field selection of high  $WUE_{PC}$  genotypes. Leaf mass area (LMA), a rough estimate of the resource investment in a unit area of leaf (Wright *et al.*, 2004), did not predict  $WUE_{PC}$  (Fig. 5A), although there was significant variation between genotypes ( $F_{10,103}=4.08$ ,  $P<0.002$ ).  $J_{max}$ , which limits photosynthesis at low light or high  $CO_2$ , corresponded only roughly to  $WUE_{PC}$  (Fig. 5B), though there were significant differences between genotypes in  $J_{max}$  (Supplementary Table S2 at *JXB* online).  $V_{cmax}$ , which typically limits photosynthesis at low to intermediate  $g_{H_2O}$ , correlated significantly to  $WUE_{PC}$  for six genotypes for which temperature responses were measured (Fig. 5C); the inclusion of three extra genotypes measured at a set temperature (unfilled symbols) confirmed the relationship. Across an  $\sim 13^\circ C$  range of leaf temperatures the genotypes displayed a temperature effect for  $V_{cmax}$  and  $J_{max}$  significantly affecting the elevation of the  $V_{cmax}$  or  $J_{max}$  to  $T_{leaf}$  relationship, but not the slope (Supplementary Fig S2., Fig S3.). Thus, the differences in  $V_{cmax}$  and  $J_{max}$  across genotypes were consistent across all  $T_{leaf}$  conditions. Consistent with this pattern,  $T_{leaf}$  did not have a significant effect on  $WUE_{intr}$  ( $F_{1,357}=0.428$ ,  $P>0.513$ ), or interact with genotype ( $F_{10,357}=0.634$ ,  $P>0.785$  for the model  $WUE_{intr} \sim \text{genotype} \times T_{leaf} + SWC + \text{error}$ ). Modelling of the measured  $V_{cmax}$  and  $J_{max}$  values using standard photosynthetic functions (von Caemmerer, 2000) demonstrated that  $V_{cmax}$  was limiting, or co-limiting, to photosynthesis at virtually all values of  $g_{H_2O}$  (data not shown). The average  $Q_{10}$  values for all genotypes combined over the measured  $22\text{--}32^\circ C$  range were 2.15 and 1.67 for  $V_{cmax}$  and  $J_{max}$ , respectively. As these compare favourably with published values, which indicate a higher  $Q_{10}$  value for  $V_{cmax}$  than  $J_{max}$  e.g.  $Q_{10}=2.21$  and 1.65 from  $25^\circ C$  to  $35^\circ C$  (von Caemmerer, 2000), the fitting procedures adopted here appear adequate. Thus, genotypes with increased  $V_{cmax}$  consistently led to  $WUE_{PC}$  advantages during mesic and drought conditions and over a  $10^\circ C$  range of temperatures.

## Discussion

### Breeding strategies relating to soybean water use

A plant could be bred to avoid drought through conservation of soil moisture by reducing stomatal conductance to water at high VPD (a constant TR at high VPD) and thus avoiding future drought. While this strategy would result in a net increase in  $WUE_{intr}$  (Fig. 1), it would be at the expense of photosynthesis during periods of high VPD. Typical stomatal conductance reductions of 33% would result in 14% reductions in photosynthesis under high VPD, well-watered conditions, and would be larger in the field (personal observation) where hotter temperatures, wind, and soil water depletion are more important. Therefore, it would be beneficial to breed such water-conserving plants with enhanced  $WUE_{PC}$  to compensate for the photosynthetic loss



**Fig. 4** Variation in intrinsic water use efficiency ( $WUE_{intr}$ ) between soybean genotypes at two levels of drought (C and D, 50% SWC; and A and B, 85% SWC), and components of  $WUE_{intr}$  caused by variation in photosynthetic capacity ( $WUE_{PC}$ ) and stomatal conductance to  $H_2O$  ( $WUE_{gH_2O}$ ). All WUE values are expressed as percentage differences ( $\% \Delta WUE$ ) relative to PI416937, the ancestral genotype of the majority of the other genotypes. Genotypes were ranked in order of greatest (N01-11136) to lowest  $g_{H_2O}$  (G00BP-59). Soybean genotypes with a constant TR at high VPD (circles) and genotypes with increasing TR at high VPD (triangles) are shown.

associated with reduced stomatal conductance at high VPD. Breeding plants with low water use during mild drought could also involve breeding for an increase in photosynthetic capacity ( $WUE_{PC}$ ), thus enabling the plant to photosynthesize under stomatal limiting conditions, where turgor-related growth inhibition may be present but water stress effects are not severe (Hsiao, 1973). This assumes that a photosynthetic capacity advantage is maintained under such drought conditions. These two strategies of water conservation and mild drought tolerance are not mutually exclusive as both could involve selection for increased photosynthetic capacity.

