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For submission to: **SI: Water Efficiency in Plants**

## **Gravimetric phenotyping of whole plant transpiration responses to atmospheric vapour pressure deficit identifies genotypic variation in water use efficiency**

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***Highlights:***

- 100 well-watered maize genotypes demonstrated a 2-fold variation in whole plant water use efficiency (WUE)
- Two categories of transpiration response to varying VPD were identified: 1) a linear increase in transpiration with low (high WUE) or high (low WUE) transpiration at all VPDs, 2) a non-linear response with a change point at low VPD (high WUE) or high VPD (low WUE)
- The phenotyping platform successfully reproduced the transpiration responses of individuals measured in whole plant chambers, accelerating the identification high WUE plants

## ***Abstract***

There is increasing interest in rapidly identifying genotypes with improved water use efficiency, exemplified by the development of whole plant phenotyping platforms that automatically measure plant growth and water use. Transpirational responses to atmospheric vapour pressure deficit (VPD) and whole plant water use efficiency (WUE, defined as the accumulation of above ground biomass per unit of water used) were measured in 100 maize (*Zea mays* L.) genotypes. Using a glasshouse based phenotyping platform with naturally varying VPD (1.5 to 3.8 kPa), a 2-fold variation in WUE was identified in well-watered plants. Regression analysis of transpiration versus VPD under these conditions, and subsequent whole plant gas exchange at imposed VPDs (0.8 to 3.4 kPa) showed identical responses in specific genotypes. Genotype response of transpiration versus VPD fell into two categories: 1) a linear increase in transpiration rate with VPD with low (high WUE) or high (low WUE) transpiration rate at all VPDs, 2) a non-linear response with a pronounced change point at low VPD (high WUE) or high VPD (low WUE). In the latter group, high WUE genotypes required a significantly lower VPD before transpiration was restricted, and had a significantly lower rate of transpiration in response to VPD after this point, when compared to low WUE genotypes. Change point values were significantly positively correlated with stomatal sensitivity to VPD. A change point in stomatal response to VPD may explain why some genotypes show contradictory WUE rankings according to whether they are measured under glasshouse or field conditions. Furthermore, this novel use of a high throughput phenotyping platform successfully reproduced the gas exchange responses of individuals measured in whole plant chambers, accelerating the identification of plants with high WUE.

*Keywords: water use efficiency, atmospheric vapour pressure deficit, phenotyping platform, whole plant transpiration*

## 1. Introduction

Many different approaches have assessed the physiological responses of crops to sub-optimal water supply such as field scale measurements of transpiration, growth and yield [1, 2], leaf level responses of container grown plants [3] and manipulating individual genes that may confer drought tolerance [4]. Tolerance or resistance to drought usually implies some improvement or maintenance of metabolic process that enable a plant to regulate cell water status and maintain leaf turgor under stressful conditions. One way to achieve this is by partially closing the stomata, which restricts transpiration and water loss, but may also decrease carbon assimilation. While some studies have focused on finding or developing specific drought tolerant genotypes (defined as having improved yield under drought conditions) [5], others have identified genotypes with greater whole plant water use efficiency [6]. Mathematically, whole plant water use efficiency (WUE) is simply the ratio of accumulated plant biomass to the amount of water used. Yield is defined as the product of the ratio of grain mass to total plant mass (harvest index), the amount of water transpired by the crop and the crop transpiration (or water use) efficiency [7]. Therefore, plants with high WUE either maintain or match yield compared to other genotypes under the same drought conditions but use less water to do so; or use the same volume of water, but have increased yield relative to other genotypes.

Most plants conserve water by restricting maximum transpiration rates as atmospheric vapour pressure deficit (VPD) increases, as a result of stomatal closure. The physiological mechanisms underlying this stomatal response have been investigated for over a century, but a dominant mechanism appears to be a negative feedback of guard cell turgor loss on transpiration rate [8]. Under high VPD, guard cell turgor may be decreased by direct evaporative losses from the guard cells and/or decreased water supply to the guard cells if root or shoot hydraulic conductance is limiting [9]. This hydropassive response occurs in all land plants, but in angiosperms is supplemented by rapid increases in leaf ABA concentrations that appear sufficient to initiate stomatal closure [10]. The dominant source of this ABA is more contentious, with increased guard cell ABA biosynthesis [11], leaf biosynthesis outside of the epidermis [10] and xylem delivery of ABA [12] all proposed as being responsible for stomatal closure under high VPD. Whether genetic variation in

transpiration response to VPD within a species reflects genetic variation in the relative importance of different regulatory mechanisms is uncertain.

Regardless of the physiological mechanisms causing restriction of transpiration at high VPD, genetic variation in the response has been observed in a number of crops such as sorghum [13, 14], C<sub>3</sub> and C<sub>4</sub> grasses [15] and maize [16, 17]. By limiting transpiration at high VPD, water may be “saved” thereby maximising soil water availability later in the growing season when drought may be more likely to occur, thereby minimising crop losses and increasing water use efficiency. Modelling studies have shown that plants with restricted transpiration rates at high VPD may have significantly increased crop yields due to more soil water being available later in the season during grain filling [18].

Previous studies with individual whole plant gas exchange chambers in which atmospheric VPD can be controlled have necessarily been limited in the number and developmental stage of genotypes for which transpirational responses to VPD can be profiled. Thus 35 maize genotypes were studied during two separate experiments each comprising 1 month. [16]. Similarly, nine sorghum genotypes were studied in a chamber (at fourth leaf stage) and in the field (following completion of vegetative development) over the course of approximately 5 weeks [13, 16]. Often, these studies have fitted a “broken-stick” regression model to transpirational responses to VPD to highlight putative “change points” in the response, but this approach may be empirical and more robust statistical procedures are warranted. Alternatively, transpirational responses to VPD can be modelled by fitting an inverse power function to the relationship and statistically comparing the components of the fitted function [19]. Since both mathematical approaches are used in the literature, their ability to characterise variation in WUE was compared.

