

## 1 **QTL and drought effects on leaf physiology in lowland *Panicum virgatum***

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28 **Abstract**

29 Switchgrass is a key component of plans to develop sustainable cellulosic ethanol production for  
30 bioenergy in the US. We sought quantitative trait loci (QTL) for leaf structure and function,  
31 using the Albany full-sib mapping population, an F<sub>1</sub> derived from lowland tetraploid parents. We  
32 also assessed both genotype × environment interactions (G×E) in response to drought and spatial  
33 trends within experimental plots, using the mapping population and check clones drawn from the  
34 parent cultivars. Phenotypes for leaf structure and physiological performance were determined  
35 under well watered conditions in two consecutive years, and we applied drought to one of two  
36 replicates to test for G×E. Phenotypes for check clones varied with location in our plot and were  
37 impacted by drought, but there was limited evidence of G×E except in quantum yield ( $\Phi_{PSII}$ ).  
38 Phenotypes of Albany were also influenced by plant location within our plot, and after correcting  
39 for experimental design factors and spatial effects we detected QTL for leaf size, tissue density  
40 (LMA), and stomatal conductance ( $g_s$ ). Clear evidence of G×E was detected at a QTL for  
41 intrinsic water use efficiency (iWUE) that was expressed only under drought. Loci influencing  
42 physiological traits had small additive effects, showed complex patterns of heritability, and did  
43 not co-localize with QTL for morphological traits. These insights into the genetic architecture of  
44 leaf structure and function set the stage for consideration of leaf physiological phenotypes as a  
45 component of switchgrass improvement for bioenergy purposes.

46  
47 **Keywords**

48 switchgrass; *Panicum virgatum*; photosynthesis; QTL; genotype × environment; water use  
49 efficiency

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50 **Introduction**

51 Concerns about fuel security and greenhouse gas emissions during the last decade led to  
52 mandated increases in fuel production from biomass sources in the United States, complemented  
53 by promotion of other renewable energy sources and technologies for greenhouse gas capture  
54 [1]. In addition to providing a novel domestic energy supply, effective implementation of biofuel  
55 production can help to offset CO<sub>2</sub> emissions from ubiquitous fossil fuel combustion technologies  
56 [2]. However, bioenergy production in the United States competes for space with agricultural  
57 and natural ecosystems [3] during a period in which there are increasing concerns about the  
58 sustainability of food crop yield increases necessary to feed growing human populations [4, 5]. It  
59 is therefore increasingly important that high efficiency bioenergy crops are developed.

60 Switchgrass (*Panicum virgatum*) and switchgrass containing mixtures of native grasses, with  
61 their capacity for high productivity and soil carbon storage on marginal lands across the United  
62 States, are leading candidates to improve efficiency and reduce pollution linked with current  
63 bioenergy production from corn [6-11]. Biologists and agronomists have made rapid progress in  
64 developing the resources necessary for improvement of switchgrass as an energy crop [11, 12]  
65 and have begun to release new high yielding varieties [13]. Most published research aimed at  
66 improvement of switchgrass has focussed on yield and biomass characteristics [6, 14, 15].  
67 Among plant physiologists, however, there is an understanding that resource use efficiencies are  
68 important when considering biomass yield in energy crops [16-18]. We therefore addressed the  
69 genetic architecture of leaf-level phenotypes in switchgrass, including water use efficiency.

70         In the study of leaf physiology, technical advances over the last forty years have seen the  
71 development of field portable systems for measuring photosynthetic performance [19-20] and  
72 detailed models that allow us to scale up predictions of environmental responses at the leaf scale  
73 to canopies and even global vegetation models [21]. Ecological datasets have also shown that  
74 plant leaves demonstrate adaptations to habitat driven by trade-offs linking leaf lifespan with  
75 photosynthetic efficiency [22, 23]. One important trade off central to leaf function in most plants  
76 is that between carbon assimilation and water loss: carbon uptake requires that stomata be open,  
77 risking desiccation of photosynthetic tissues because of inevitable water loss through  
78 transpiration [24]. Both photosynthetic performance and rates of water loss are strongly driven

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79 by abiotic factors [25, 26] but leaf function is also maintained by structural and biochemical  
80 differences that are linked with genetic variation among individuals [27-29]. From a crop  
81 improvement perspective it is important to note that natural selection has acted to oppose  
82 maximization of canopy and stand level photosynthetic efficiency because of conflicts with  
83 competitive interactions, leaving opportunities for intervention to improve efficiency in plant  
84 productivity [5]. It is also clear that we do not yet understand how adaptations evident within and  
85 among plant communities map to intraspecific variation that underpins evolutionary lability of  
86 leaf physiological traits [27, 30, 31]. Understanding the genetic architecture of leaf phenotypes  
87 and their plasticity is therefore essential, both to help address gaps in our basic understanding of  
88 plant performance and to inform approaches to the improvement of efficiency in plant biomass  
89 production.

90 In switchgrass, intraspecific variation in photosynthetic performance has been studied for  
91 decades [32, 33]. Classic physiological studies addressed differences in leaf performance  
92 between ecologically differentiated upland and lowland switchgrass populations with distinct  
93 vegetative phenotypes and ploidy levels [32, 34]. Evidence for local adaptation [35, 36] has also  
94 led to more recent experiments focussed on inter-population variation in productivity and  
95 physiological performance [6, 33, 37-40]. Results from these experiments support differences in  
96 seasonal patterns of photosynthetic performance that complement adaptive variation in  
97 phenology [33, 39, 40]. Our recent, detailed studies of leaf physiological traits among ecotypic  
98 variants of switchgrass suggest that they are genetically determined and linked with local  
99 adaptation in the species [39]. Here, we focus instead on genetic variability in leaf phenotypes of  
100 lowland populations. This variation is important because it provides the raw materials for local  
101 adaptation among populations and because it will influence the outcome of crop improvement  
102 strategies based on lowland germplasm.

103 Switchgrass breeding for bioenergy purposes is being facilitated by existing genetic  
104 resources and cutting edge technologies for genomics and transgenics [12, 41] including the  
105 development of genetic maps [42-47]. QTL mapping is an important component of switchgrass  
106 improvement programs both because it identifies the native genetic variability available to  
107 breeders and because information from QTL studies can be utilized directly in marker assisted  
108 selection approaches. The first published QTL studies using switchgrass have focused on

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5 109 phenotypes for biomass, morphology, and flowering time [48-50]. Though a number of  
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7 110 switchgrass mapping populations have now been produced, the first high density linkage map  
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9 111 was developed for the Albany population (ALB, developed in Albany, CA [47]). A single  
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11 112 generation F<sub>1</sub>, the ALB population allows detection of QTL for genetic variation segregating  
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13 113 within parents selected from two highly productive lowland cultivars: Alamo-A4 (male), and  
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15 114 Kanlow-K5 (female); we have already demonstrated that there is segregating variation in ALB  
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17 115 for leaf coloration, and for agronomic traits including biomass yield [49].

18 116 We asked whether the lowland switchgrass parents of ALB and two check clones drawn  
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20 117 from the parent cultivars show genetic variation in leaf physiology and structure. We also asked  
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22 118 whether genetic variation for leaf phenotypic responses to drought (G×E) could be detected in  
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24 119 lowland switchgrass, and whether phenotypes were plastic in response to local abiotic gradients  
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26 120 within our experiment. We mapped QTL for leaf phenotypes under well watered conditions  
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28 121 during two growing seasons, and tested for G×E by applying a controlled drought treatment  
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30 122 under a rain-out shelter.

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## 32 33 124 **Materials and Methods**

### 34 35 125 *Rain-out shelter and plant material*

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38 126 To facilitate drought experiments our work was conducted under a rain-out shelter (Windjammer  
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40 127 Cold Frame, International Greenhouse Company, Danville, IL, USA) located at the University of  
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42 128 Texas Brackenridge Field Laboratory in Austin, TX (N 30.2845, W -97.7809) [49]. The  
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44 129 footprint of the shelter's steel frame is 18.3 x 73 m, and the shelter is covered with a clear 240  
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46 130 μm polyethylene roof that reduces photosynthetically active radiation by ~10%. The walls (2.1  
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48 131 meters) and eaves (4.2 meters) of the shelter are open to allow free air circulation.

49 132 To allow paired comparisons of droughted and well watered plants we installed an  
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51 133 irrigation system designed by Charles Swanson, Texas A&M University that allowed  
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53 134 independent control of watering in odd and even rows in our experiment. We inserted 3.2 mm  
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55 135 thick hollow plastic sheets (Regal Plastics, Austin, TX) to a depth of 1.2 m, roughly every 2.1 m  
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57 136 along the length of the shelter, providing 34 isolated rows, each of which was irrigated by three  
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59 137 parallel strands of drip tape (T-Tape, John Deere; internal diameter 10 mm, flow rate 4.16 m<sup>3</sup>

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5 138 m<sup>-1</sup>, drippers 0.42 m apart). Drip tapes ran the length of each row and were separated by 0.42 m.  
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7 139 Pressure regulators maintained pressure below 69 kPa, and solenoid valves allowed independent  
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9 140 application of water to odd and even rows.

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11 141 Sixteen plants were positioned in each of the 34 rows in our experiment, with roughly 0.9  
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13 142 m spacing between them. Plants on the perimeter, the first and last row in the field and plants at  
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15 143 the ends of other rows, were switchgrass plants from a variety of cultivars and were not  
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17 144 measured during experiments: their purpose was to minimize edge effects. Interior plants (14  
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19 145 plants × 32 rows = 448 plants) were two independently randomised replicates of 192 lines from  
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21 146 ALB (384 plants) respectively placed into odd and even rows, and 32 clonal replicates of both  
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23 147 Kanlow-398209 and Alamo-AP13 (64 plants). Alamo-AP13 and Kanlow-398209 are not the  
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25 148 parental lines for ALB (male, Alamo-A4; female, Kanlow-K5), but we incorporated them in our  
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27 149 experiment as checks to help identify environmental gradients under the shelter influencing  
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29 150 phenotypes. One plant from each of these two clones was planted in every row in the experiment  
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31 151 at randomised positions.

