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The wheat *Seven in Absentia* gene is associated with increases in biomass and yield in hot climates

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Abstract

Wheat productivity is severely reduced by high temperatures. Breeding of heat tolerant cultivars can be achieved by identifying genes controlling physiological and agronomical traits with high temperature and using these to select superior genotypes, but no gene underlying genetic variation for heat tolerance has previously been described.. We completed the positional cloning of *qYDH.3BL*, a quantitative trait locus (QTL) on bread wheat chromosome 3B associated with increased yield in hot and dry climates. The delimited genomic region contained 12 putative genes and a sequence variant in the promoter region of one gene - *seven in absentia, TaSINA*. This was associated with the QTL's effects on early vigour, plant biomass and yield components in two distinct wheat populations grown under various growth conditions. Near isogenic lines carrying the positive allele at *qYDH.3BL* under-expressed *TaSINA* and had increased vigour and water use efficiency early in development, as well as increased biomass, grain number and grain weight following heat stress. A survey of worldwide distribution indicated that the positive allele became widespread from the 1950s through the CIMMYT wheat breeding programme but, to date, has been selected only in breeding programmes in Mexico and Australia.

Keywords: *Triticum aestivum*, positional cloning, E3 ligase, drought, heat stress, grain

Significance statement

Wheat is the world's most widely grown crop and a staple of human diet. Even brief episodes of high temperature in the growing season cause severe yield reductions. Finding and deploying genes for heat stress tolerance in new varieties is a priority for food security with climate change. We narrowed a genetic locus to a small genomic region where genetic variation was present only in one gene was that showed clear differences of expression and improved yield

and physiology under stress in the populations. Using diagnostic markers to track the positive haplotype in nearly 750 accessions, we found many regions where the allele could be used in breeding programmes to increase wheat's heat tolerance.

Introduction

As one of the world's major crops providing 20 % of human food, bread wheat (*Triticum aestivum* L.) has a vital role in food security[1]. It is also the most widely produced crop, grown worldwide in a variety of climates including harsh environments. Wheat productivity is affected by growing season drought and heat stresses, often in combination, which can cause almost complete yield loss in some cases[2,3]. Due to the increasing occurrence of drought and heat stress, particularly in the Mediterranean region, the USA, India and Australia, which are amongst the largest producers of bread wheat in the world, improving yield under these stresses is a priority[4,5]. Targeting crop productivity in regions affected by drought and heat is believed to be the best strategy to reach the 1.6 % yield improvement per year required to meet the food needs of an increasing world population[6].

One way to deliver new, high yielding varieties is to discover and deploy genes associated with grain yield variation in stress-prone environments through breeding. Many genetic studies have focussed on the identification of quantitative trait loci (QTL) associated with yield variation under abiotic stress and, in wheat, several genes have been identified for tolerance of salinity, cold, aluminium and boron toxicity[7–10]. Genetic variation in wheat yields in dry and hot conditions has been extensively studied using bi-parental populations[11–27] but no genes underlying QTL for heat and drought tolerance have yet been successfully identified. Grain yield is a complex trait, highly influenced by the environment and, although QTL for yield have been identified, only a few have been used in breeding programmes due to inconsistent performance of the QTL in different environmental conditions.

We focussed on a QTL located on chromosome 3BL, *qYDH.3BL*, identified in a doubled-haploid (DH) population from the cross between the Australian wheats RAC875 and Kukri. These lines were selected for their contrasting physiological responses in Mediterranean-like climates: RAC875 is a water conservative line, whereas Kukri depletes water more quickly under stress[28]. In the RAC875 x Kukri DH population, a multi-environment analysis of 21 field trials showed a strong genotype by environment (G x E) interaction at the locus[29]. The QTL was expressed in the deep soil of northern Mexico with the RAC875 allele positively contributing to yield, thousand grain weight and early vigour. The Kukri allele at *qYDH.3BL* was associated with increased yield in southern Australian trials, but only when plants were irrigated[11,29]. Parent et al.[30] subsequently observed that the allele effect at *qYDH.3BL* was dependent on growth temperatures. In this study, we fine mapped the QTL to a short region of 690 Kbp sequence which includes 12 putative genes. Furthermore, we identified sequence variants and differential expression of a *SINA* gene consistent with the positive effects of one allele on early growth in unstressed conditions and plant biomass following heat stress. We identified and tracked the allele in a worldwide collection of wheat accessions to enable its future use.