While the need for high throughput methods to determine transpiration responses to changes in atmospheric VPD, and thus WUE for multiple species or genotypes within a species, has previously been identified [20], the throughput of current techniques is inadequate to study bi-parental mapping populations or panels for genome-wide association studies. The aims of this study were three fold: (i) identify genetic variation in WUE of 100 well-watered genetically diverse maize genotypes using a glasshouse based phenotyping platform, (ii) demonstrate that high frequency measurements of gravimetric transpiration in a high throughput phenotyping platform could quickly (over a duration of two weeks) determine transpirational responses to VPD and (iii) model transpirational response to atmospheric VPD

to statistically define these relationships, thereby discriminating genetic variation in model parameters (eg. change points) in the response.

## **2. Methods and materials**

### **2.1 Plant material**

Maize (*Zea mays* L.) was chosen since it (i) is an important EU crop (nine million hectares are grown across the EU every year), (ii) currently subjected to significant water deficits and (iii) has considerable genetic resources that are well adapted to European conditions and are suitable for drought analysis ([www.dropsproject.eu](http://www.dropsproject.eu)). The panel of 100 maize genotypes (Numbers 3001-3100, Figure 3) was selected to have a narrow flowering date (within six days). This panel consisted of public accessions of dent and flint types crossed to a common tester and had been developed and studied over several years [For more information on plant material please see 21, 22].

### **2.2 Determining whole plant WUE and transpiration responses to naturally varying atmospheric VPD on a phenotyping platform**

One hundred genotypes of maize (three seeds per genotype) were pre-germinated on filter paper (Whatman #1), which had been moistened with distilled water and placed in a petri dish. The dishes were covered with foil and left in the dark in the glasshouse for 36 hrs. Once germinated, seeds were placed, one per pot (two pots per genotype and per experiment), into rectangular (60 x 60 x 300 mm) pots (1.1 L) filled with a well-watered (to pot capacity) peat-based substrate (Levingtons M3, Everris Ltd, Suffolk, UK), and covered with approximately 15 mm of substrate. Pots were then covered with thin, black plastic lids to reduce water loss from the substrate during germination. Seedlings emerged three days later at which point the lids were removed. Plants grew for 14 days after emergence (leaf four stage), and were watered once per day at 09:00 with 60 mL tap water for the first seven days, then 120 mL tap water for the next seven days.

At 14 days, one plant of each genotype was placed on the phenotyping platform and grown under well-watered conditions (daily replacement of 100 % of the water transpired measured gravimetrically) in five replicate experiments (n=5, Figure 1). The platform was housed in a naturally lit climate-controlled glasshouse (dimensions 3 m wide x 4 m long) with



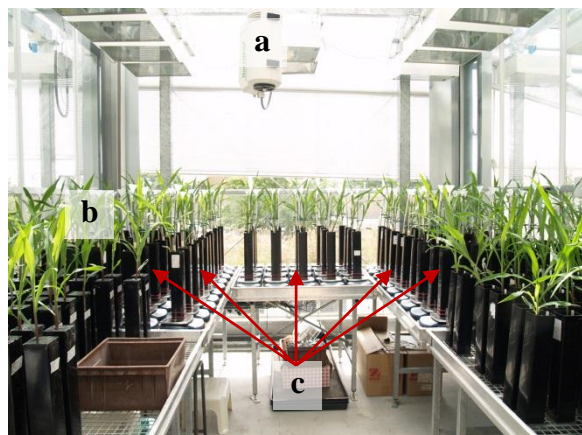
supplementary lighting (supplied by Osram 600 W daylight bulbs, OSRAM, Munich, Germany) for 14 hours per day at the Lancaster Environment Centre. The additional lighting provided  $350 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR at pot height, increasing to  $728 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR at canopy height at the start of each experiment. Day/night minimum temperature was set to 22/16 °C. The platform consisted of 100 balances (0.1g resolution, Ohaus Scout Pro, Ohaus, Switzerland) that automatically logged weight every minute (averaged every 15 minutes). Environmental conditions were monitored throughout each experiment by three independent sensors: 1) a central glasshouse sensor (Hortimax Ektron II C with integrated Hortimax PAR sensor, HortiMaX B. V, The Netherlands) which logged hourly PAR ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), T (°C), RH (%) and CO<sub>2</sub> (ppm). 2) An EGM 4 (PP Systems, Hitchin, UK) placed at canopy height logged PAR ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), T (°C), RH (%) and CO<sub>2</sub> (ppm) every 30 mins and 3) five data loggers (Tinytag Plus 2, Gemini Data Loggers (UK) Ltd, West Sussex, UK) spaced equidistantly every 20 balances at bench height recorded (T (°C) and RH (%), every 15 mins. The 15 minute Tinytag RH and T data was averaged and used to calculate VPD within each block of 20 genotypes.

Plant position was randomised based on an alpha square design to take into account variation in environmental conditions across the glasshouse. Glasshouse environmental conditions showed similar variability during all five replicate phenotyping experiments as measured by the EGM and Tinytags placed at canopy and bench height respectively (temperature 23-39/18-22°C day/night, RH 19-60/33-71% day/night, CO<sub>2</sub> 424 – 443/441 – 458 ppm, light 540 – 728  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR, **Error! Reference source not found.**). One plant per genotype was harvested 14 days after emergence to determine initial shoot fresh and dry weight (g) immediately before the experiments began and the remaining plants placed onto the balances for a further seven days. Plants were kept well-watered by maintaining the substrate gravimetric water content (GWC, defined as the weight of soil water divided by the weight of dry soil in the pot) between 1.6 and 2.6 g g<sup>-1</sup> (equivalent to a soil matric potential of -1 to -10 kPa) based on pot weights. Moisture release characteristics of the substrate were previously determined [23]. At the end of the experiment plants were harvested for above ground biomass and leaf area (Li-3050A, LiCOR, NE, USA).

Whole plant transpiration (Tr) was calculated every 24 hours as  $\text{Tr} = (\text{FW}_n \text{ (g)} + \text{water added (g)} - \text{FW}_{n+1} \text{ (g)})$  (where n = day and FW = pot + soil + plant weight). Total water use (WU) over the duration of the experiment was determined as  $\text{WU} = (\text{starting FW (g)} + \text{water added (g)}) - \text{final fresh weight (g)}$  and water use efficiency (WUE) was determined as  $\text{WUE} =$

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above ground dry weight (g) / water used (L). Both were calculated over the duration of the trial (14 days).