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33 152 The ALB population was shipped to Austin in the summer of 2010. It was divided to  
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35 153 produce two clonal replicates of the 192 lines, which were grown in pots until planting during  
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37 154 the third week of October 2010. As described, one replicate was planted in odd numbered rows  
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39 155 and the other in even numbered rows. During establishment water was applied using a hose twice  
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41 156 a week from planting until late November, then once a week until our irrigation system was  
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43 157 completed in early March 2011. Odd numbered rows were well watered except during a drought  
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45 158 treatment in July 2011. Even numbered rows were continuously watered during growing seasons.  
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47 159 Growing season irrigation in the even rows supplied 90% of expected plant water requirements  
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49 160 [49].

## 50 161

### 51 162 *Phenotyping*

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53 163 Leaf traits were measured in three large experiments over the course of two years. Experiment A  
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55 164 was carried out in the first year of growth (12<sup>th</sup>-15<sup>th</sup> July 2011), with the aim of providing a  
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57 165 baseline experiment in which all plants were well watered. Experiment B closely followed  
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59 166 Experiment A with the aim of detecting QTL demonstrating G×E: the odd replicate of ALB was  
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5 167 allowed to dry down, the even remained watered, and physiological performance was measured  
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7 168 over the 26<sup>th</sup>-29<sup>th</sup> July 2011. Finally, in Experiment C (22<sup>nd</sup>-25<sup>th</sup> May 2012) we aimed to detect  
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9 169 QTL in well watered second year plants early in the growing season.  
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13 171 *Experiment A: baseline measurements*  
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15 172 Our aim in this experiment was to obtain baseline measurements prior to drought, thus ~33 mm  
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17 173 of water was added to the entire experiment on the evening of the 10th, followed by an additional  
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19 174 ~8-12 mm on the evenings of the 12th, 13th and 14th of July. We sampled the 32 rows of plants  
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21 175 in four blocks of eight adjacent rows, each block being randomly allocated to a day within the  
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23 176 experiment. Pre-dawn, we sheathed a youngest fully emerged leaf blade on each plant in a plastic  
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25 177 bag and immediately detached it above the ligule using sharp scissors. We stored the bagged leaf  
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27 178 blades in a cool box and refrigerator, before scanning them (Epson Perfection V37, Epson  
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29 179 America, Long Beach, CA) and placing them into coin envelopes for drying. We determined leaf  
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31 180 areas using ImageJ software [51], and, after drying the leaves for at least 48 h at 65 °C,  
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33 181 determined their dry mass using an analytical balance (AB104-S, Mettler-Toledo, LLC,  
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35 182 Columbus OH). We calculated leaf mass per area (LMA) as dry mass/leaf area. Within each  
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37 183 sampling block we randomly assigned two rows to each of four LI-6400XT portable  
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39 184 photosynthesis systems (LI-COR Inc., Lincoln, NE) equipped with integrated modulated  
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41 185 fluorometers (LI-6400-40), and between 11 am and 2:30 pm we used either one, or two (as  
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43 186 necessary to fill the gas exchange cuvette) young fully emerged leaves to determine leaf gas  
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45 187 exchange (net CO<sub>2</sub> assimilation,  $A$ ; stomatal conductance to water,  $g_s$ ; and intrinsic water use  
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47 188 efficiency,  $iWUE = A/g_s$ ) and chlorophyll fluorescence (effective quantum yield,  $\Phi_{PSII} =$   
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49 189  $(F_m' - F_s)/F_m'$ ; efficiency of energy harvesting by oxidized PSII reaction centers in the light,  
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51 190  $F_v'/F_m' = (F_m' - F_o')/F_m'$ ; and photochemical quenching,  $q_p = (F_m' - F_s)/(F_m' - F_o')$ ); we measured  
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53 191 flag leaves (subtending emerging or fully emerged flowers) in all but three cases. Based on  
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55 192 weather station measurements from the site and an initial reading taken before measurements  
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57 193 began, we fixed light levels in LI-6400XT cuvettes to match the expected average photosynthetic  
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59 194 photon flux density (PPFD) during the measurement period (mean±sd: 1620±18  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).  
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61 195 We also fixed block temperatures, resulting in cuvette air temperatures of 37.3±1.38 °C  
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5 196 (mean±sd). We maintained reference CO<sub>2</sub> concentrations in the open system at 410 μmol mol<sup>-1</sup>  
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7 197 using CO<sub>2</sub> mixers (LI-6400-01), which resulted in cuvette CO<sub>2</sub> concentrations of 393±6.6 μmol  
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9 198 mol<sup>-1</sup> (mean±sd). Finally, we did not control relative humidity of incoming air; cuvette values  
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11 199 for relative humidity were 54±12 % (mean±sd).

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14 201 *Experiment B: drought experiment*

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17 202 Our aim in Experiment B was to investigate G×E in leaf physiological performance as responses  
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19 203 to drought. We imposed drought on odd rows and maintained watering of even rows. Drought  
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21 204 was imposed by restricting watering, which allowed plants to deplete soil moisture. All rows  
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23 205 were watered with ~63 mm on the 17<sup>th</sup> and 18<sup>th</sup> of July 2011. Subsequently, only the even rows  
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25 206 were irrigated with ~34 mm on July 23<sup>rd</sup>, and ~21 mm on both the 26<sup>th</sup> and 28<sup>th</sup> of July. We used  
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27 207 volumetric water content (VWC, %) in the top 20 cm of soil, measured at four evenly spaced  
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29 208 positions along each row using a Hydrosense soil moisture probe (Campbell Scientific, Inc.,  
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31 209 Logan, UT), to determine when to initiate phenotyping and to account for variable rates of soil  
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33 210 drying across our site. We began phenotyping on July 26<sup>th</sup>, when VWC in the even rows of the  
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35 211 experiment averaged 21±7.2 % (mean±sd, N = 64) compared with 5±2 % in odd rows,  
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37 212 consistent with odd-row soil water potentials below wilting point (Fig. S1). We began  
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39 213 phenotyping in pairs of adjacent odd and even rows where average soil moisture was lowest,  
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41 214 giving rows with higher soil moisture contents additional time to dry down. We measured eight  
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43 215 rows of plants per day for four days, pairs of adjacent odd and even rows being randomly  
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45 216 allocated to one of four LI-6400XT photosynthesis systems. We completed photosynthesis  
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47 217 measurements as in Experiment A then, at around 2:30 pm each day, selected an independent set  
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49 218 of leaves for determination of midday water potentials (Ψ<sub>m</sub>). We sheathed leaf blades in Ziploc  
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51 219 bags (containing damp paper towels to halt transpiration), immediately excised them, stored  
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53 220 them in cool boxes, and removed them to the on-site laboratory for measurement using one of  
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55 221 two Scholander-type pressure bombs (PMS-1000, PMS Instrument Company, Albany, OR)  
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57 222 attached to cylinders of compressed nitrogen.

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6 224 *Experiment C: minimizing day effects*

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8 225 Because preliminary analyses of Experiment A and B did not show much evidence for genetic  
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10 226 effects, Experiment C was designed to determine whether QTL for physiological traits could be  
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12 227 detected in second year plants early during the growing season. We had observed larger  
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14 228 differences between the check clones Alamo-AP13 and Kanlow-398209 in preliminary  
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16 229 measurements made in June 2011 (unpublished data) than in Experiments A and B during July.  
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18 230 Evidence for spatial and temporal effects in our 2011 measurements also suggested a need for a  
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20 231 stratified, rather than random, sampling approach. We therefore carried out Experiment C in May  
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22 232 2012, following tiller emergence in February and March. To minimize temporal effects within  
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24 233 each mapping population we measured the even rows on May 22<sup>nd</sup> and 23<sup>rd</sup>, and the odd rows on  
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26 234 May 24<sup>th</sup> and 25<sup>th</sup>. We measured eight rows per day, two from every quarter of the length of the  
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28 235 shelter. Rows were randomly assigned to four LI-6400XT photosynthesis systems paired with  
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30 236 two Scholander pressure bombs. We measured pre-dawn water potential ( $\Psi_{pd}$ ) using one leaf  
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32 237 blade from each plant, which was sheathed in plastic, excised using sharp scissors, and measured  
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34 238 at the field site within 30 minutes. Gas exchange measurements always used two leaves, and  
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36 239 were made 2 min 30 s after closing the cuvette, a period determined to be adequate for re-  
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38 240 equilibration of gas concentrations. (The fluorometer function of one LI-6400-40 malfunctioned,  
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40 241 so we discarded chlorophyll fluorometry data from this experiment.) We standardized for  
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42 242 phenology wherever possible by using youngest fully emerged leaves from vegetative tillers or  
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44 243 tillers yet to reach anthesis: 93% of measurements were made using pairs of tillers yet to reach  
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46 244 anthesis. We matched cuvette conditions (mean $\pm$ sd: PPFD, 1203 $\pm$ 4.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; air  
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48 245 temperature, 31 $\pm$ 0.27  $^{\circ}\text{C}$ ) to expected light and temperature conditions as in Experiments A and  
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50 246 B. To reduce variability in the driving force for transpiration that underpins measurements of  $g_s$ ,  
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52 247 we controlled water concentration in the reference channel at 32.9 $\pm$ 0.97  $\text{mmol mol}^{-1}$  (mean $\pm$ sd).  
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54 248 During each measurement of photosynthesis we tagged one of the two measured tillers. Within  
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56 249 30 minutes of photosynthesis measurements the youngest fully emerged leaf blade from the  
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58 250 tagged tiller was sheathed in plastic, excised and collected into a cool box, and measured for  $\Psi_m$ .  
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60 251 Immediately after we had determined  $\Psi_m$  we measured lamina area using a LI-3000A Portable  
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62 252 Leaf Area Meter (LI-COR Inc.). Leaves were then dried and LMA was calculated as above.  
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253 Because pre-dawn water potentials showed limited variability and a highly non-normal  
254 distribution they were not analysed as a quantitative trait, but we did use them to standardize  
255 midday water potentials by calculating the hydrodynamic gradient ( $\Delta\Psi = \Psi_m - \Psi_{pd}$ ).