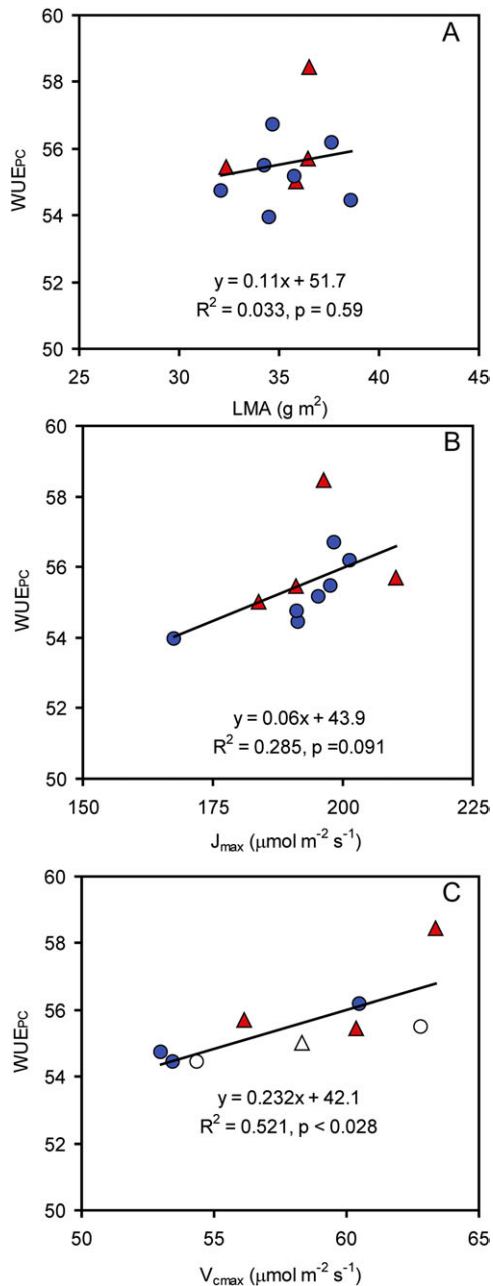
Fortunately, it appears that for soybean genotypes photosynthetic capacity is a constitutive trait, which does not interact with drought or temperature and is thus a potentially useful characteristic for breeders. However, the rankings of genotypes relative to TR do vary with VPD (Sadok and Sinclair, 2009a). This combination of differentially varying traits offers much choice for breeders. As photosynthetic capacity is independent of the presence of the constant TR at high VPD trait, this signifies that reduced productivity is not necessarily a consequence of breeding for water conservation under high VPD, and that the selection strategies suggested here may be successful.

Three possible breeding strategies present themselves when choosing a genotype of interest for breeding relative to a

reference genotype, and based upon gas exchange measurements made under well-watered conditions. First, one could select for high photosynthetic rates (which it is assumed translate into some productivity advantage) through either increased  $g_{H_2O}$  or photosynthetic capacity. It is important to note that this strategy corresponds to the plants in region (2) in Fig. 3, and can be consistent with increases or decreases in  $WUE_{intr}$ . It seems likely that such plants, while using the same amount as (e.g. N96-7031 and G00BP-78) or more water than (e.g. N01-11136) the reference genotype (PI416937) or the standard cultivar Biloxi would have the potential for higher productivity and thus be generally desirable for non-water-limiting conditions. While it is difficult to extrapolate from greenhouse data as to which genotypes would be useful in the field, it does seem evident that genotypes with increasing TR at high VPD exemplify this selection strategy (Fig. 3). Past work suggests that this strategy was employed for Canadian short season soybeans over the last half-century (Morrison et al., 1999), although it is not necessarily the case that photosynthetic increases will increase productivity (Ford et al., 1983).