**Figure 1: Lancaster Environment Centre phenotyping platform.** Balance readouts were automatically logged to a computer every minute and averaged every 15 minutes. One central glasshouse sensor (position a), one PP Systems EGM probe at canopy height (position b) and 5 Tinytag Plus 2 data loggers placed at bench height every 20 balances (position c) continuously monitor glasshouse conditions every hour, 30 minutes, and 15 minutes respectively. Preliminary tests had shown limited environmental differences between the two sides of the platform (data not shown).

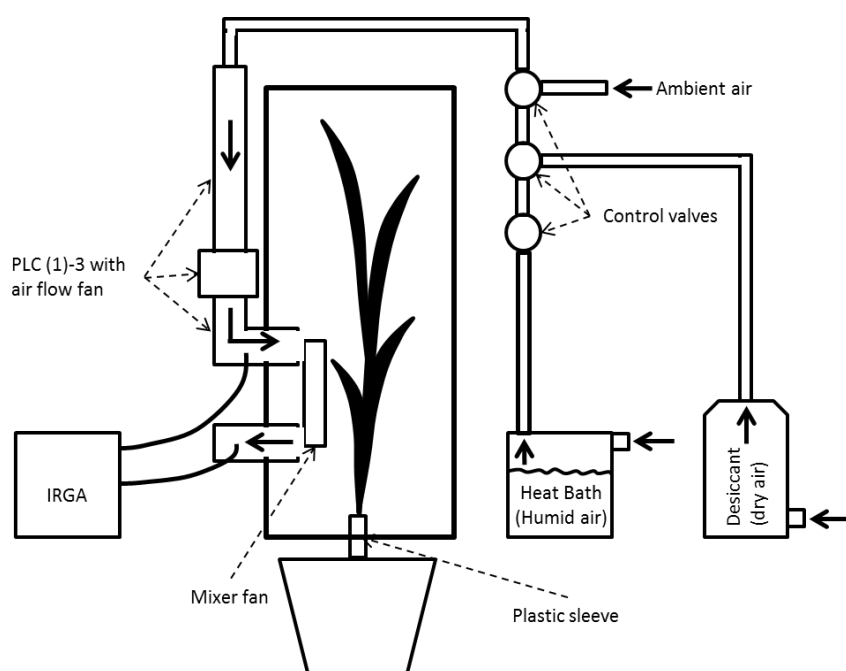
### 2.3 Transpirational responses to artificially changing atmospheric VPD

Seeds of *Zea mays* L (genotypes 3036, 3047, 3075 and 3093) were sown directly into 1.5 L pots containing the same substrate as above. The emerging shoot was grown through a 50 mm long section of 21 mm diameter plastic sleeving to facilitate sealing into the whole plant gas exchange chamber. Plants were maintained as given above but with supplementary lighting for 12 hours per day.

The chamber was constructed from 5 mm clear Perspex (230 mm wide x 630 mm high x 230 mm deep) that had a total volume of 30 L (Figure 2). A sealable slot in the base of the chamber, that fitted the 21 mm diameter sleeving, allowed the plant to be isolated from the substrate within the gas exchange chamber. The chamber was fitted to a PLC (1)-3 whole canopy gas exchange system that regulated air flow through the chamber and included a chamber air mixing fan and measured transpiration rate with a CIRAS-1 infra-red gas analyser (both PP Systems, Amesbury, MA, USA) that sampled air entering and leaving the chamber. VPD within the chamber was controlled by supplying humidified, dried or ambient

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air to the chamber. Air was humidified by passing air over a heat bath or dried by passing air through a 2 L plastic bottle containing silica gel desiccant. Different VPDs were generated by controlling the ratio of ambient, humidified or dried air flowing into the chamber via a series of valves. A single 400 W Osram daylight bulb above the canopy chamber supplied 600 – 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR at canopy height and air flow through the chamber was set to 30 L  $\text{min}^{-1}$ . Temperature and relative humidity (RH) within the chamber was recorded with a digital hygrometer (Testo 608-H1).



**Figure 2: Schematic of whole plant gas exchange chamber.** Solid arrows indicate the direction of air flow through the system

To record the transpiration response to VPD in the whole plant chamber, the plastic pipe around the stem of the plant was sealed with a two-part silicone elastomer (Sylgard 184, Dow Corning, Midland, USA) and the plant enclosed within the chamber and acclimatised for ~30 minutes at ambient humidity. Plants were then exposed to air of ~70% RH to generate the lowest VPD. Humidity was then dropped in approximate 10% RH steps to generate a total of seven different VPDs. Before recording, transpiration was allowed to stabilise for 15 minutes after each lowering of VPD as initial experiments indicated that transpiration remained stable after this period of time. Temperature within the chamber was typically 28.5-31°C and did not fluctuate more than 1.5°C within any experiment. All genotypes except 3007 were

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measured at the same developmental stage as on the phenotyping platform. Genotype 3007 was measured seven days earlier due to its early vigour and size. Experiments were conducted 10:30 to 16:00 GMT to reduce diurnal effects. Preliminary testing of the chamber with two contrasting WUE and CP genotypes showed no hysteresis in transpirational response to increasing and decreasing VPD.

## 2.4 ABA Analyses

Fresh leaf tissue was snap frozen in liquid N<sub>2</sub>, freeze dried for 36 hours, then ground to a fine powder and extracted in distilled water on a shaker overnight at -4°C. The concentration of foliar abscisic acid (ABA) was determined by radioimmunoassay using the monoclonal antibody AFRCMAC 252 [24]. A spike dilution test [25] on aqueous extracts of maize leaves revealed a low level of interfering immunoreactive compounds.

## 2.5 Statistics

To compare WUE across each replicate phenotyping platform experiment, the data were normalised by averaging all 500 plants. Each replicate experiment was then averaged separately and a ratio of the individual average (n = 100) to the combined average (n = 500) was calculated. WUE for each genotype was then scaled according to the ratio for that experiment. Two-way analysis of variance with post hoc Waller-Duncan test (ratio of type I to type II error set to 70) was used to determine significant differences for individual traits and G x E interactions (IBM SPSS v20). In addition a paired t-test was used to determine significant differences between contrasting genotypes. Sampling date and genotype were included as factors. Using the residual variance ( $R_{var}$ ) and an estimate of the genotypic variance ( $G_{var}$ ) calculated by the statistical test used, broad sense heritability ( $H^2$ ) for the parameter of interest was calculated as ( $G_{var} / (G_{var} + (R_{var}/n))$ ), where n is the number of replicates.