256

257 *Data processing*

258 Rapidly made measurements of physiological traits usually require quality control for unusual  
259 values linked with operator error. We therefore inspected bivariate plots of leaf traits and  
260 removed clear outliers prior to statistical analysis. For Experiments A and B we removed  
261 measurements from five individuals with leaf intercellular CO<sub>2</sub> concentrations ( $c_i$ ) outside a  
262 physiologically reasonable range of 0-400  $\mu\text{mol mol}^{-1}$ . In addition we removed one individual  
263 with  $\Psi_m = -4.65$  MPa (33% greater than the highest retained value), and two individuals with  
264  $\Phi_{PSII} > 0.37$  (>21% greater than the highest retained value) from the Experiment B dataset. There  
265 were no similarly unique values measured in Experiment C, but on the basis of substantial  
266 deviations from linear relationships between traits we excluded data for  $g_s$  from three plants  
267 where values were outside of the usual range given  $A$  (which strongly influenced iWUE), and  
268 one plant where leaf area was unusual given leaf mass (which strongly influences LMA). To  
269 ensure that analyses from all experiments were comparable we further removed data for clones  
270 that were not duplicated within the field or that were missing from any of Experiments A, B, or  
271 C. After these exclusions, data was retained for 165 of the original 192 ALB genotypes (86%),  
272 30 clonal replicates of Kanlow-398209, and 32 clonal replicates of Alamo-AP13.

273

274 *Effects of genotype and environment on phenotypes*

275 Among the ALB we evaluated the relative importance of environmental gradients for different  
276 phenotypes and corrected for the effects of experimental factors using generalized least squares  
277 models (*gls* function in nlme 3.1-120, with `glsControl(opt="optim")`; [52]). For Experiments A  
278 and B, using maximum likelihood as a criterion, we fit the model  $X_{ij} = \alpha_i + \theta_j + \gamma_k + \varepsilon_{ijk}$ , where  $\alpha_i$   
279 are the odd and even replicates,  $\theta_j$  a fixed effect of the day within the experiment, and, where  
280 appropriate,  $\gamma_k$  is a fixed effect of equipment used in the experiment (LI-6400XT machines or  
281 Scholander pressure bombs depending on the trait). For Experiment C, odd and even rows were

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282 fit using separate models because they had been measured consecutively. To determine the  
283 significance of spatial effects we used likelihood ratio tests to compare models fit using restricted  
284 maximum likelihood that either assumed a normal error distribution or corrected for correlations  
285 due to distances among plants. Within-plot spatial correlations were modelled as a component of  
286 measurement error,  $\varepsilon_{ijk}$ , as  $\sigma^2 \times \gamma(r, d)$ , where if  $r > 0$ ,  $\gamma(r, d) = (1-n) \times (1-1.5(r/d)+0.5(r/d)^3)$ ,  
287 and if  $r \geq d$ ,  $\gamma(r,d) = 0$ :  $r$ , is distance;  $d$ , a range;  $n$ , a nugget (spherical autocorrelation structure  
288 [52]). Phenotypes corrected for both experimental factors and spatial patterning were extracted  
289 as normalized residuals from our *gls* models:  $(X - \bar{X}) / \sqrt{(\sigma^2 \gamma(r, d))}$ , where  $X - \bar{X}$  are the  
290 raw residuals (observed – fitted).

291       Because we had only two replicates of the ALB population, we indexed the degree of  
292 genetic determination among ALB genotypes as repeatability, i.e., the Pearson correlation  
293 coefficient for the clonal replicates.

294       Owing to greater replication we were able to use *gls* to determine effects of genotype (G),  
295 environment (E), genotype  $\times$  environment interactions (G $\times$ E) and plot-scale spatial trends for  
296 Alamo-AP13 and Kanlow-398209. We fit the fixed effects model  $X_{ij} = \alpha_i + \beta_j + \alpha_i\beta_j + \varepsilon_{ij}$ , using  
297 maximum likelihood:  $X_{ij}$  are phenotypes,  $\alpha_i$  are the two genotypes;  $\beta_j$  are the odd and even rows  
298 in the experimental design;  $\alpha_i\beta_j$  interaction terms; and  $\varepsilon_{ij}$  the residual. Because we did not fit  
299 effects of days and observers in these models we were able to fit the same model for all three  
300 experiments, but note that  $\beta_j$  in Experiment C incorporated day effects that were a component of  
301  $\varepsilon_{ij}$  in Experiments A and B. Significance of fixed effects and the spherical autocorrelation  
302 structure were tested as for ALB. Predicted means for Alamo-AP13 and Kanlow-398209 in odd  
303 and even rows were obtained as linear combinations of coefficients and corresponding standard  
304 errors using the package *contrast* [53].

305  
306 *QTL mapping*

307 We implemented QTL mapping using the R package *qtl* [54]. Prior to QTL mapping we  
308 constructed our outbred linkage map using OneMap [55] and raw marker genotyping data  
309 available from an original mapping study that used ALB [47] (Details of map construction were  
310 given in a previous publication [49]). Our primary analysis used *scanone* to implement Haley-

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311 Knott regression, but we also carried out non-parametric analyses to account for observations of  
312 heteroskedasticity, skewed distributions and occasional outliers (Tables S1 & S2). Thresholds for  
313 rejection of the null hypothesis of no QTL at  $P < 0.05$ , and  $P < 0.1$  were estimated using 1000  
314 permutations. We used *makeqtl* and *fitqtl* to estimate 1.5 LOD drop confidence intervals and  
315 percent variance explained. We mapped using the odd and even replicates separately for all three  
316 experiments for consistency, since in Experiment B we fit QTL separately for watered and  
317 droughted rows. We also used a post-hoc analysis to determine whether QTL-linked markers  
318 showed significant effects of genotype, environment (odd versus even replicate) and/or  $G \times E$ .  
319 Because many QTL-linked markers were not fully informative our post-hoc analysis used 500  
320 imputed genotype draws from *simgeno* to repeat ANOVA analyses, and we report summaries of  
321 the distribution of P-values from these 500 ANOVA. We also used the 500 draw set of imputed  
322 genotypes to estimate genotype level effects for QTL using *effectplot* and to link phenotypes  
323 with genotype assignments using *plotpxg*.

324

## 325 Results

### 326 *Effects of genotype and environment: Alamo-AP13 and Kanlow-398209*

327 We found few significant differences between the Alamo-AP13 and Kanlow-398209 genotypes  
328 (Fig. 1; Table 1). Among 24 phenotypes significant effects of genotype (Table 1) were found for  
329 leaf area and LMA in Experiments A (Fig. 1h & i) and C (Fig. 1w & x), leaf mass in Experiment  
330 A (Fig. 1g), and  $F_v/F_m'$  in Experiments A and B (Fig. 1e & n). Differences between the  
331 genotypes in  $A$  and  $g_s$  were only marginally non-significant ( $0.05 < P < 0.058$ ) in Experiment C  
332 (Table 1; Fig 1q & s).

333 The drought treatment imposed in Experiment B (Fig. 1j-p) decreased  $\Psi_m$  (Fig. 1p), gas  
334 exchange ( $A$ ,  $g_s$ ; Fig 1j & k), and photosynthetic performance ( $\Phi_{PSII}$ ,  $F_v/F_m'$ ,  $q_p$ ; Fig. 1m-o) in  
335 both Alamo-AP13 and Kanlow-398209 (Table 1). The only trait for which no significant effect  
336 of drought was detected was  $iWUE$  (Fig. 1l), and only one trait showed significant  $G \times E$  ( $\Phi_{PSII}$ ;  
337 Fig. 1m); however, decreases in  $A$ ,  $g_s$ ,  $\Phi_{PSII}$ , and  $q_p$  were usually greater for Alamo-AP13 than  
338 Kanlow-398209 (Fig. 1). The marginally significant  $G \times E$  effect on  $\Phi_{PSII}$  ( $P = 0.046$ ) was  
339 detected against a background of marginal  $G \times E$  effects for other traits; only  $q_p$  showed  $P < 0.1$

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5 340 for G×E in Experiment A but  $A$ ,  $g_s$  and  $q_p$  all showed  $P < 0.082$  in Experiment B (Table 2).  
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7 341 Differences between the odd and even replicates were also detected for four phenotypes:  $A$ ,  $g_s$ ,  
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9 342  $\Delta\Psi$ , and LMA (Fig 1q, r, u, and x), in Experiment C (Table 1), probably as a result of  
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11 343 consecutive phenotyping of the odd and even rows rather than chronic effects of the previous  
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13 344 year's drought treatment.

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15 345 Tests of spatial effects supported plasticity of Alamo-AP13 and Kanlow-398209 in  
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17 346 response to location under the shelter for 8 of the 24 phenotypes (Table 2). The traits linked with  
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19 347 significant spatial patterns were: leaf area, and leaf mass, in Experiment A;  $A$ ,  $\Phi_{PSII}$ ,  $q_p$ , and  $\Psi_m$ ,  
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21 348 in Experiment B; then leaf area, and LMA, in Experiment C.