Results

The QTL *qYDH.3BL*, originally found in the spring wheat RAC875 x Kukri DH population[29] was also present in a Drysdale x Gladius recombinant inbred line (RIL) population[31]. The analysis of these two populations across multiple trials in Australia and Mexico showed that at this locus the allele from RAC875 and Drysdale increased yield relative to the Kukri and Gladius allele (Fig. 1a, b). The multi-environment analysis of both populations showed that the high confidence QTL interval was flanked by the markers AWG43_1 and AWG38 (Fig. 1a, b). The RAC875 allele was positively associated with a 10 % yield increase (Fig. 1b). We fine mapped *qYDH.3BL* in RAC875 by anchoring the interval defined by the multi-environment

100 analysis onto the v1.0 reference sequence of cv. Chinese Spring (IWGSC RefSeq v1.0)[32]. We
101 then aligned the whole genome shotgun sequencing data of the four parental lines[33] against the
102 reference genome to identify sequence polymorphisms. Comparison between the genotypes
103 RAC875-Drysdale vs Kukri-Gladius enabled us to identify new sequence variants. Seventy-
104 four single nucleotide polymorphism (SNP)-based markers (named AWG and ADW hereafter)
105 and InDel markers were added to the previously described RAC875 x Kukri RIL map[29]. The
106 interval delimited by AWG43_1 and AWG38 defined a ~1.5 Mbp sequence in the reference
107 sequence assembly, containing 29 gene models, 17 described as high confidence genes (HC)
108 and 12 as low confidence genes (LC) (Fig. 1c). We found that this interval was smaller, only
109 ~1 Mbp, in the RAC875 genome (Fig. 1c, Supplementary Fig. 1).
110 The QTL was highly significant for yield in Mexican field trials with heat treatment (by late
111 planting with irrigation by flooding)[29]. These trials were conducted in the northern part of
112 Mexico in the Sonora region where the climate is hot and dry: temperatures at flowering time
113 averaged 25 - 28°C for conventional sowing and 30 - 35°C for late sowing; there is no rainfall
114 during the growing season and water is provided by irrigation; the soil profile is deep with an
115 effective soil depth of 110 - 120 cm[34]. The QTL was most significant when flood irrigation and
116 heat stress were applied to the crop which suggested that the QTL gave an advantage in deep
117 soil where water is stored at depth.
118 To identify the genes underlying the QTL, higher genetic map resolution was required and new
119 recombinants needed to be grown in conditions where the QTL has the strongest effects. As
120 southern Australian soils are shallow, we used a deep soil-mimic platform to test the hypothesis
121 that a deep soil profile, characteristic of the Mexican environments, triggered *qYDH.3BL*
122 expression. Forty-four and 20 RAC875 x Kukri RILs previously grown in Mexican field trials[29]
123 were planted in the deep soil platform in August and September 2014, respectively; late
124 planting dates enabled us to phenotype the plants under combined drought and heat stress

125 (Supplementary Fig. 2 and 3). The analysis of the September trial showed a significant positive
126 effect of the RAC875 allele on most measured traits, increasing early vigour, stem biomass,
127 plant height, spike length and biomass, grain and spike number per plant, spikelets per spike,
128 chlorophyll content and seminal roots number, by at least 19 % (Table 1). No effects were
129 observed on stomatal density, nor on carbon and nitrogen isotope discrimination in mature
130 grains (data not shown). These results showed that *qYDH.3BL*'s effects in the deep soil -mimic
131 platform were similar to those observed in Mexican field trials. A quantitative analysis of soil
132 water potential and air temperature effects on *qYDH.3BL* had previously found that the allele
133 effect was correlated to temperatures[30]. The RAC875 allele increased individual seed weight,
134 biomass and harvest index when temperatures were above 25˚C around flowering time. As
135 expected, we found that the RAC875 allelic effects were larger under the hotter conditions
136 experienced by plants sown in September (Table 1) compared to those planted in August
137 (Supplementary Fig. 4).
138 Out of 2,000 RAC875 x Kukri RILs, we found 30 lines with recombination breakpoints in the
139 interval delimited by the AWG43_1 and AWG38 markers and grew them in the deep soil-
140 mimic platform with heat stress to fine map *qYDH.3BL*. In 2015, maximum temperatures at
141 flowering time ranged between 34.8˚C and 38.8˚C (Supplementary Fig. 2). Spike length and
142 biomass, stem biomass and early vigour were significantly higher in plants carrying the
143 RAC875 allele at the ADW594 – ADW577 interval (Fig. 2a). Forty-four Drysdale x Gladius
144 RILs were also phenotyped in dry and hot conditions, using the deep soil-mimic platform.
145 The single-marker analysis showed the positive allele from Drysdale associated with
146 increases in flag leaf length and single grain weight that narrowed the interval in this
147 population to markers AWG 43_1 and AWG38 (Fig. 2b).
148 The eight recombination breakpoints in the QTL region in the 30 RAC875 x Kukri RILs
149 enabled us to fine map *qYDH.3BL* to a ~690 Kbp sequence interval delimited by markers