To determine whether genotypes had a significant change point in transpiration response to changing VPD, three steps were taken. Firstly, for each genotype, a linear regression was fitted through all the replicate experiment data of transpiration vs average Tinytag VPD for the final 24 hours immediately prior to harvest. The equation of the line was then used to calculate the residuals. Secondly, a third order polynomial was plotted through the residuals and the equation of the third order line used to calculate the value of VPD at which the

maximum residual transpiration occurred. This was the potential change point (CP) in transpiration. Finally, piecewise analysis was carried out in which the null hypothesis was that the linear regression would have a significantly different slope before and after the change point, if the change point was significant. A piecewise model was fitted in which VPD was centred around the change point (VPD-CP), the data were split into pre and post CP using dummy variables (Int = 0 before CP and 1 post CP) and VPD = 0 pre CP, and VPD-CP post VPD. A regression model (IBM SPSS v20), was then run with transpiration as the dependent variable, and VPD-CP, Int and VPD as independent variables. If the change in slope above the CP was not significant the null hypothesis was rejected and a linear response of transpiration to changing VPD was accepted. If the change in the slope above the potential CP was significant, the null hypothesis was accepted. This indicated that the genotype restricted transpiration at high VPDs, and the change in transpiration occurred at the VPD value determined from the analysis of residuals. The above steps were repeated once for each of the 100 genotypes, and then again for the data collected from the whole plant gas exchange chamber.

A comparative analysis evaluating the stomatal sensitivity to VPD ( $\Phi$ ) was also performed [19]. For each genotype, firstly a linear regression was fitted through all the replicate data of transpiration vs VPD within the VPD range 1.5 to 2.5 kPa. This was then used to calculate the transpiration rate at 3.5 kPa assuming no change in stomatal conductance had occurred. Secondly, an exponential rise to maximum regression was fitted through all transpiration vs VPD data (range 1.5 – 3.8 kPa) for each genotype. This was used to calculate the actual transpiration rate at 3.3 kPa. Finally,  $\Phi$  was calculated as the actual transpiration rate at 3.5 kPa divided by the rate of transpiration that would have occurred had there been no change in stomatal conductance to increasing VPD [19].

## 2.6 Principle Component and Discriminant Analysis

Principle component analysis (PCA) and discriminant analysis were used to determine whether change point could predict the whole plant water use efficiency of the maize genotypes tested. PCA identifies those input variables (the principle components) that explain the majority of the variance between individual cases. Multiple input variables may be significantly correlated to one principle component thereby reducing the number of variables required to explain the variation between cases. Data were log transformed to minimise scaling differences between variables. Only components with Eigenvalues > 0.8 were

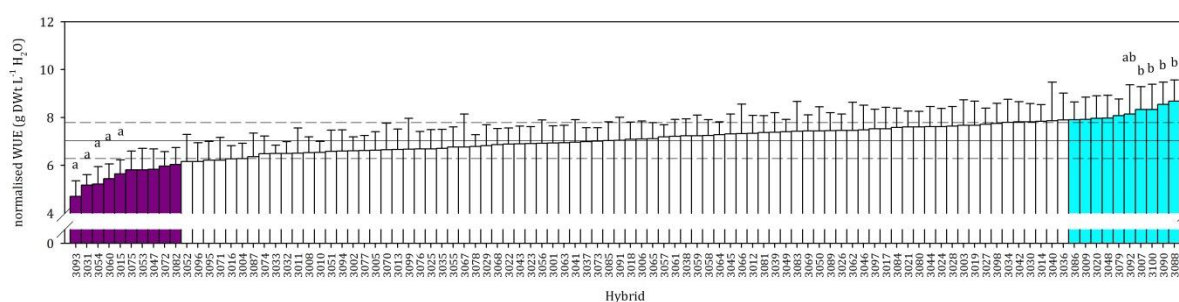
extracted. Once the principle components were identified, the data were grouped in five data sets related to their whole plant WUE as determined by the platform experiments.

Discriminant analysis on the log transformed grouped data were carried out in order to determine the most significant factors that separated the groups. The first function separated the groups as far as possible, with the second function then separating the groups further but being uncorrelated to the first function. The model was set to randomly select 70 % of cases to build the model, with the remaining cases used only to test the effectiveness of the model at assigning a genotype to the correct WUE group.

### 3. Results

#### 3.1 Genotypic variation in whole plant water use efficiency in maize

In the panel of 100 genotypes, whole plant WUE of the most efficient genotype (W95115-H, 3088) was approximately twice that of the least efficient (I224, 3093 (Figure 3). When calculated across all platform experiments, the heritability ( $H^2$ ) of WUE based on genotype and biomass was 0.78 and 0.9 respectively. Across the whole panel, WUE was most strongly correlated with shoot dry weight ( $R^2 = 0.54$ ,  $P < 0.001$ ) and water use ( $R^2 = 0.37$ ,  $P = 0.014$ ) (Table 1).