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24 350 *Effects of genotype and environment: ALB*

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26 351 Correlations between clonal replicates (repeatabilities, Pearson's  $\rho$ ) indicate the importance of  
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28 352 genetic effects over environmental effects and measurement error. In ALB we found that  
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30 353 repeatabilities tended to be greater for leaf structural traits (0.12 to 0.35) than physiological traits  
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32 354 ( $-0.05$  to  $0.17$ : negative values were not significantly different from 0; Table 2). Importantly,  
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34 355 when we corrected for experimental factors (additive effects of odd-even, day of measurement,  
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36 356 and observer, as well as spatial autocorrelation, Table 4) by calculating  $\rho$  among normalized  
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38 357 residuals we found that  $\rho$  increased for 20 of 24 phenotypes, and was statistically significant ( $P <$   
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40 358  $0.05$ ) for 13 phenotypes compared with only seven significant tests using the raw data (Table 2).

41 359 Repeatabilities were not markedly different in Experiment B compared with Experiments  
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43 360 A and C (Table 2), suggesting that additive genetic differences were comparable under well  
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45 361 watered conditions and drought. As expected, drought significantly decreased values for all  
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47 362 photosynthetic performance phenotypes and  $\Psi_m$  (Fig. 2j-p). Drought had smaller impacts on  
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49 363  $iWUE$  (8% decrease; Fig. 2l) and  $F_v'/F_m'$  (6% decrease; Fig. 2n) than other phenotypes, which  
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51 364 showed decreases ranging from 36% ( $q_p$ ; Fig. 2o) to 64% ( $g_s$ ; Fig. 2k). Significant differences  
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53 365 between odd and even rows were also observed for two phenotypes in Experiment A (Table 3),  
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55 366 but these were linked with very small effects:  $+0.03\%$ ,  $F_v'/F_m'$ ;  $-0.5\%$   $iWUE$  (Fig. 2e & c). We  
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57 367 did not directly compare the odd and even replicates in Experiment C because odd-even  
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59 368 comparisons were conflated with day effects.

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5 369 By explicitly accounting for spatial effects as a component of error we significantly  
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7 370 improved model inference for 53% of phenotypes from ALB (17/32 tests; Table 3), a greater  
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9 371 frequency than for Alamo-AP13 and Kanlow-398209 (33%, 8/24 tests; Table 1). This difference  
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11 372 between the mapping population and the clonal lines likely reflects their different densities  
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13 373 within the experiment (Alamo-AP13 and Kanlow-398209 filled 26% and ALB 74% of the  
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15 374 regularly spaced planting) and suggests that spatial effects on phenotypes acted at relatively fine  
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17 375 scales (~0-5 m) within our plot. Because we measured different suites of traits in each of our  
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19 376 three experiments it is difficult to assess how consistent spatial effects were for individual  
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21 377 phenotypes, but of the phenotypes measured in both 2011 and 2012, leaf areas (Experiments A &  
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23 378 C) and leaf water status ( $\Psi_m$ , Experiments B & C;  $\Delta\Psi$ , Experiment C) showed spatial patterning  
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25 379 in both years (Table 3). By contrast, leaf gas exchange ( $A$ ,  $g_s$ , iWUE) and leaf mass showed  
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27 380 significant spatial variability in 2011 but not 2012, and LMA showed significant spatial effects  
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29 381 only in 2012 (Table 3).

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### 31 383 *QTL*

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33 384 Using normalized residuals we detected nine QTL with  $P < 0.1$ , five of which were significant  
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35 385 with  $P < 0.05$  (Fig. 3; Table 4). QTL for LMA (Experiment A odd replicate only, LG 5b, LOD =  
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37 386 5.14) and leaf mass (Experiment C odd and even replicates, LG 1b, LOD  $\geq 5.25$ ), both structural  
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39 387 traits, were most strongly supported. The next most strongly supported QTL was for iWUE  
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41 388 (Experiment B odd replicate only, LG 9a, LOD = 4.66) and the only other QTL with  $P < 0.05$   
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43 389 was for  $q_P$  (Experiment A even rows only, LG 5b, LOD = 4.62). We detected four QTL in the  
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45 390 marginal range ( $0.05 < P < 0.1$ ), two for  $g_s$  (Experiment A even replicate only, LG 2b,  
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47 391 LOD=4.06; Experiment B even replicate only, LG 3a, LOD = 3.93), a pair of co-localising QTL  
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49 392 for  $\Phi_{PSII}$  and  $q_P$  (Experiment A even replicate only, LG 5b, LOD  $\geq 4$ ), and a QTL for leaf area  
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51 393 that co-localised with the more strongly supported QTL for leaf mass (Experiment C even  
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53 394 replicate only, LG 1b, LOD = 4.07). Consistent with LOD scores and corresponding P-values,  
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55 395 the percentage of additive variance explained by QTL (Table 4) was greatest for leaf structure  
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57 396 phenotypes (10.8-14.1%) and less than 10.8% for all of the physiological phenotypes except  
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59 397 iWUE (12.26%).

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399 *G×E and parental effects at QTL*

400 We found limited evidence to support G×E in check clones, showed that repeatabilities were  
401 improved for a number of traits when correcting for experimental effects, and found that the  
402 majority of QTL were detectable in one or other of the two replicates of ALB. We therefore  
403 tested for genotype, environment (even versus odd replicates), and G×E effects at each of our  
404 QTL using marker regression. We had also been surprised to find a QTL for iWUE in  
405 Experiment B because repeatabilities for that phenotype were particularly low. So, we also  
406 aimed to determine whether that QTL was linked with significant G×E, which could explain low  
407 scores for repeatability. We accounted for the effect of uncertainty in genotyping at marker and  
408 pseudomarker locations by repeating ANOVA tests of G, E and G×E for 500 imputed genotype  
409 sets and report means and percentiles of P-values we obtained.

410 Our analysis showed that using normalized residuals fully corrected for any offsets  
411 between the odd and even replicates in our experiments (E, mean  $P \geq 0.365$ ; Table 5). We also  
412 found that there was strong support for additive effects of genotype underpinning QTL for the  
413 structural traits LMA and leaf mass (G, mean  $P < 0.0001$ ; G×E, mean  $P \geq 0.207$ ; Table 5), while  
414 QTL for physiological traits showed mixed outcomes. Two co-localizing QTL on LG 5b, for  
415  $\Phi_{PSII}$  and  $q_p$ , showed no significant effects at the marker level (mean  $P \geq 0.168$ ; Table 5).  
416 Although some imputed genotype sets for these two QTL did support significant effects of G (5<sup>th</sup>  
417 percentile  $P \leq 0.029$ ) some also supported significant G×E (5<sup>th</sup> percentile  $P \leq 0.021$ ; Table 5) and  
418 these two QTL were not supported by alternative mapping approaches using raw trait values  
419 and/or non-parametric techniques (Tables S1 and S2). At both markers linked with QTL for  $g_s$   
420 additive effects of genotype were significant (mean  $P \leq 0.018$ ); however, while sww2747 on LG  
421 3a showed no strong support for significant G×E (mean  $P = 0.071$ ; Table 5), despite being  
422 detected in the absence of drought in Experiment A sww1517 on LG 2b did show significant  
423 G×E (mean  $P = 0.047$ ; Table 6). The strongly supported QTL for iWUE (LG 9a, Experiment B)  
424 also showed significant G×E (mean  $P = 0.005$ ; Table 5); it was detected only under drought.

425 Segregating variation from both parents contributed to QTL and G×E effects. Among  
426 QTL that our ANOVA tests supported as primarily additive (Table 5, Fig. 4): the QTL for odd

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427 mass and leaf area on LG 1b segregated from Kanlow-K5 (Fig. 4a-f), while markers for LMA on  
428 LG 5b (sww332c) and  $g_s$  on LG3a (sww2747) showed less clear cut phenotype-genotype  
429 linkages (Fig. 4g-j). These QTL for LMA and  $g_s$  showed segregation from Alamo-A4 that was  
430 stronger in combination with one Kanlow-K5 allele than with the other (Fig. 4g-j; at least a small  
431 fraction of genotype calls at both of these markers provided support for marginal G×E effects: 5<sup>th</sup>  
432 percentile  $P \leq 0.062$ ). Significant G×E for  $g_s$  at sww1517 on LG 2b was linked with among  
433 genotype effects in the even replicate (Fig. 5a) where the QTL was detected, and no differences  
434 among genotypes in the odd replicate (Fig. 5b). For individuals in the even replicate with the  
435 second Kanlow-K5 allele at sww1517, values of  $g_s$  were smaller, but there was also a clear  
436 pattern of reduced variation in  $g_s$  among individuals containing one of the Alamo-A4 alleles (Fig.  
437 5a-b). This heteroskedasticity in phenotypic values for  $g_s$  had no obvious explanation arising  
438 from our experimental design, and was challenging from a data analysis perspective: non-  
439 parametric analysis did not support the QTL (Table S2). Finally, G×E in iWUE at nfsg107 (LG  
440 9a), detected when drought was applied in Experiment B, clearly arose through segregation from  
441 the Alamo-A4 parent: no effect was observed under well watered conditions (Fig. 5c) and  
442 differences in iWUE under drought arose between individuals carrying different alleles from  
443 Alamo-A4 (Fig 5d).

444  
445 **Discussion**

446 Using the ALB lowland switchgrass mapping population we found evidence for QTL influencing  
447 leaf structure and performance. Repeatabilities tended to be greater for leaf structural phenotypes  
448 than for leaf performance phenotypes, and we located robust QTL for leaf mass on LG 1b and  
449 tissue density on LG 5b. In check clones, comparisons between droughted and well watered  
450 plants provided only limited evidence for G×E, but 1/3 phenotypes showed spatial variation  
451 indicating plasticity in response to abiotic gradients. After correcting for spatial effects on ALB  
452 we found a QTL on LG 9a that influenced iWUE and was expressed only in response to drought,  
453 further demonstrating G×E. This evidence for heritable variation and G×E gives insights into the  
454 genetic architecture underpinning leaf performance and suggests that leaf phenotypes should be  
455 considered as responsive to selection implemented for crop improvement. In addition to evidence



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456 for plasticity linked with spatial variation in our plots, significant variability in leaf phenotypes  
457 linked with observers and days within experiments emphasized the responsiveness of leaf  
458 phenotypes to abiotic drivers, which presents a major challenge for large scale phenotyping of  
459 physiological traits.