The second selection strategy is to breed plants that conserve water but also maintain some photosynthetic capacity advantage relative to the reference genotype [selection of genotypes in region (3) of Fig. 3]. Although plants





**Fig. 5** Relationships of photosynthetic capacity, estimated as WUE at a common  $g_{H_2O}$  ( $WUE_{PC}$ ), to average leaf mass area (A), the rate of RuBP regeneration ( $J_{max}$ ) (B), and the maximal rate of Rubisco carboxylation ( $V_{cmax}$ ) (C).  $J_{max}$  and  $V_{cmax}$  values were corrected to a common leaf temperature of 31.3°C (the average leaf temperature at which  $WUE_{PC}$  was measured), using fitted linear or power temperature response functions.  $WUE_{PC}$  was calculated by using photosynthetic values derived from Fig. 3 assuming the same stomatal conductance to  $H_2O$  ( $0.498 \text{ mol m}^{-2} \text{ s}^{-1}$ , the average value for PI416937 at 85% SWC). Open symbols in (C) represent average data for leaves measured within 1°C of 30°C ( $n \geq 3$ ), and temperature corrected using the fitted power response function used for the other genotypes. Soybean genotypes with a constant TR at high VPD (circles) and genotypes with increasing TR at high VPD (triangles) are shown.

selected for these traits may avoid drought through water conservation during mesic periods, this would be associated with a reduction in productivity as there is only a partial photosynthetic compensation through higher photosynthetic capacities. The third selection strategy involves breeding genotypes for region (1) of Fig. 3, where reduced water use is associated with potential for higher productivity through higher photosynthetic capacities. While no soybean genotypes tested in this study filled this space, one may envisage a progeny line from a cross of N96-7031 and Benning, with a combination of the low stomatal conductance of Benning and the high photosynthetic capacity of both parents falling into region (1). Indeed, the observed 8.3% increase in photosynthetic capacity for N96-7031 would compensate for photosynthetic decreases caused by a constitutive 20% decrease in  $g_{H_2O}$ . Genotypes in region (4) would offer little benefits to breeders, as they would have reduced photosynthetic rates relative to the reference genotype's  $A$  to  $g_{H_2O}$  relationship. Note that some of the areas within region (4)—areas above the dashed line, but below the curve—actually have greater  $WUE_{intr}$  than PI416937, but would have depressed photosynthetic rates relative to a genotype with equivalent photosynthetic capacity to PI416937, and reduced  $g_{H_2O}$ . This illustrates the point that increased  $WUE_{intr}$  relative to the reference genotype is not necessarily associated with advantageous changes in the components of  $WUE_{intr}$ . These considerations are also valid at a whole-plant level: a high  $WUE_{wholeplant}$  genotype is only of interest if it has a high productivity (Sinclair and Muchow, 2001), a fact not immediately obvious from the value of  $WUE_{wholeplant}$ . The breeding strategies suggested here are obviously contingent upon a number of assumptions. Do leaf-level processes directly scale with crop-level productivity and water use? Are photosynthetic advantages under well-watered conditions representative of drought or varying environmental conditions?

In practice, variation in transpiration, and  $g_{H_2O}$ , is likely to have a greater effect on  $WUE_{intr}$  than photosynthetic capacity when comparing genotypes within a crop species (Blum, 2005, 2009), and measured under controlled conditions (no variation in nitrogen or light). This is due to the large curvature of the  $A$  to  $g_{H_2O}$  response for soybean, and the typical high position of photosynthetic operating points on this response in well-watered crops (Fig. 2). This curvature results in seemingly beneficial increasing  $WUE_{intr}$  under mild drought or reduced  $g_{H_2O}$  at high VPD (Fig. 2), but it is important to note that it is also associated with decreasing  $A$ . A number of other studies confirm that  $WUE_{wholeplant}$  can be increased under mild drought (Hubick and Farquhar, 1989), while others find little change (Hubick *et al.*, 1986; Liu and Stutzel, 2004). This variation may relate to the position of the typical operating point of these species on the  $A$  to  $g_{H_2O}$  curve. Obviously, reductions in leaf-level transpiration are concomitant with mild drought-induced productivity decreases, and therefore undesirable. Thus it is important for mild-drought stressed— $g_{H_2O}$ -limited—plants, or genotypes with constitutive traits conferring low  $g_{H_2O}$ , to have increased photosynthetic capacities to compensate for this effect.