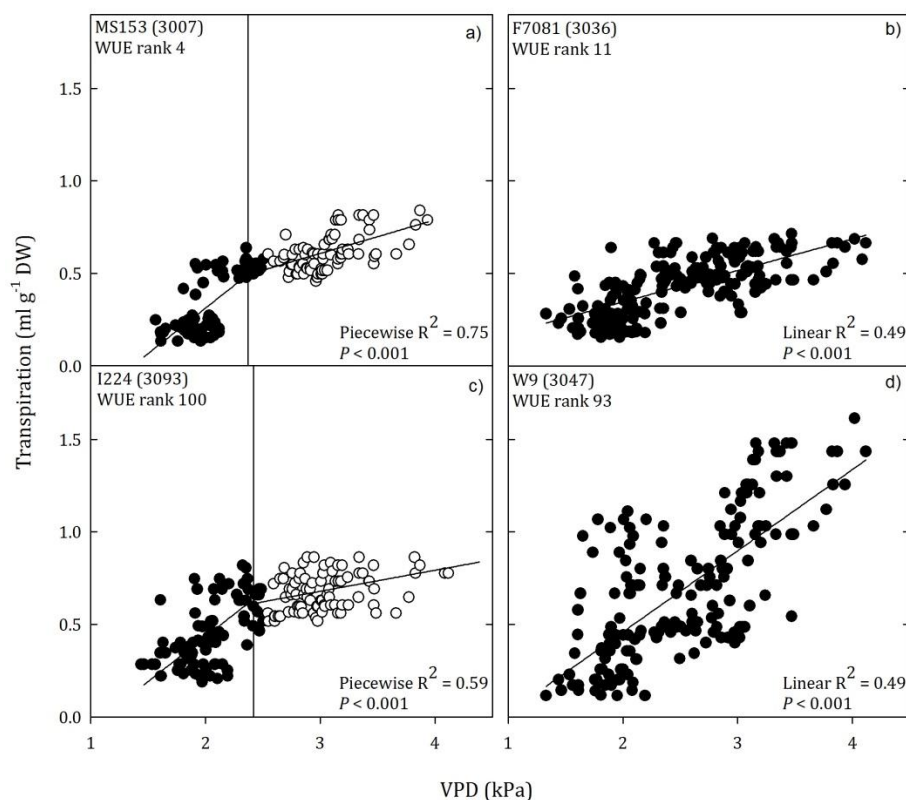
**Figure 3: Variation in whole plant water use efficiency of 100 maize genotypes under well-watered conditions.** Solid horizontal line indicates mean and dashed horizontal lines represent  $\pm 1$  SD of all five replicate experiments. Bars show means ( $n = 4$  or  $5$ )  $\pm 1$  SD for individual genotypes. Letters identify genotypes with significantly different whole plant water use efficiencies (defined as shoot dry weight per unit of water used).

### 3.2 Some genotypes showed restricted transpiration in response to increasing VPD

Transpiration data were all normalised for shoot dry weight. The 100 maize genotypes fell into one of four categories in terms of their response to naturally changing atmospheric VPD when analysed via the phenotyping platform. High water use efficiency genotypes had either a highly significant ( $P < 0.001$ ) change point (CP) in transpiration (e.g. Figure 4, a) as VPD increased, or showed a significantly reduced transpiration rates with increasing VPD. The average rate of increase in transpiration with increasing VPD for the top 20 % WUE ( $= 0.2 \text{ ml g}^{-1} \text{ DW kPa}^{-1}$ ) compared to low WUE genotypes over all VPD ranges (average rate of increase in transpiration with increasing VPD for the lowest 20 % WUE  $= 0.3 \text{ ml g}^{-1} \text{ DW kPa}^{-1}$ , significant difference in slope  $P = 0.01$ ) (e.g. Figure 4, b). Unexpectedly, some low WUE genotypes also showed restricted transpiration at high VPD (e.g. Figure 4, c).

However, the transpiration rate change was not as clearly defined ( $0.001 < P < 0.05$ ) and the VPD range which initiated the change in transpiration response was significantly lower ( $P = 0.007$ ) when comparing the highest WUE genotypes with CP (mean change point  $= 2.4 \text{ kPa}$ ), to the lowest WUE genotypes with CP (mean change point  $= 2.7 \text{ kPa}$ ). In all, 51% of genotypes, irrespective of high or low WUE, demonstrated a change point in transpiration in response to increasing VPD (Table S1. Supplementary Data).

In addition, stomatal sensitivity to changing VPD was significantly positively correlated with CP ( $R^2 = 0.238$ ,  $P < 0.001$ ). Thus, as the VPD required to restrict transpiration response increased, stomata showed a concomitant increasing insensitivity to changing VPD (Table S1, Supplementary Data).



**Figure 4: Example transpiration responses to naturally varying glasshouse VPD of four maize genotypes with contrasting WUE and transpiration response to VPD.** High WUE genotypes 3007 (MS153,  $\Phi = 0.70$ ) with (a) and 3036 (F7081,  $\Phi = 0.78$ ) without (b) a change point in transpiration and low WUE genotypes 3093 (I224,  $\Phi = 0.75$ ) with (c) and 3047 (W9,  $\Phi = 1.0$ ) without (d) a change point in transpiration in response to VPD. Closed circles indicate transpiration rates prior to the change point, open circles indicate transpiration rates post change point. Vertical line indicates value of VPD at which change point occurs.

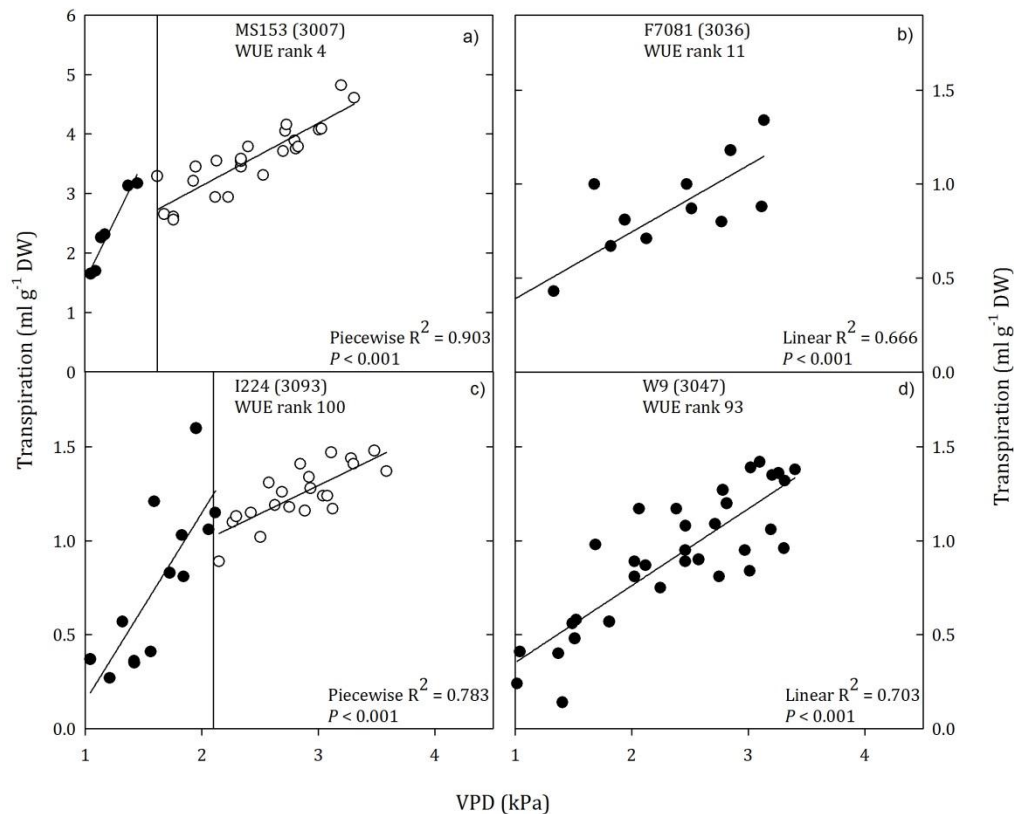
Transpiration of four genotypes with contrasting WUE and responses to VPD (as identified from the phenotyping platform) were tested further using the whole plant transpiration chamber (Figure 5). These had the same transpiration response, at similar VPD points, to artificially changing atmospheric VPD, as previously identified. Due to its size and initial rate of growth, genotype 3007 was tested at a younger developmental stage (seven days earlier) and had increased rates of transpiration when compared to the platform data (Figure 4a). It also responded significantly sooner to a decrease in VPD ( $1.7 \pm 0.1$  kPa in the chamber