460  
461 *QTL*

462 Of the QTL we detected, those for leaf size on LG 1b and LMA on LG 5b were the most  
463 strongly supported. The QTL on LG 1b co-localizes with QTL for base tiller width, internode  
464 width and 4<sup>th</sup> leaf length and area that we detected in parallel experiments using ALB [49]. It  
465 was a result of segregating variation in Kanlow-K5, in a region of the genome that is covered by  
466 maps for both parents [42, 47]. By contrast, the QTL we detected for LMA at 146 cM on LG 5b  
467 is novel, and segregation from Alamo-A4 was implicit in its location: in the original male and  
468 female maps for ALB that our map is derived from no information was available for the Kanlow-  
469 K5 (female) parent beyond 84 cM of LG 5b [47]. Interestingly, the tip of LG 5b is also not  
470 covered in the NF × GA map [57], more recent genotyping-by-sequencing maps for ALB [42],  
471 or a novel four-way cross that incorporates the Alamo-AP13 genotype as a male parent [45].  
472 These results suggest that there may be a low level of polymorphism in the genome of cv. Alamo  
473 individuals adjacent to the QTL for LMA, but we also note that a QTL for SLA (1/LMA)  
474 segregating in the AP13 × Dacotah parent of the novel four-way cross was located on LG 5b  
475 within 50 cM (100-110 cM) of the QTL we found in ALB [45].

476         The QTL we detected for iWUE on LG 9a was also linked with segregation in Alamo-  
477 A4, and falls within a region covered by the Kanlow map. Given the evidence for G×E at this  
478 QTL it is interesting that our confidence intervals showed some marginal overlap with QTL for  
479 biomass (25.4 and 32 cM) and plant height (74 cM) previously detected as showing G×E in the  
480 Alamo parent of NF×GA [48]. However, the peak LOD for our iWUE QTL fell outside the  
481 confidence regions given for the NF×GA QTL [48]. Notwithstanding the difficulties of drawing  
482 direct comparisons between maps for these crosses, if our QTL for iWUE is associated with a  
483 novel genetic element it may be closely linked with loci known to affect biomass and yield in  
484 other switchgrass mapping populations. Attempts to improve biomass and yield related traits in

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5 485 switchgrass through, e.g., marker assisted selection on LG 9a loci might, therefore, result in  
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7 486 unintended selection for leaf physiological responses to drought.

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9 487 We found several additional QTL for physiological traits. Two QTL explained variation  
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11 488 in  $g_s$ . Like the QTL for  $iWUE$  both of these were originally detected in only one of the two  
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13 489 replicates of ALB. In one case a lack of effects in the second replicate drove significant  $G \times E$   
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15 490 despite similar watering treatments and the QTL, which was linked with heteroskedasticity  
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17 491 among genotypes, was not supported in secondary non-parametric QTL analyses. In the other  
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19 492 case, similar effects across the two replicates were supported by our marker regression analysis  
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21 493 but those effects were small and appeared to be influenced by both parents. The pattern of  
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23 494 heritable variation for this second QTL for  $g_s$  (sww2747) is therefore consistent with  
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25 495 transgressive segregation. We were unable to confirm patterns of segregation for phenotypes  
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27 496 because parental genotypes were not available, but we have previously demonstrated  
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29 497 transgressive segregation for several physiological traits in the close relative of switchgrass,  
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31 498 *Panicum hallii* [58]. Determining whether stabilizing selection tends to constrain the evolution of  
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33 499 traits showing transgressive segregation may help to determine whether the rarer, more extreme  
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35 500 phenotypes arising from crosses could be useful tools for crop improvement.

36  
37 501 Another parallel with *Panicum hallii* is the lack of any evidence for co-localization of  
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39 502 physiological QTL with QTL for leaf structural traits [58]. Thus, by contrast with the evidence  
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41 503 that QTL for  $iWUE$  and biomass yield on LG 9a might show moderate linkage, most aspects of  
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43 504 leaf performance seem likely to be genetically independent of leaf structural properties. This  
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45 505 result fits with the finding that evolution of leaf phenotypes is generally less constrained by  
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47 506 genetic correlation and more constrained by selection against ecologically unfit trait  
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49 507 combinations [27]. It has been proposed that there is considerable scope for crop improvement  
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51 508 because ecologically unsuitable trait combinations that decrease intraspecific competitive ability,  
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53 509 and therefore individual fitness, may improve performance in an agricultural setting [5]. Finally,  
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55 510 although our Haley-Knot analysis of normalized residuals identified QTL for  $\Phi_{PSII}$  and  $q_P$  we  
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57 511 found no support for those two QTL using marker regression based on a set of imputed  
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59 512 genotypes: the method used to deal with uncertainty in genotyping assignments at these loci  
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61 513 played an important role in determining outcomes.

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5 515 *Relevance of G×E in leaf phenotypes*  
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8 516 The QTL we located for iWUE (LG 9a) was not detected by approximate tests of additive  
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10 517 genetic variation through calculation of repeatabilities, because it was detectable only under  
11  
12 518 drought. One allelic variant segregating from the Alamo-A4 parent was linked with decreased  
13  
14 519 iWUE under drought. Greater iWUE represents greater capacity for net CO<sub>2</sub> assimilation (*A*)  
15  
16 520 relative to stomatal conductance to H<sub>2</sub>O (*g<sub>s</sub>*). Gas exchange measurements from our check clones  
17  
18 521 illustrate how shifts in iWUE can be obtained as a result of subtle differences in the response of  
19  
20 522 *A* and *g<sub>s</sub>* to drought: under watered conditions we found that Alamo-AP13 showed higher *A* and  
21  
22 523 *g<sub>s</sub>* than Kanlow-398209, while under drought *A* and *g<sub>s</sub>* were much more similar between the two  
23  
24 524 clonal genotypes and mean values were slightly lower for Alamo-AP13. Higher iWUE was  
25  
26 525 observed for Kanlow-398209 under both droughted and watered conditions, but the difference  
27  
28 526 was exacerbated by drought. When comparing these clonal lines then, the plants with the more  
29  
30 527 conservative photosynthetic strategy exhibited lower *A* and *g<sub>s</sub>* under well watered conditions and  
31  
32 528 were better able to maintain leaf-level efficiency when challenged by drought. A similar pattern  
33  
34 529 may explain differences in performance among ALB lines that depended on the Alamo-A4 allele  
35  
36 530 linked with *nfsg107*. We found no evidence for QTL influencing *A* and *g<sub>s</sub>* under drought, but  
37  
38 531 plants with lower water use efficiency under drought had similar efficiency under well watered  
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40 532 conditions and may have shown differences in gas exchange that were below the detection  
41  
42 533 threshold for QTL in an F<sub>1</sub> design. While breeding for improved water use efficiency in crops  
43  
44 534 requires consideration of variation in plant structure and phenology [59] as well as iWUE, the  
45  
46 535 detection of a QTL for iWUE segregating in Alamo germplasm represents a potential step  
47  
48 536 towards genetic approaches to determine the importance of resource use efficiency in  
49  
50 537 switchgrass [60].

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52 538 Although the 14 to 16 clonal replicates of Alamo-AP13 and Kanlow-398209 are  
53  
54 539 illustrative with respect to iWUE in ALB, they were insufficient to detect significant G×E driven  
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56 540 by our drought treatment. Putting this in context, the QTL for iWUE in the lowland ALB was  
57  
58 541 detected with N ~ 40 per genotype. A requirement for large sample sizes, indicating low  
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60 542 statistical power, is consistent with the high degrees of similarity among the plants in our  
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62 543 experiments, all of which are derived from highly productive southern lowland tetraploid  
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544 ecotypes. Greater phenotypic differences are found between northern and southern varieties of  
545 switchgrass [6, 33, 37, 39, 61], or between upland and lowland populations [32, 34]. QTL  
546 mapping applied to crosses that incorporate this strong genetic differentiation among ecotypes  
547 are likely to provide much greater power to rapidly detect loci with large effects on physiological  
548 performance or that underpin G×E.

549         Despite similarities between Alamo-AP13 and Kanlow-398209 in their physiological  
550 responses to drought we did detect genetic differences in leaf structural traits, and we found  
551 evidence for differences in the efficiency of energy harvesting and quantum yield ( $F_v'/F_m'$  and  
552  $\Phi_{PSII}$ ) that included the only significant G×E term in our analysis, for  $\Phi_{PSII}$ . Our results indicated  
553 that the drought we imposed placed limits on gas exchange and decreased the proportion of light  
554 energy utilized in photochemistry ( $q_p$  declined). That effect was linked with a significantly  
555 greater decrease in  $\Phi_{PSII}$  of Alamo-AP13 than of Kanlow-398209 under drought: Alamo-AP13  
556 showed greater, but non-significant, reductions in  $g_s$  and  $q_p$  compared with Kanlow-398209. If  
557 improved photosynthetic performance of lowland derived genotypes in drought prone  
558 environments is considered useful, assessment of genetic variation for photoprotection [62] or  
559 strategies for avoidance of excess irradiance, e.g., leaf rolling [63-65] may be important.