*The nature of variation in photosynthetic capacity*

During the transition from mesic conditions to those of high VPD or mild drought, stomatal conductance is likely to become increasingly limiting to the supply of CO<sub>2</sub> for photosynthesis. The major biochemical limitations to photosynthesis, V<sub>cm<sub>ax</sub></sub> and J<sub>max</sub>, are less likely to change directly with VPD although between-genotype variation in these traits is a likely cause of differences in photosynthetic capacity. However, high VPD is dependent on high temperatures, which affect whether V<sub>cm<sub>ax</sub></sub> or J<sub>max</sub> is limiting to photosynthesis (Hikosaka, 1997; Hikosaka *et al.*, 1999). Therefore, it is necessary to determine both the cause of the observed differences in photosynthetic capacity and the variation of photosynthetic capacity limitations with temperature. From the measured temperature response data it was possible to use standard photosynthetic models to determine which process was limiting to photosynthesis under given temperature and g<sub>H<sub>2</sub>O</sub> conditions (von Caemmerer, 2000). The majority of photosynthesis measurements were on the portion of the CO<sub>2</sub> response limited by V<sub>cm<sub>ax</sub></sub> across the observed range of g<sub>H<sub>2</sub>O</sub>. Thus, the consistency of the photosynthetic advantages across a broad range of g<sub>H<sub>2</sub>O</sub> is partially due to the lack of limitation by J<sub>max</sub> under mesic conditions that would be associated with transitions to V<sub>cm<sub>ax</sub></sub> limitation under drought. Consistent with this finding is the correlation between WUE<sub>PC</sub> and V<sub>cm<sub>ax</sub></sub>, but not J<sub>max</sub> (Fig. 5). Thus, V<sub>cm<sub>ax</sub></sub> appears to be the major source of variation in photosynthetic rates between soybean genotypes for a given g<sub>H<sub>2</sub>O</sub>. It is possible that the consistent V<sub>cm<sub>ax</sub></sub> limitation is an artefact of the study being conducted in greenhouse conditions, resulting in greater leaf light capture capacity than may occur in the field. However, Rubisco-limited photosynthesis in the current study is consistent with Bernacchi *et al.* (2005) who found that field-grown soybeans were largely Rubisco or co-limited for much of the year, with increasing J<sub>max</sub> over the growth season.

A second source of consistent photosynthetic capacity advantages may be due to the similarity of V<sub>cm<sub>ax</sub></sub> and J<sub>max</sub> temperature responses between genotypes. Any differences in the temperature responses of J<sub>max</sub> and V<sub>cm<sub>ax</sub></sub> would be important as they determine the co-limitation point of the CO<sub>2</sub> response curve, and therefore which factor limits photosynthesis. A third source of variation in *A* over a range of g<sub>H<sub>2</sub>O</sub> may be shifts from stomatal to metabolic limitations of photosynthesis under drought conditions (Medrano *et al.*, 2002; Ennahli and Earl, 2005; Lawlor and Tezara, 2009). This did not appear to be the case for the plants measured during the drought (based upon photosynthetic modelling of observed C<sub>i</sub>, *A*, V<sub>cm<sub>ax</sub></sub>, and J<sub>max</sub>; data not shown). The magnitude of the stomatal and metabolic limitations increased similarly with decreasing g<sub>H<sub>2</sub>O</sub> and did not appear to differ between genotypes. As differential metabolic limitations may dramatically change the shape of the *A* to g<sub>H<sub>2</sub>O</sub> relationship at low g<sub>H<sub>2</sub>O</sub> (Ripley *et al.*, 2010), it is important that there are no systematic genotype differences. Metabolic limitations are likely to become important under severe drought—a state which the water-saving genotypes are more able to avoid.

*Extension of these breeding strategies to the field*

Inherent in these strategies are a number of assumptions concerning the scaling up of leaf-level processes to the canopy (Richards, 2000; Long *et al.*, 2006): for instance, that the crop's total transpiring leaf area remains unchanged during drought, or that the leaf is well coupled with environmental conditions (Sinclair *et al.*, 1984). Thus, while knowledge of components of WUE<sub>intr</sub> may be a useful tool to identify potential breeding genotypes, whole-plant and field validations of how well these traits scale up to canopy conditions are vital.