compared to 2.4 +/- 0.2 kPa on the platform), and had a lower  $\Phi$  (0.55 in the chamber compared to 0.70 on the platform). However, even at this earlier developmental stage, genotype 3007 still clearly demonstrated a change point in transpiration and higher stomatal sensitivity in response to increasing VPD in the chamber. For genotype 3093, a chamber CP value of approximately 2.3 +/- 0.1 kPa was similar to a value of 2.4 +/- 0.2 kPa when tested with the platform. Likewise, a chamber  $\Phi$  of 0.68 was similar to the platform  $\Phi$  of 0.75. Similar to the platform data, genotypes 3036 and 3047 showed a linear response and similar stomatal sensitivity to transpiration in the chamber. Genotype 3036 demonstrated a lower rate of transpiration on the platform (0.17 ml g<sup>-1</sup> DW, Figure 4b) when compared to the chamber (0.36 ml g<sup>-1</sup> DW, Figure 5b), but had comparative  $\Phi$  (0.74 compared to 0.78 for chamber and platform respectively) whereas genotype 3047 demonstrated a similar rate of transpiration and  $\Phi$  in both (0.41 ml g<sup>-1</sup> DW and 0.44 ml g<sup>-1</sup> DW, and  $\Phi$  = 0.91 and 1.0 for the chamber and platform respectively, Figures 4d and 5d).

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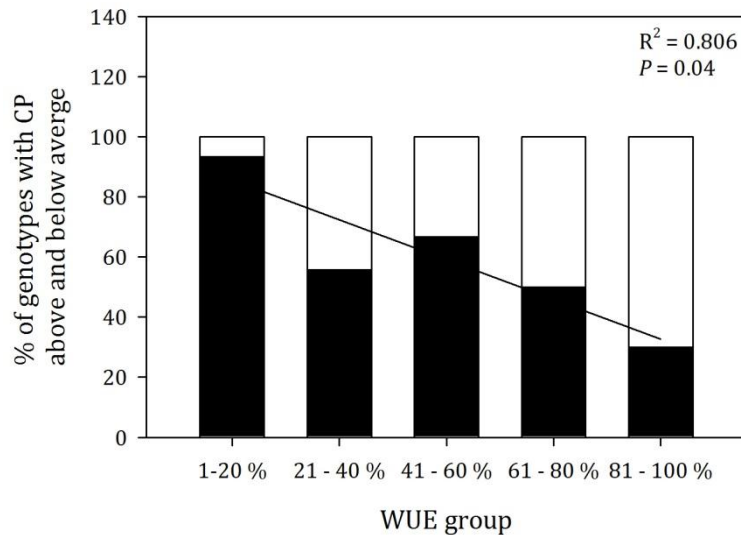
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**Figure 5: Whole plant transpiration responses to artificially changing atmospheric VPD.** Transpiration responses to changing VPD are shown for the high WUE genotypes 3007 (MS153,  $\Phi = 0.55$ ) with (a) and 3036 (F7081,  $\Phi = 0.74$ ) without (b) a change point in transpiration and low WUE genotypes 3093 (I224,  $\Phi = 0.68$ ) with (c) and 3047 (W9,  $\Phi = 0.91$ ) without (d) a change point in transpiration in response to VPD. Closed circles indicate transpiration rates prior to the change point, open circles indicate transpiration rates post change point. Vertical line indicates value of VPD at which change point occurs.

The average VPD value at which a CP in transpiration occurred was  $2.5 \pm 0.1$  kPa. Most (93 %) genotypes in the top 20% WUE group had a change point in their transpiration response to VPD (Figure 6) that occurred at VPDs less than 2.5 kPa. In contrast only 30% of those in the least efficient group had a CP before this VPD point. Thus there was a significant linear decrease in CP (equating to a significant decline in stomatal sensitivity to atmospheric VPD) with increasing whole plant WUE ( $P = 0.04$ ). It is also interesting to note that,

irrespective of CP value, on average 50 % of genotypes within each of the lower four WUE groups had change points in transpiration in response to increasing VPD. In contrast, this increased to 75 % of genotypes for the top 20 % (Figure 6).



**Figure 6: Decreasing sensitivity of genotypes with a change point to increasing VPD as WUE decreases.**

Black bars indicate the percentage of genotypes within each WUE group with a change point in transpiration in response to decreasing VPD below 2.5 kPa. Open bars indicate those genotypes within each WUE group with a change point in transpiration above 2.5 kPa. Linear regression (solid black line) indicates a significant decrease in number of genotypes per group with above average response to decreasing VPD.

### 3.3 Principle component and discriminant analysis suggest that including change point improves prediction of whole plant WUE

Whole plant WUE was significantly correlated with above ground plant fresh and dry weight, water use, soil water content, leaf area and foliar abscisic acid concentration for all genotypes irrespective of transpiration response to VPD. For those genotypes without a change point, the slope of the linear regression of transpiration vs VPD was significantly correlated with whole plant WUE (Table 1).