560  
561 *Experimental design factors influencing leaf phenotypes*

562 Repeatabilities were lower for photosynthetic and leaf water status phenotypes than for structural  
563 traits. The repeatabilities we observed are consistent with values from the literature for the  
564 heritability of  $A$  and LMA [27]. They are also consistent with the expectation that leaf  
565 performance is strongly entrained to variations in light and temperature that occur both within  
566 and between days and at seasonal scales [16, 39]. The intrinsic variability in physiological  
567 phenotypes between days drove our decisions to improve spatial and temporal blocking and  
568 reduce the number of days spent measuring each replicate of ALB in our Experiment C in 2012.  
569 Daytime measurements alone in Experiment C required four LI-6400XT photosynthesis systems  
570 and two pressure bombs along with skilled operators, and three or more technical assistants to  
571 collect leaf material, determine leaf areas, and package leaf material for subsequent  
572 determination of dry mass. That effort was useful because it decreased the frequency of

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573 significant measurement effects on photosynthetic phenotypes. Nonetheless, both ALB and the  
574 check clones continued to exhibit plasticity in leaf areas and leaf water status within our plot.  
575 The spatial scales of a few meters over which these patterns were observed present considerable  
576 challenges for QTL experiments with large perennial grasses that demand distribution of  
577 hundreds of genotypes across an experimental site. Despite considerable efforts made during the  
578 construction of our rainout shelters to homogenise and evenly distribute topsoil across the site,  
579 fine-grained variation in abiotic drivers of performance remained influential. Because adjustment  
580 of leaf area is a common mechanism for acclimation in plant hydraulics [56], that both leaf areas  
581 and water potentials were repeatedly linked with spatial patterning in our plot suggests  
582 heterogeneous water availability may have been a driver for leaf phenotypic plasticity through  
583 hydraulic adjustment.

584         Given strong evidence for within-plot spatial variation in leaf area in both 2011 and 2012,  
585 we were surprised to find that within-plot variation in LMA was significant only in 2012.  
586 Progress of the switchgrass plants towards establishment may have influenced this pattern, but  
587 leaves measured in 2012 were primarily collected from vegetative tillers, rather than the  
588 flowering tillers we had sampled in 2011. Repeatabilities for raw values of LMA might therefore  
589 have been influenced by the way our sampling strategy represented tiller developmental status.  
590 Indeed, measurements in 2012 were carried out earlier during the growth season to capture a  
591 more homogeneous set of leaves and tillers and to better fit with the timing of preliminary  
592 measurements in 2011 that had indicated significant differences in photosynthetic performance  
593 between check clones. In combination with improved stratification of our sampling effort, the  
594 timing of sampling in 2012 resulted in decreased P-values for comparisons of  $A$  and  $g_s$  between  
595 Alamo-AP13 and Kanlow-398209. Thus, our results provide some support for greater  
596 differences between these lowland cultivars during the early phases of the growing season and  
597 complement other demonstrations of seasonal variation in performance among switchgrass  
598 cultivars [33, 39].

599  
600 *Conclusions*

601         We were able to detect QTL for leaf physiological performance in a lowland switchgrass

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602 F<sub>1</sub> despite low estimates of heritability. This demonstrates that individual lowland switchgrass  
603 plants harbor genetic variability for physiological performance. Our findings also support the  
604 important insight that, in addition to careful experimental control for abiotic effects, G×E can be  
605 a crucial influence on QTL detection for physiological traits. Heritable variation in leaf structure  
606 and function in switchgrass should therefore be considered when breeding for bioenergy.  
607 Evidence suggests that leaf traits are often under independent genetic control, and that  
608 coordinated trait variation linked with adaptation to local conditions, as demonstrated at the  
609 intraspecific level in switchgrass [39], is generated by the influence of natural selection on trait  
610 combinations [27]. In a crop improvement setting there is, therefore, potential for selection of  
611 novel combinations of leaf traits that could complement progress in the improvement of yield  
612 and biomass properties [13]. Although we found relatively few QTL for leaf phenotypes in ALB,  
613 we expect that greater power to detect genetic effects in switchgrass will be obtained from  
614 crosses that fully exploit known phenotypic differences linked with local adaptation [36, 45, 49].

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787 **Table 1** P-values testing for genetic and environmental effects on leaf phenotypes of switchgrass clones  
 788 regularly interspersed in 'even' and 'odd' replicates of the ALB F<sub>1</sub> switchgrass population growing in  
 789 Austin, Texas.

<sup>1</sup> Experiment	Phenotype	<sup>2</sup> Genotype	<sup>2</sup> Environment	<sup>2</sup> Genotype × Environment	<sup>3</sup> Autocorrelation
A	Mass	<b>0.007</b>	0.06	0.373	<b>0.0004</b>
	Area	<b>0.032</b>	0.066	0.444	<b>&lt;0.0001</b>
	LMA	<b>&lt;0.0001</b>	0.41	0.637	0.088
	<i>A</i>	0.149	0.203	0.552	0.978
	<i>g<sub>s</sub></i>	0.185	0.521	0.921	0.999
	<i>iWUE</i>	0.230	0.985	0.324	0.576
	$\Phi_{PSII}$	0.087	0.263	0.143	0.998
	$F_v'/F_m'$	<b>0.008</b>	0.238	0.37	0.999
	q <sub>P</sub>	0.293	0.338	0.074	0.792
B	<i>A</i>	0.128	<b>&lt;0.0001</b>	0.054	<b>0.028</b>
	<i>g<sub>s</sub></i>	0.107	<b>&lt;0.0001</b>	0.082	0.093
	<i>iWUE</i>	0.147	0.273	0.276	0.703
	$\Phi_{PSII}$	<b>0.005</b>	<b>&lt;0.0001</b>	<b>0.046</b>	<b>0.015</b>
	$F_v'/F_m'$	<b>&lt;0.0001</b>	<b>0.0002</b>	0.281	0.654
	q <sub>P</sub>	0.367	<b>&lt;0.0001</b>	0.054	<b>0.0005</b>
	$\Psi_m$	0.194	<b>&lt;0.0001</b>	0.693	<b>&lt;0.0001</b>
C	Mass	0.147	0.638	0.187	0.219
	Area	<b>0.033</b>	0.737	0.274	<b>0.036</b>
	LMA	<b>0.019</b>	<b>0.011</b>	0.391	<b>0.021</b>
	<i>A</i>	0.058	<b>0.015</b>	0.528	0.999
	<i>g<sub>s</sub></i>	0.051	<b>0.014</b>	0.164	0.999
	<i>iWUE</i>	0.698	0.229	0.437	0.999
	$\Delta\Psi$	0.201	<b>0.03</b>	0.121	0.210
	$\Psi_m$	0.826	0.067	0.308	0.392

<sup>1</sup>Experiments: A, odd and even replicates watered July 2011; B, even replicate watered and odd replicate droughted July 2011; C, odd and even replicates watered May 2012

<sup>2</sup>Wald tests (all F<sub>1,58</sub>): Genotype, Alamo-AP13 vs. Kanlow-398209; Environment, even vs. odd

<sup>3</sup>Likelihood ratio tests ( $\chi^2_1$ )

Values in bold are statistically significant

790 **Table 2** Similarity between clonal replicates (correlation, Pearson's  $\rho$ ) for phenotypes measured  
 791 from 165 F<sub>1</sub> lowland switchgrass genotypes in the ALB mapping population, and the impact of  
 792 using normalized residuals to correct for experimental effects (day, observer and spatial  
 793 correlation).

	Experiment A June 2011		Experiment B June 2011		Experiment C May 2012	
Phenotype	$\rho$	Corrected $\rho$	$\rho$	Corrected $\rho$	$\rho$	Corrected $\rho$
Mass	0.12	<b>0.24***</b>	-	-	<b>0.35***</b>	<b>0.35***</b>
Area	0.08	<b>0.22**</b>	-	-	<b>0.25***</b>	<b>0.28***</b>
LMA	<b>0.31***</b>	<b>0.33***</b>	-	-	<b>0.18**</b>	<b>0.22**</b>
<i>A</i>	0.12	<b>0.16*</b>	0.10	0.12	0.04	<b>0.15*</b>
<i>g<sub>s</sub></i>	0.11	<b>0.17*</b>	0.09	<b>0.16*</b>	0.07	<b>0.14*</b>
<i>iWUE</i>	0.04	0.03	0.01	0.02	<b>0.13*</b>	<b>0.14*</b>
<i>F<sub>v</sub>'/F<sub>m</sub>'</i>	0.05	0.06	0.07	0.10	-	-
$\Phi_{PSII}$	0.01	0.06	<b>0.14*</b>	<b>0.16*</b>	-	-
qp	0.06	0.09	0.12	0.11	-	-
$\Delta\Psi$	-	-	-	-	0.04	0.04
$\Psi_m$	-	-	-0.05	0.08	0.05	0.07

Bold: statistically significant using a one-tailed t-test ( $H_1, r > 0$ )  
 \*0.01 < P < 0.05, \*\*0.001 < P < 0.01, \*\*\*P < 0.001

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**Table 3** Significance and magnitude of experimental design factors and spatial correlations affecting leaf phenotypes of 165 ALB F<sub>1</sub> switchgrass genotypes grown in Austin, Texas.