These results are also predicated upon a positive relationship between photosynthesis and seed yield under breeding conditions. Past studies have shown varying correlations between photosynthesis and seed yield in soybean: with selection for *A* being unrelated to yield (Ford *et al.*, 1983), and other studies demonstrate that canopy photosynthesis may relate to yield (Wells *et al.*, 1982; Ashley and Boerma, 1989). While photosynthesis appears to be a heritable trait (Wiebold *et al.*, 1981; Secor *et al.*, 1982), the probable low potential for increasing leaf-level photosynthetic capacity may constrain the potential for differences in yield that one may find after generations of breeding for high *A* (Ashley and Boerma, 1989). However, the genotypes selected here showed sufficient genetic variability in photosynthetic capacity, through increases in V<sub>cm<sub>ax</sub></sub> (Fig. 5), to allow some compensation for considerable reductions in g<sub>H<sub>2</sub>O</sub>, suggesting that there is some potential for breeding soybeans with higher photosynthetic rates. Further increases in photosynthetic rates and yields might be possible via a combination of selection for decreased resistance to CO<sub>2</sub> via increased stomatal conductance (Morrison *et al.*, 1999), and possibly through alternative mechanisms such as increased mesophyll conductance to CO<sub>2</sub> (Barbour *et al.*, 2010). Regardless, it seems that it is necessary to ask the opposite question: 'Does reduced photosynthesis, associated with limited water use when TR is constant at high VPD or g<sub>H<sub>2</sub>O</sub> low under mild drought cause reductions in grain yield?' If so, increases in photosynthetic capacity that compensate for stomatal limitations to photosynthesis may allow water-saving genotypes to retain the high yields of less water-conservative genotypes.

Field validation of this work is required before these breeding strategies are applied to the particular genotypes studied here. It is expected that for soybean the principle of photosynthetic capacity being non-interactive with mild drought would hold in field situations, particularly if V<sub>cm<sub>ax</sub></sub> limits photosynthesis. These breeding strategies may not be entirely applicable to other crop species, as: (i) the shape of the *A* to g<sub>H<sub>2</sub>O</sub> relationship may be more linear, e.g. *Panicum virgatum* and *Miscanthus × giganteus* (Dohleman *et al.*, 2009), resulting in a weak link between g<sub>H<sub>2</sub>O</sub> and WUE<sub>intr</sub>; (ii) photosynthesis may be limited by processes other than Rubisco carboxylation (e.g. RuBP regeneration), leading to interactions in photosynthetic capacity with drought or other environmental conditions; and (iii) increases in photosynthetic capacity may depend upon

greater leaf nitrogen contents, a possible trade-off when these strategies are applied to non-nitrogen fixers or when there is pressure to reduce fertilizer inputs (Sinclair and Purcell, 2005).

### Conclusion

$WUE_{\text{intr}}$  can be conveniently measured as the ratio  $A/g_{H_2O}$  or by carbon isotope discrimination. However, as has been shown here,  $WUE_{\text{intr}}$  is comprised of two components that vary independently of each other, one of which is interactive with drought. Thus we suggest that breeding soybean genotypes for drought tolerance, escape or avoidance requires more than an analysis of leaf-level  $WUE_{\text{intr}}$ . Instead, the components of  $WUE_{\text{intr}}$ : variation in photosynthetic capacity and stomatal conductance to  $H_2O$ , distinguish between the separate reasons for  $WUE_{\text{intr}}$  variation, and allow better choice of genotypes for these breeding strategies. Large variations in these traits were found, with variation in  $WUE_{\text{intr}}$  primarily being driven by  $g_{H_2O}$ , but also by considerable variation in photosynthetic capacity. Crucially, this independent variation did not lead to a trade-off; genotypes with moderately low  $g_{H_2O}$  could have equally high photosynthetic rates due to high photosynthetic capacities. Finally, photosynthetic capacity advantages were maintained under all conditions, suggesting that breeding for this trait would increase  $WUE_{\text{intr}}$  under a broad spectrum of environments.

### Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Rapid screening of the carboxylation capacity of Rubisco ( $V_{\text{cmax}}$ ) and the maximal rate of RuBP regeneration ( $J_{\text{max}}$ ) was achieved for many replicates and genotypes as illustrated. Five points were measured at  $C_{\text{is}} < 200 \mu\text{mol mol}^{-1}$  and the slope of these was taken as an estimate of the derivative of the classical Rubisco-limited photosynthesis function at a  $C_i$  of  $\Gamma^*$ , the photo-compensation point. This estimate was used to solve for  $V_{\text{cmax}}$  assuming an infinite internal conductance to  $\text{CO}_2$  and the temperature corrections for Rubisco kinetic constants from von Caemmerer (2000).  $J_{\text{max}}$  was estimated as four times the maximum rate of  $\text{CO}_2$  and light-saturated photosynthesis at  $> 2000 \mu\text{mol mol}^{-1} \text{CO}_2$ . Thus, only one and five measurements were needed to provide high-throughput estimates of  $J_{\text{max}}$  and  $V_{\text{cmax}}$ , allowing 178 and 91 leaves to be measured, respectively. Filled points represent the points used for estimation of  $V_{\text{cmax}}$  and  $J_{\text{max}}$ , open points represent reference points measured for a full  $\text{CO}_2$  response curve, but not used in the analysis.

**Figure S2.** Differences between genotypes in the relationship of maximum Rubisco carboxylation capacity ( $V_{\text{cmax}}$ ) to leaf temperature for three genotypes with constant TR at high VPD (circles) and three genotypes with increasing TR at high VPD (triangles) under well-watered conditions. Each panel shows measurements made for a soybean genotype (filled symbols) and points for all genotypes combined (open

squares). The fitted relationships (a power function) are shown for each genotype (solid line) and relative to the relationship for PI416937 (dashed line). Statistical differences and standard errors of the mean are shown in Table S2.

**Figure S3.** Differences between genotypes in the relationship of maximum RuBP regeneration capacity ( $J_{\text{max}}$ ) to leaf temperature for all 11 genotypes under well-watered conditions. Each panel shows measurements made for a soybean genotype (filled symbols) and points for all genotypes combined (open squares). The fitted relationships (a linear function) are shown for each genotype (solid line) and relative to the relationship for PI416937 (dashed line). Statistical differences and standard errors of the mean are shown in Table S2. Soybean genotypes with a constant TR at high VPD (circles) and genotypes with increasing TR at high VPD (triangles) are shown.

**Table S1.** Minimum adequate linear models for stomatal conductance to  $H_2O$  ( $g_{H_2O}$ ), and photosynthetic rate ( $A$ ) measured during a drought experiment on 11 soybean genotypes

**Table S2.** Minimum adequate linear model for the maximal rate of Rubisco carboxylation ( $V_{\text{cmax}}$ ), and RuBP regeneration ( $J_{\text{max}}$ ), measured on well-watered plants

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### References

- Ashley DA, Boerma HR. 1989. Canopy photosynthesis and its association with seed yield in advanced generations of a soybean cross. *Crop Science* **29**, 1042–1045.
- Barbour MM, Warren CR, Farquhar GD, Forrester G, Brown H. 2010. Variability in mesophyll conductance between barley genotypes, and effects on transpiration efficiency and carbon isotope discrimination. *Plant, Cell and Environment* **33**, 1176–1185.
- Bernacchi CJ, Morgan PB, Ort DR, Long SP. 2005. The growth of soybean under free air  $[\text{CO}_2]$  enrichment (FACE) stimulates photosynthesis while decreasing *in vivo* Rubisco capacity. *Planta* **220**, 434–446.
- Blum A. 2005. Drought resistance, water-use efficiency, and yield potential—are they compatible, dissonant, or mutually exclusive? *Australian Journal of Agricultural Research* **56**, 1159–1168.
- Blum A. 2009. Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. *Field Crops Research* **112**, 119–123.