**Table 1: Correlations of all variables tested with whole plant WUE for all genotypes.** Variables listed are above ground plant fresh weight (FW final), dry weight (DW final), water use (WU), soil water content (SWC), leaf area (LA), foliar abscisic acid concentration (ABA), change point VPD value (CP number) and linear regression gradient of those genotypes without a change point (Gradient no CP). Significant correlations are highlighted in bold text, N = sample number.

Correlations		DW final	WU	WUE	SWC	LA	ABA	CP number	Gradient no CP
FW final	Pearson Correlation	<b>.766</b>	<b>.550</b>	<b>.594</b>	<b>-.546</b>	<b>.556</b>	<b>.267</b>	.054	-.203
	Sig. (2-tailed)	<b>.000</b>	<b>.000</b>	<b>.000</b>	<b>.000</b>	<b>.000</b>	<b>.009</b>	.706	.166
	N	<b>100</b>	<b>100</b>	<b>99</b>	<b>100</b>	<b>100</b>	<b>95</b>	51	48
DW final	Pearson Correlation		<b>.753</b>	<b>.769</b>	<b>-.693</b>	<b>.702</b>	<b>.383</b>	-.177	<b>-.337</b>
	Sig. (2-tailed)		<b>.000</b>	<b>.000</b>	<b>.000</b>	<b>.000</b>	<b>.000</b>	.213	<b>.019</b>
	N		<b>100</b>	<b>99</b>	<b>100</b>	<b>100</b>	<b>95</b>	51	<b>48</b>
WU	Pearson Correlation			<b>.245</b>	<b>-.329</b>	<b>.738</b>	<b>.251</b>	.029	-.239
	Sig. (2-tailed)			<b>.014</b>	<b>.001</b>	<b>.000</b>	<b>.014</b>	.839	.101
	N			<b>99</b>	<b>100</b>	<b>100</b>	<b>95</b>	51	48
WUE	Pearson Correlation				<b>-.643</b>	<b>.340</b>	<b>.325</b>	<b>-.284</b>	<b>-.460</b>
	Sig. (2-tailed)				<b>.000</b>	<b>.001</b>	<b>.001</b>	<b>.046</b>	<b>.001</b>
	N				<b>99</b>	<b>99</b>	<b>94</b>	<b>50</b>	<b>48</b>
SWC	Pearson Correlation					<b>-.389</b>	<b>-.301</b>	.162	.149
	Sig. (2-tailed)					<b>.000</b>	<b>.003</b>	.255	.311
	N					<b>100</b>	<b>95</b>	51	48
LA	Pearson Correlation						<b>.287</b>	.023	-.194
	Sig. (2-tailed)						<b>.005</b>	.870	.185
	N						<b>95</b>	51	48
ABA	Pearson Correlation							-.190	-.143
	Sig. (2-tailed)							.200	.339
	N							47	47

To determine which of the correlated parameters best predicted whole plant WUE, principle component analysis was performed. PCA indicated that, for genotypes with a change point in transpiration, initial dry weight, water use, foliar abscisic acid concentration and value of VPD at the change point were most strongly correlated with the four main principle

components (Eigenvalues > 0.8). Using these principle components, discriminant analysis suggested that initial dry weight explained 38 % of the total variance, water use explained 20 % of the total variance, change point explained 17 % of the total variance and foliar abscisic acid explained 14 % of the total variance. For the genotypes not used to initialise the model (the 30 % not randomly selected by the model), including change point improved the model's ability to correctly assign a genotype to a WUE group from 44 % to 56 %.

For genotypes without a change point, PCA indicated that final dry weight, foliar abscisic acid concentration and slope of transpiration versus VPD were most strongly correlated with the three main principle components (Eigenvalues > 0.8). Discriminant analysis showed that 47 % of the total variance was explained by dry weight, 21 % was explained by foliar abscisic acid concentration and 14 % was associated with the slope of transpiration versus VPD. In this group, the model only correctly assigned a genotype to the correct WUE group 52 % of the time and this value was not improved by including slope of transpiration versus VPD.

#### **4. Discussion**

Although genotypes with high WUE can show enhanced grain yield in water limited environments (Condon et al. 2004), rapidly identifying these varieties is difficult, especially in C4 species where surrogate measurements of WUE such as carbon isotope discrimination are of limited use [26]. The maize panel showed a 2-fold variation in WUE (Figure 3). This is consistent with previous reviews which have shown a 3-fold variation for wheat, and rice, and a 2-fold variation for cotton seed and maize [27]. Since measurement of plant biomass and water use was labour-intensive, we examined different variables that could explain variation in WUE.

Although the effect of VPD on plant transpiration responses and the resulting impact on WUE was suggested 30 years ago [28], only in the last five years has large intra-specific variation in transpiration restriction under high VPD been documented in peanut [29], sorghum [30], wheat [31] and soybean [32]. Of the 100 maize genotypes studied, 51 had significant ( $P < 0.05$ ) change points in transpiration (Figure 4a, c) with some also showing almost complete restriction of transpiration after this point, similar to previous studies [13, 33]. In addition, all high WUE genotypes with a change point demonstrated a significantly greater restriction in transpiration (a significantly lower slope) above the change point than the lower WUE genotypes with a change point (Figure 4, Table S1 Supplementary Data). The environmental conditions during plant development may affect the VPD value of the change point. Previous studies have shown a range of 1.6 – 3.9 kPa for sorghum depending on whether they were grown in the field or in a chamber [13]. A possible reason for this difference is plant developmental stage: fourth leaf stage during the chamber experiments versus complete vegetative development in the field [13]. Our data for maize fit within the upper range of change points previously identified for this species (2.3 – 3.0 kPa, at a growth temperature of 23 – 39 °C within the glasshouse and 28.5 – 31 °C within the whole plant gas exchange chamber), and well within the range identified for other cereal crops. The only genotype to fall below 2.3 kPa in this study was genotype 3007 which, due to its size, was tested at a younger developmental stage in the chamber. This genotype demonstrated a change point in transpiration at 1.7 kPa (Figure 5). This response of genotype 3007 at the two developmental stages for the platform and the chamber suggests that change point varies with developmental stage.