<sup>1</sup> Experiment	Phenotype	<sup>2</sup> Odd vs. Even			<sup>2</sup> Day		<sup>3</sup> Autocorrelation
		P-values	P-values	Range of means/ grand mean (%)	P-values	Range of means/ grand mean (%)	
A	Mass	0.553	-	-	0.919	6.4	< <b>0.0001</b>
	Area	0.842	-	-	0.852	9.6	< <b>0.0001</b>
	LMA	0.354	-	-	<b>0.011</b>	5	0.933
	<i>A</i>	0.060	<b>0.011</b>	18.9	< <b>0.0001</b>	23.2	0.107
	<i>g<sub>s</sub></i>	0.4	0.199	15.1	< <b>0.0001</b>	32.8	<b>0.0002</b>
	<i>iWUE</i>	<b>0.027</b>	<b>0.0008</b>	8	< <b>0.0001</b>	10.4	<b>0.0002</b>
	F <sub>v</sub> /F <sub>m</sub> '	<b>0.004</b>	< <b>0.0001</b>	8.4	< <b>0.0001</b>	8.7	<b>0.002</b>
Φ <sub>PSII</sub>	0.064	<b>0.0009</b>	14.1	< <b>0.0001</b>	18.2	0.182	
	q <sub>p</sub>	0.428	<b>0.0007</b>	11.5	<b>0.0001</b>	9.9	0.303
B	<i>A</i>	< <b>0.0001</b>	0.426	55.9	< <b>0.0001</b>	27.8	< <b>0.0001</b>
	<i>g<sub>s</sub></i>	< <b>0.0001</b>	0.092	59.7	< <b>0.0001</b>	35.1	<b>0.0003</b>
	<i>iWUE</i>	<b>0.0002</b>	<b>0.009</b>	3.4	0.344	4	0.199
	F <sub>v</sub> /F <sub>m</sub> '	< <b>0.0001</b>	< <b>0.0001</b>	5.5	<b>0.0007</b>	8.7	<b>0.003</b>
	Φ <sub>PSII</sub>	< <b>0.0001</b>	0.119	30.7	< <b>0.0001</b>	27.2	0.051
	q <sub>p</sub>	< <b>0.0001</b>	0.39	25.3	< <b>0.0001</b>	19.2	0.052
	Ψ <sub>m</sub>	< <b>0.0001</b>	<b>0.0008</b>	6.4	<b>0.004</b>	10.6	< <b>0.0001</b>
C - even	Mass	-	-	-	0.934	0.4	0.75
	Area	-	-	-	0.082	11.3	< <b>0.0001</b>
	LMA	-	-	-	0.088	4.6	< <b>0.0001</b>
	<i>A</i>	-	< <b>0.0001</b>	23.5	0.084	4.6	0.745
	<i>g<sub>s</sub></i>	-	<b>0.047</b>	18.5	<b>0.046</b>	9.5	0.226
	<i>iWUE</i>	-	<b>0.005</b>	13	<b>0.046</b>	5.4	0.057
	†ΔΨ	-	0.988	0.1	0.306	3.7	<b>0.003</b>
†Ψ <sub>m</sub>	-	0.422	2.7	0.172	4.4	<b>0.004</b>	
C - odd	Mass	-	-	-	0.136	7.6	0.129
	Area	-	-	-	0.024	12.7	<b>0.01</b>
	LMA	-	-	-	0.026	7.4	< <b>0.0001</b>
	<i>A</i>	-	< <b>0.0001</b>	30.3	0.234	5.5	0.13
	<i>g<sub>s</sub></i>	-	<b>0.002</b>	33.3	0.256	7.2	0.07
	<i>iWUE</i>	-	<b>0.031</b>	12.7	< <b>0.0001</b>	13.5	0.217
	†ΔΨ	-	0.762	1.2	0.379	3.5	<b>0.0009</b>
†Ψ <sub>m</sub>	-	0.508	2.2	0.785	0.9	<b>0.005</b>	

<sup>1</sup>Experiment A, July 2011 odd and even replicates watered; Experiment B, July 2011 even replicate watered and odd replicate droughted; Experiment C, odd and even replicates watered similarly but measured consecutively and tested independently.

<sup>2</sup>Wald F-tests, numerator d.f.: Odd vs. Even = 1; Observer = 3, except Ψ<sub>m</sub> and ΔΨ = 1; Day Experiments A & B = 3, Day Experiment C = 1.

<sup>3</sup>Likelihood ratio tests (χ<sup>2</sup><sub>1</sub>)

Values in bold are statistically significant

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**Table 4** QTL for leaf phenotypes in ALB F<sub>1</sub> switchgrass grown in Austin, Texas, mapped separately in two replicates (odd and even rows) using phenotypes corrected for additive experimental effects (odd-even, day of experiment, where relevant observer) and spatial autocorrelation, i.e., normalized residuals; QTL with P < 0.1 based on permutation testing are shown by experiment and linkage group (LG).

Experiment <sup>1</sup>	LG	Replicate	Phenotype	Position (cM)	1.5 LOD interval (cM)	Marker <sup>2</sup>	LOD <sup>3</sup>	Percent variation explained
A	2b	even	g <sub>s</sub>	52.8	0-66	sww1517	4.06 <sup>+</sup>	10.77
	5b	even	Φ <sub>PSII</sub>	32.0	0-72	-	4 <sup>+</sup>	10.63
	5b	even	q <sub>p</sub>	48.2	2-72	sww1252	4.62*	12.16
	5b	odd	LMA	146.0	134-147	-	5.14*	13.43
B	3a	even	g <sub>s</sub>	87.2	66-121	sww2747	3.93 <sup>+</sup>	10.44
	9a	odd (dry)	iWUE	55.2	24.0-96	nfs107	4.66*	12.26
C	1b	even	mass	34	24-62	sww2596	5.43**	14.13
	1b	even	area	41.3	24-73.9	sww1855	4.07 <sup>+</sup>	10.8
	1b	odd	mass	45.7	22-66	sww2970	5.25**	13.71

<sup>1</sup>Experiments were: A, odd and even replicates watered July 2011; B, even replicate watered and odd replicate droughted July 2011; C, odd and even replicates watered May 2012.  
<sup>2</sup>Missing values indicate localisation to a pseudomarker position.  
<sup>3</sup>+0.05 ≤ P < 0.1, \*0.01 ≤ P < 0.05, \*\*0.001 ≤ P < 0.01, \*\*\*P < 0.001 (LOD threshold ranges: P=0.1, 3.8-4.09; P=0.05, 4.12-4.46; P=0.01, 4.73-5.58; P=0.001, 5.2-7.53).

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804**Table 5** Single marker tests of QTL, Environment ('odd' versus 'even' replicate), and QTL × Environment effects at markers and pseudomarkers corresponding to peak LOD scores in ALB

Experiment	LG	<sup>1</sup> Phenotype	Marker/ pseudomarker position	P-values: mean (2.5, 97.5 percentile)		
				<sup>2</sup> Genotype	<sup>2</sup> Environment	<sup>2</sup> Genotype × Environment
A	2b	<i>g<sub>s</sub></i>	sww1517	<b>0.018</b> (0.017, 0.023)	0.974 (0.974, 0.974)	<b>0.047</b> (0.039, 0.048)
	5b	Φ <sub>PSII</sub>	32 cM	0.382 (0.029, 0.862)	0.887 (0.886, 0.888)	0.308 (0.012, 0.807)
	5b	qp	sww1252	0.168 (0.004, 0.622)	0.912 (0.911, 0.913)	0.334 (0.021, 0.844)
	5b	LMA	146 cM	<b>1×10<sup>-4</sup></b> (4×10 <sup>-7</sup> , 7×10 <sup>-4</sup> )	0.995 (0.995, 0.995)	0.207 (0.062, 0.374)
B	3a	<i>g<sub>s</sub></i>	sww2747	<b>0.001</b> (5×10 <sup>-4</sup> , 0.002)	0.795 (0.795, 0.796)	0.071 (0.053, 0.096)
	9a	iWUE	nfsg107	<b>0.013</b> (4×10 <sup>-4</sup> , 0.063)	0.929 (0.929, 0.931)	<b>0.005</b> (1×10 <sup>-4</sup> , 0.022)
C	1b	mass	sww2596	<b>4×10<sup>-9</sup></b> (2×10 <sup>-9</sup> , 2×10 <sup>-8</sup> )	0.99 (0.99, 0.99)	0.553 (0.553, 0.722)
	1b	area	sww1855	<b>7×10<sup>-6</sup></b> (1×10 <sup>-7</sup> , 2×10 <sup>-5</sup> )	0.365 (0.361, 0.369)	0.897 (0.761, 0.961)
	1b	mass	sww2970	<b>2×10<sup>-5</sup></b> (2×10 <sup>-9</sup> , 5×10 <sup>-5</sup> )	0.991 (0.99, 0.991)	0.518 (0.322, 0.674)

<sup>1</sup>Normalized residuals, correcting for additive experimental effects (odd-even, day of measurement, and where relevant observer) and spatial autocorrelation  
<sup>2</sup>P-values from ANOVA applied to 500 imputed genotype classifications (marker sww2596 was fully informative and 498/500 imputed genotype sets matched exactly, so P-values are maximum and minimum not percentiles)  
**Bold: Mean P < 0.05**

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8 807 **Fig. 1** Leaf physiological phenotypes for Alamo-AP13 (filled symbols, solid line) and Kanlow-  
9 808 398209 (open symbols, dashed line), including response to drought (center column). Generalized  
10 809 least squares means and standard errors (N = 14-16) are shown for: (a,j,q)  $A$ , net CO<sub>2</sub>  
11 810 assimilation; (b,k,r)  $g_s$ , stomatal conductance to water; (c,l,s) iWUE, intrinsic water use  
12 811 efficiency; (d,m)  $\Phi_{PSII}$ , quantum efficiency of photosystem II; (e,n)  $F_v'/F_m'$ , light adapted  
13 812 efficiency of energy harvesting by open photosystem II reaction centers; (f,o)  $q_p$ , photochemical  
14 813 quenching of chlorophyll fluorescence; (p,t)  $\Psi_m$ , midday leaf water potential; (u)  $\Delta\Psi$ , midday  
15 814 hydrodynamic gradient; (g,v) leaf mass; (h,w) leaf area; (i,x) LMA, leaf mass per area.  
16 815 Significance values for statistical tests are presented in Table 1.