- Boyer JS.** 1982. Plant productivity and environment. *Science* **218**, 443–448.
- Brisson N, Ollio A, Clastre P.** 1993. Daily transpiration of field soybeans as related to hydraulic conductance, root distribution, soil potential and midday leaf potential. *Plant and Soil* **154**, 227–237.
- Bunce JA.** 1984. Identifying soybean lines differing in gas-exchange sensitivity to humidity. *Annals of Applied Biology* **105**, 313–318.
- Centritto M, Lauteri M, Monteverdi MC, Serraj R.** 2009. Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. *Journal of Experimental Botany* **60**, 2325–2339.
- Condon AG, Farquhar GD, Richards RA.** 1990. Genotypic variation in carbon isotope discrimination and transpiration efficiency in wheat-leaf gas-exchange and whole plant studies. *Australian Journal of Plant Physiology* **17**, 9–22.
- Condon AG, Richards RA, Rebetzke GJ, Farquhar GD.** 2002. Improving intrinsic water-use efficiency and crop yield. *Crop Science* **42**, 122–131.
- Crawley MJ.** 2005. *Statistics: an introduction using R*. Chichester: John Wiley and Sons Ltd.
- Dohleman FG, Heaton EA, Leakey ADB, Long SP.** 2009. Does greater leaf-level photosynthesis explain the larger solar energy conversion efficiency of *Miscanthus* relative to switchgrass? *Plant, Cell and Environment* **32**, 1525–1537.
- Ennahli S, Earl HJ.** 2005. Physiological limitations to photosynthetic carbon assimilation in cotton under water stress. *Crop Science* **45**, 2374–2382.
- Evans JR.** 1989. Photosynthesis and nitrogen relationships in leaves of C<sub>3</sub> plants. *Oecologia* **78**, 9–19.
- Fletcher AL, Sinclair TR, Allen LH.** 2007. Transpiration responses to vapor pressure deficit in well watered 'slow-wilting' and commercial soybean. *Environmental and Experimental Botany* **61**, 145–151.
- Ford DM, Shibles R, Green DE.** 1983. Growth and yield of soybean lines selected for divergent leaf photosynthetic ability. *Crop Science* **23**, 517–520.
- Harley PC, Weber JA, Gates DM.** 1985. Interactive effects of light, leaf temperature, CO<sub>2</sub> and O<sub>2</sub> on photosynthesis in soybean. *Planta* **165**, 249–263.
- Hikosaka K.** 1997. Modeling optimal temperature acclimation of the photosynthetic apparatus in C<sub>3</sub> plants with respect to nitrogen use. *Annals of Botany* **80**, 721–730.
- Hikosaka K, Murakami A, Hirose T.** 1999. Balancing carboxylation and regeneration of ribulose-1,5-bisphosphate in leaf photosynthesis: temperature acclimation of an evergreen tree. *Quercus myrsinaefolia*. *Plant, Cell and Environment* **22**, 841–849.
- Hsiao TC.** 1973. Plant responses to water stress. *Annual Review of Plant Physiology and Plant Molecular Biology* **24**, 519–570.
- Hubick K, Farquhar G.** 1989. Carbon isotope discrimination and the ratio of carbon gained to water lost in barley cultivars. *Plant, Cell and Environment* **12**, 795–804.
- Hubick KT, Farquhar GD, Shorter R.** 1986. Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (*Arachis*) germplasm. *Australian Journal of Plant Physiology* **13**, 803–816.
- Hufstetler EV, Boerma HR, Carter TE, Earl HJ.** 2007. Genotypic variation for three physiological traits affecting drought tolerance in soybean. *Crop Science* **47**, 25–35.
- Jasienski M, Bazzaz FA.** 1999. The fallacy of ratios and the testability of models in biology. *Oikos* **84**, 321–326.
- King CA, Purcell LC, Brye KR.** 2009. Differential wilting among soybean genotypes in response to water deficit. *Crop Science* **49**, 290–298.
- Lawlor DW, Tezara W.** 2009. Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. *Annals of Botany* **103**, 561–579.
- Liu F, Stutzel H.** 2004. Biomass partitioning, specific leaf area, and water use efficiency of vegetable amaranth (*Amaranthus* spp.) in response to drought stress. *Scientia Horticulturae* **102**, 15–27.
- Long SP, Zhu XG, Naidu SL, Ort DR.** 2006. Can improvement in photosynthesis increase crop yields? *Plant, Cell and Environment* **29**, 315–330.
- Medrano H, Escalona JM, Bota J, Gulias J, Flexas J.** 2002. Regulation of photosynthesis of C<sub>3</sub> plants in response to progressive drought: stomatal conductance as a reference parameter. *Annals of Botany* **89**, 895–905.
- Morrison MJ, Voldeng HD, Cober ER.** 1999. Physiological changes from 58 years of genetic improvement of short-season soybean cultivars in Canada. *Agronomy Journal* **91**, 685–689.
- Müller I, Schmid B, Weiner J.** 2000. The effect of nutrient availability on biomass allocation patterns in 27 species of herbaceous plants. *Perspectives in Plant Ecology, Evolution and Systematics* **3**, 115–127.
- Pettigrew WT, Turley RB.** 1998. Variation in photosynthetic components among photosynthetically diverse cotton genotypes. *Photosynthesis Research* **56**, 15–25.
- Purcell LC, Edwards JT, Brye KR.** 2007. Soybean yield and biomass responses to cumulative transpiration: questioning widely held beliefs. *Field Crops Research* **101**, 10–18.
- R\_Development\_Core\_Team.** 2010. *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.
- Richards RA.** 2000. Selectable traits to increase crop photosynthesis and yield of grain crops. *Journal of Experimental Botany* **51**, 447–458.
- Ripley B, Frole K, Gilbert M.** 2010. Differences in drought sensitivities and photosynthetic limitations between co-occurring C<sub>3</sub> and C<sub>4</sub> (NADP-ME) Panicoid grasses. *Annals of Botany* **105**, 493–503.
- Sadok W, Sinclair TR.** 2009a. Genetic variability of transpiration response to vapor pressure deficit among soybean (*Glycine max* [L.] Merr.) genotypes selected from a recombinant inbred line population. *Field Crops Research* **113**, 156–160.
- Sadok W, Sinclair TR.** 2009b. Genetic variability of transpiration response to vapor pressure deficit among soybean cultivars. *Crop Science* **49**, 955–960.