Highly WUE genotypes altered their transpiration behaviour in response to decreasing VPD at lower VPDs ( $P = 0.007$ ) than lower WUE genotypes (2.4 kPa versus 2.7 kPa respectively, Figure 6). Genotype 3007 was one of the highest WUE genotypes (ranked 4 out of 100) and

had one of the lowest change points (ranked 6 out of 51) in the platform experiments (Figure 4, Table S1). These data suggest that higher WUE genotypes respond to increasing VPD earlier than the low WUE genotypes and thus conserve more water for later in the growing season [13, 14, 16]. Similarly, highly WUE genotypes also showed increased stomatal sensitivity to changing VPD (average 0.78) than the lowest WUE genotypes (average 0.83), as determined by the ratio of stomatal sensitivity ( $\Phi$ ) [19] (Table S1, supplementary information). However, while lower CP values were significantly correlated with lower  $\Phi$  ( $R^2 = 0.238$ ,  $P < 0.001$ ),  $\Phi$  showed no correlation with whole plant WUE (Table S1, supplementary information). Although it is beyond the scope of this study to mechanistically explain genotypic variation in transpiration response, it has been suggested that  $\Phi$  may be controlled by hydraulic feedback mechanisms, in conjunction with VPD stimulated modulation of ion fluxes [9, 19]. The lack of correlation between WUE and  $\Phi$  in the present study suggest alternative control mechanisms are active under the environmental conditions used here.

Collectively, shoot biomass and water use, foliar abscisic acid concentration and, for genotypes with a change point, the VPD value at which a change point in transpiration occurred explained up to 89 % of the variance in whole plant WUE. The remaining 11 % were explained by slight variations in SWC and in the sensitivity of transpiration response to VPD before and after CP. Previous studies have shown a strong relationship between intrinsic WUE and foliar ABA concentration in wild-type and transgenic tomatoes overexpressing an ABA biosynthesis gene [34]. In the current study, foliar ABA concentration showed a weak but significant positive linear relationship ( $R^2 = 0.325$ ,  $P < 0.001$ ) with increasing WUE. This relatively weak relationship may arise from the fact that whole plant WUE is an integrated measure calculated over the duration of the experiment

whereas foliar ABA concentration is an instantaneous measure and responds quickly to changes in SWC and other hydraulic and chemical signals [35].

Interestingly, including change point improved the discriminant models' ability to correctly assign a genotype to the correct WUE group from 44 % to 56 %. This supports the theory that accounting for transpiration responses to changing VPD, and selecting genotypes with the ability to restrict transpiration at high VPD are central to refining predictions of WUE thereby potentially improving crop yields [14, 20]. For example, modelling studies suggested that selecting sorghum cultivars that exhibited restricted transpiration rates under high VPD could increase yields by up to 13 % in dry seasons [14]. Similarly, sorghum genotypes with restricted transpiration rates under high VPD had higher water use efficiency and grain and stover production [36, 37]. To the best of our knowledge, this study is the first time transpiration responses to VPD have been investigated on this scale with 100 genotypes for any cereal crop, thereby providing empirical data to model how these responses affect maize yields in water limited environments.

In conclusion, these data highlight the large (two-fold) natural genotypic variation in whole plant WUE within 100 maize genotypes under well-watered conditions. They also strongly suggest that selecting genotypes that restrict transpiration under high VPD could significantly improve whole plant WUE. Moreover, measuring transpiration rate *versus* VPD can be achieved on a high throughput phenotyping platform instead of restricted to individual whole plant gas exchange chambers. Further work should determine the consistency of the transpiration response to VPD under different environmental conditions (eg. when the soil is dry), and whether the genotypes identified as highly water use efficient with a change point in transpiration produce comparable or greater yields under environmentally stressful conditions than those without a change point.

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### ***Table legends***

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### **Figure legends**

**Figure 1:** Lancaster Environment Centre phenotyping platform. Balance readouts were automatically logged to a computer every minute and averaged every 15 minutes. One central glasshouse sensor (position a), one PP Systems EGM probe at canopy height (position b) and 5 Tinytag Plus 2 data loggers placed at bench height every 20 balances (position c) continuously monitor glasshouse conditions every hour, 30 minutes, and 15 minutes respectively. Preliminary tests had shown limited environmental differences between the two sides of the platform (data not shown).

**Figure 2:** Schematic of whole plant gas exchange chamber. Solid arrows indicate the direction of air flow through the system

**Figure 3:** Variation in whole plant water use efficiency of 100 maize genotypes under well-watered conditions. Solid horizontal line indicates mean and dashed horizontal lines represent  $\pm 1$  SD of all five replicate experiments. Bars show means ( $n = 4$  or  $5$ )  $\pm 1$  SD for individual genotypes. Letters identify genotypes with significantly different whole plant water use efficiencies (defined as shoot dry weight per unit of water used).

**Figure 4:** Example transpiration responses to naturally varying glasshouse VPD of four maize genotypes with contrasting WUE and transpiration response to VPD. High WUE genotypes 3007 (MS153,  $\Phi = 0.70$ ) with (a) and 3036 (F7081,  $\Phi = 0.78$ ) without (b) a change point in transpiration and low WUE genotypes 3093 (I224,  $\Phi = 0.75$ ) with (c) and 3047 (W9,  $\Phi = 1.0$ ) without (d) a change point in transpiration in response to VPD. Closed circles indicate transpiration rates prior to the change point, open circles indicate transpiration rates post change point. Vertical line indicates value of VPD at which change point occurs.

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Transpiration responses to changing VPD are shown for the high WUE genotypes 3007 (MS153,  $\Phi = 0.55$ ) with (a) and 3036 (F7081,  $\Phi = 0.74$ ) without (b) a change point in transpiration and low WUE genotypes 3093 (I224,  $\Phi = 0.68$ ) with (c) and 3047 (W9,  $\Phi = 0.91$ ) without (d) a change point in transpiration in response to VPD. Closed circles indicate transpiration rates prior to the change point, open circles indicate transpiration rates post change point. Vertical line indicates value of VPD at which change point occurs.

**Figure 6:** Decreasing sensitivity of genotypes with a change point to increasing VPD as WUE decreases. Black bars indicate the percentage of genotypes within each WUE group with a change point in transpiration in response to decreasing VPD below 2.5 kPa. Open bars indicate those genotypes within each WUE group with a change point in transpiration above 2.5 kPa. Linear regression (solid black line) indicates a significant decrease in number of genotypes per group with above average response to decreasing VPD.

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