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19 816 **Fig. 2** Leaf physiological phenotypes for two replicates of the ALB F<sub>1</sub> mapping population,  
20 817 including response to drought (center column). Generalized least squares means and standard  
21 818 errors (N = 165 F<sub>1</sub>) are shown for: (a,j,q)  $A$ , net CO<sub>2</sub> assimilation; (b,k,r)  $g_s$ , stomatal  
22 819 conductance to water; (c,l,s) iWUE, intrinsic water use efficiency; (d,m)  $\Phi_{PSII}$ , quantum  
23 820 efficiency of photosystem II; (e,n)  $F_v'/F_m'$ , light adapted efficiency of energy harvesting by open  
24 821 photosystem II reaction centers; (f,o)  $q_p$ , photochemical quenching of chlorophyll fluorescence;  
25 822 (p,t)  $\Psi_m$ , midday leaf water potential; (u)  $\Delta\Psi$ , midday hydrodynamic gradient; (g,v) leaf mass;  
26 823 (h,w) leaf area; (i,x) LMA, leaf mass per area. Significance values for statistical tests are  
27 824 presented in Table 3.

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30 825 **Fig. 3** Linkage map for ALB [50] and locations of peak LOD scores and 1.5 LOD intervals for  
31 826 normalized residuals of leaf physiological phenotypes. QTL are labelled with phenotype,  
32 827 replicate (even or odd) and Experiment (A, odd and even replicates watered July 2011; B, odd  
33 828 replicate droughted and even replicate watered July 2011; C, odd and even replicates watered  
34 829 May 2012). QTL on each linkage group are plotted in order of P-values, with the lowest P-values  
35 830 closest to the linkage group: black indicates  $P < 0.05$ , gray  $0.05 \leq P < 0.1$ . Phenotypes: mass, leaf  
36 831 lamina mass; area, leaf lamina area;  $g_s$ , stomatal conductance to water;  $\Phi_{PSII}$ , quantum efficiency  
37 832 of photosystem II;  $q_p$ , photochemical quenching; LMA, leaf lamina mass per leaf lamina area;  
38 833 iWUE, intrinsic water use efficiency.

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835 **Fig. 4** Phenotypes by genotype, at five markers linked with QTL in ALB with no support for  
836 significant G×E. Marker names shown on the y-axis indicate the linkage group-marker-  
837 phenotype combination. Phenotypes are plotted as clouds of normalized residuals for all 165 F<sub>1</sub>,  
838 alongside means and s.e.m.; open symbols represent individuals from the 'odd' replicate, filled  
839 symbols the 'even' replicate. Replicates were watered similarly except (i-j) where drought was  
840 imposed on the 'odd' replicate. Parental genotypes, shown on the x-axis, were Alamo-A4 (A) and  
841 Kanlow-K5 (K), subscripts indicate alleles assigned by imputation.

842 **Fig. 5** Phenotypes by genotype at two markers linked with QTL in ALB where marker regression  
843 supported significant G×E. Marker names shown on the y-axis indicate the linkage-group-  
844 marker-phenotype combination. Phenotypes are plotted as clouds of normalized residuals for 165  
845 F<sub>1</sub>, alongside means and s.e.m.; open symbols represent the 'odd' replicate, filled symbols the  
846 'even' replicate. Replicates were watered similarly in a-b, and drought was imposed on the 'odd'  
847 replicate in c-d. Parental genotypes were Alamo-A4 (A) and Kanlow-K5 (K); alleles assigned by  
848 imputation at each marker are indicated by subscripts.

Figure 1

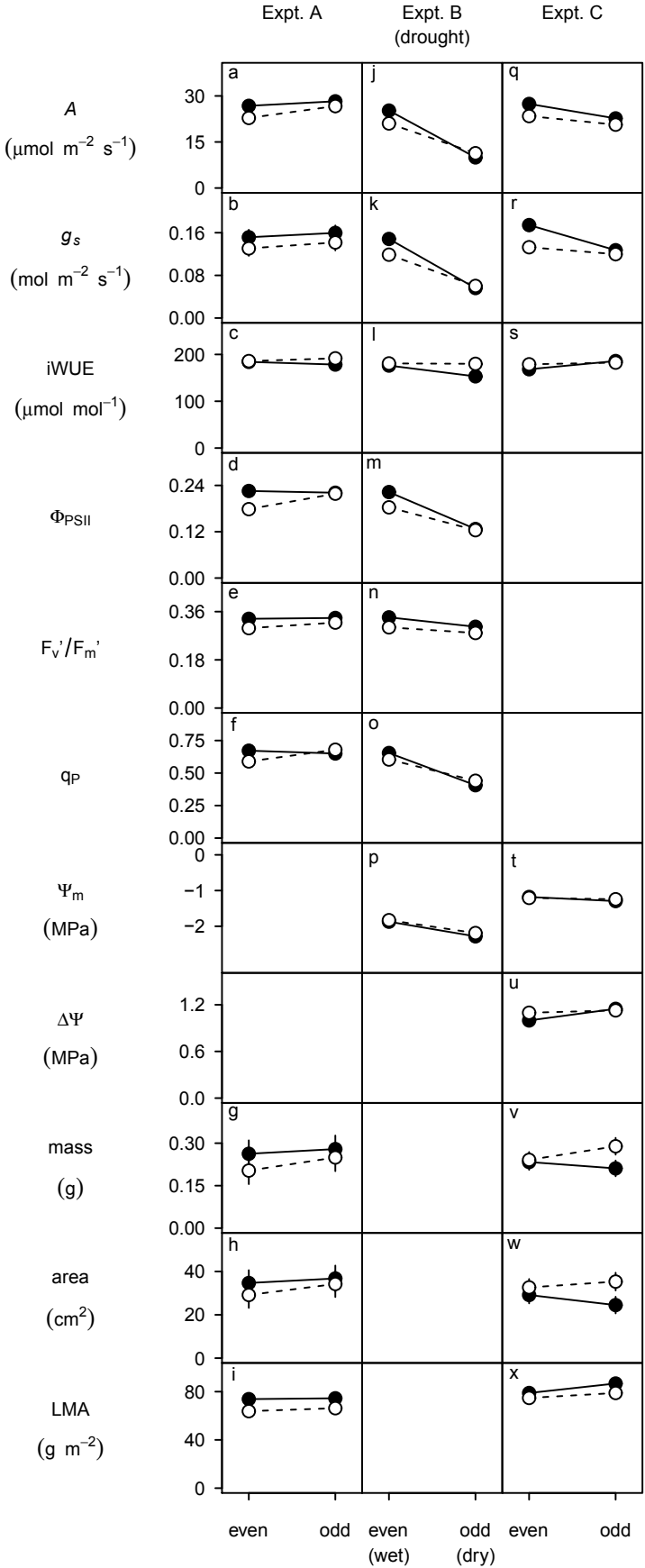


Figure 2

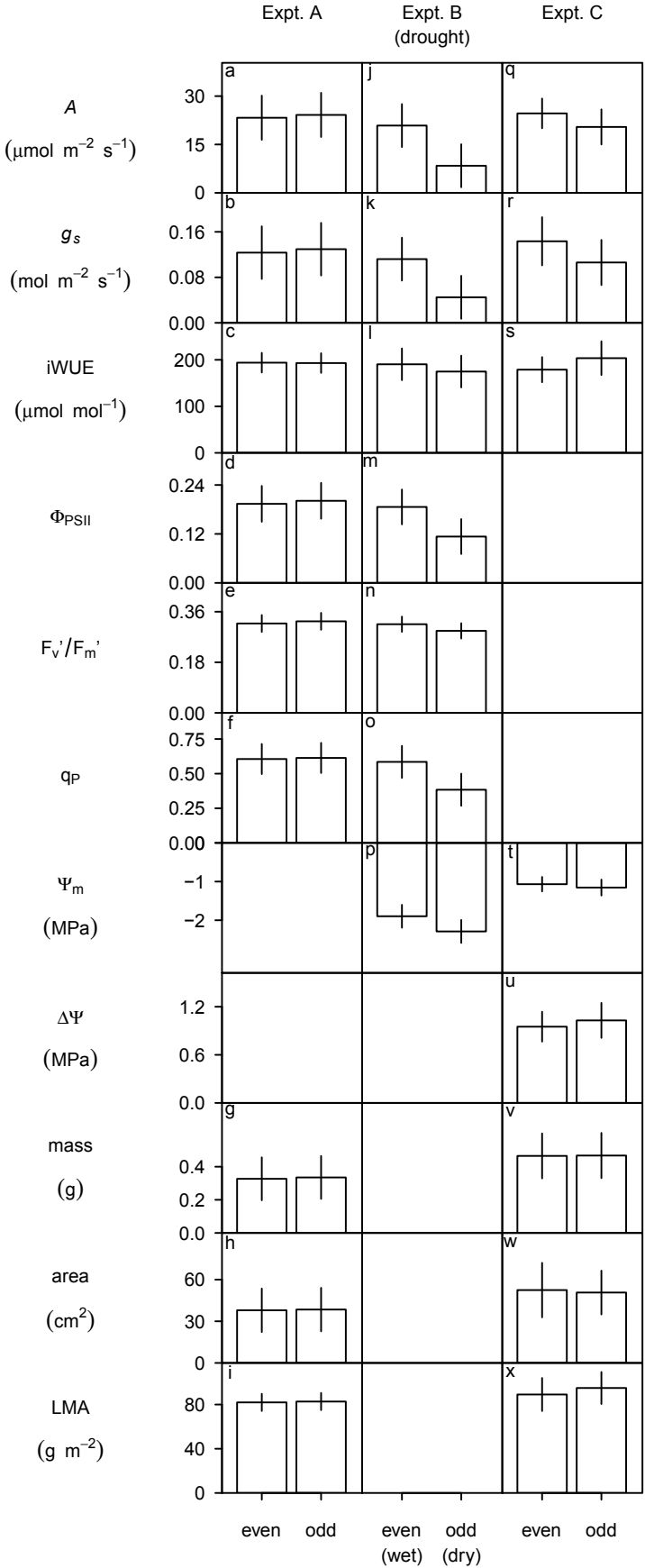


Figure 3

[Click here to download Figure 0616QTLNOnly.eps](#)