- Secor J, McCarty DR, Shibles R, Green DE.** 1982. Variability and selection for leaf photosynthesis in advanced generations of soybeans. *Crop Science* **22**, 255–259.
- Seibt U, Rajabi A, Griffiths H, Berry JA.** 2008. Carbon isotopes and water use efficiency: sense and sensitivity. *Oecologia* **155**, 441–454.
- Sinclair TR, Hammer GL, van Oosterom EJ.** 2005. Potential yield and water-use efficiency benefits in sorghum from limited maximum transpiration rate. *Functional Plant Biology* **32**, 945–952.
- Sinclair TR, Messina CD, Beatty A, Samples M.** 2010. Assessment across the United States of the benefits of altered soybean drought traits. *Agronomy Journal* **102**, 475–482.
- Sinclair TR, Muchow RC.** 2001. System analysis of plant traits to increase grain yield on limited water supplies. *Agronomy Journal* **93**, 263–270.
- Sinclair TR, Purcell LC.** 2005. Is a physiological perspective relevant in a 'genocentric' age? *Journal of Experimental Botany* **56**, 2777–2782.
- Sinclair TR, Tanner CB, Bennett JM.** 1984. Water-use efficiency in crop production. *Bioscience* **34**, 36–40.
- Sinclair TR, Zwieniecki MA, Holbrook NM.** 2008a. Changes in plant–soil hydraulic pressure gradients of soybean in response to soil drying. *Annals of Applied Biology* **152**, 49–57.
- Sinclair TR, Zwieniecki MA, Holbrook NM.** 2008b. Low leaf hydraulic conductance associated with drought tolerance in soybean. *Physiologia Plantarum* **132**, 446–451.
- Snyder F.** 1960. Evaporation on the Great Lakes. *International Association of Hydrological Sciences Publication* **53**, 364–376.
- Specht JE, Chase K, Macrander M, Graef GL, Chung J, Markwell JP, Germann M, Orf JH, Lark KG.** 2001. Soybean response to water: a QTL analysis of drought tolerance. *Crop Science* **41**, 493–509.
- Virgona JM, Farquhar GD.** 1996. Genotypic variation in relative growth rate and carbon isotope discrimination in sunflower is related to photosynthetic capacity. *Australian Journal of Plant Physiology* **23**, 227–236.
- von Caemmerer S.** 2000. *Biochemical models of leaf photosynthesis*. Collingwood: CSIRO Publishing.
- Wang GL.** 2005. Agricultural drought in a future climate: results from 15 global climate models participating in the IPCC 4th assessment. *Climate Dynamics* **25**, 739–753.
- Wells R, Schulze LL, Ashley DA, Boerma HR, Brown RH.** 1982. Cultivar differences in canopy apparent photosynthesis and their relationship to seed yield in soybeans. *Crop Science* **22**, 886–890.
- Wiebold WJ, Shibles R, Green DE.** 1981. Selection for apparent photosynthesis and related leaf traits in early generations of soybeans. *Crop Science* **21**, 969–973.
- Wright IJ, Reich PB, Westoby M.** 2003. Least-cost input mixtures of water and nitrogen for photosynthesis. *American Naturalist* **161**, 98–111.
- Wright IJ, Reich PB, Westoby M, et al.** 2004. The worldwide leaf economics spectrum. *Nature* **428**, 821–827.