150 ADW594 and ADW577 which contained 12 annotated genes in the Chinese Spring reference
151 sequence (Table 2). We also performed a local assembly of the RAC875 genomic sequence
152 where no additional or missing annotated genes were detected in the interval compared to the
153 Chinese Spring reference sequence (Supplementary Fig. 1).
154 As no further recombinants were found in our RIL collections, we developed near-isogenic
155 lines (NIL) from heterozygous inbred families with recombination breakpoints spanning the
156 QTL interval using RAC875 x Kukri RILs with residual heterozygosity between AWG43_1
157 and AWG38 (Fig. 1d). Following crossing, these families segregate for the locus in contrasting
158 pairs but are otherwise near isogenic. In 2017, the lines were phenotyped under combined
159 drought and heat treatment in the deep soil-mimic platform for yield components and main
160 tiller sap flow, a proxy for plant transpiration[35]. To avoid the confounding effects of phenology,
161 we selected NIL2, 3 and 4 with similar flowering time as the RILs previously studied. In NIL2
162 family, the lines carrying the RAC875 allele had increased spike and stem biomass, increased
163 grain weight per plant and increased average number of spikelets per spike compared to those
164 carrying the Kukri allele. In NIL3 family, the lines carrying RAC875 allele had increased single
165 grain weight compared to those carrying the Kukri allele. In NIL4 family, the lines carrying
166 the RAC875 allele had increased spike and stem biomass, grain number, average number of
167 spikelets per spike and spike number compared to those carrying the Kukri allele (Table 3).
168 Mass sap flow was, on average, significantly lower in all NILs carrying the RAC875 allele
169 compared to those carrying the Kukri allele at the QTL over the period (Fig. 3). The regression
170 of normalized sap flow mass against mean daily temperatures was significant ($P= <0.05$) in all
171 lines even though the proportion of the variance explained was low (adj. $R^2 = 0.11–0.33$).
172 Whilst the difference was not significant early on during temperate days (average temperature
173 20.5°C, Fig. 3a) or later in development (grain filling) during several cool days (average
174 temperature 17.5°C, Fig. 3b), there was a significant difference in sap flow between RAC875-

175 allele NILs versus Kukri-allele NILs during heat stress (average temperature 31.5°C) (Fig. 3c).
176 At this time, sap flow in NILs carrying the RAC875 allele was always lower than in NILs
177 carrying the Kukri allele as the RAC875-allele lines failed to increase sap flow in response to
178 increasing temperature in comparison with the alternative allele.
179 To understand whether variation in water use was related to the observed increase in early
180 vigour for the RAC875 allele at *qYDH.3BL*, we grew the NIL3 and 4 allele pair plants on an
181 automated, gravimetric watering system with real-time imaging of early vegetative growth and
182 measurement of water used per plant in unstressed conditions. Early vigour was increased in
183 NIL4 lines carrying the RAC875 allele (Fig. 4) consistent with the allelic effect observed in
184 the RIL experiments in deep soil (Table 1) and the increase in final biomass observed for this
185 NIL (Table 3), but the effect was not significant in NIL3. In unstressed conditions, NIL4 with
186 the RAC875 allele also had increased water use compared with the Kukri allele in complete
187 contrast with its reduced sap flow during heat stress. The water use index (kpixel/ ml H_2O) of
188 NIL4 was increased in plants carrying the RAC875 compared with the Kukri allele, showing
189 that this line's early increase in biomass did not demand increased water use in unstressed
190 conditions, *i.e.* that the RAC875 allele at the locus conferred both increased early vigour and
191 increased water use efficiency in unstressed conditions.
192 We then analysed the sequence of the 12 gene models present in the QTL interval delineated
193 by ADW594 and ADW577 markers using whole genome sequencing datasets of RAC875,
194 Kukri, Gladius and Drysdale. Using shotgun sequencing data of the four parental lines, no non-
195 synonymous variation was identified in the protein sequences encoded by the 12 genes at the
196 locus when the RAC875-Drysdale vs Kukri-Gladius translated sequences were compared.
197 Therefore, functional variation was most likely to lie at the gene expression level. We next
198 studied the expression profile of these gene models in the four NIL pairs that contrasted for
199 yield components in deep soil. We did not find any evidence of gene expression for the four

200 low confidence gene models, either in the wheat-expression database[36] (http://www.wheat-
201 expression.com) or in our in-house database[37] that displays RNA-seq expression data in five
202 tissues at three developmental stages[38] (Table 2). We concluded that these sequences were
203 likely pseudogenes. As expected, we found evidence of gene expression for the eight genes
204 annotated as high confidence (Table 2).
205 We measured the expression of these eight genes in the four NIL pairs, expecting a difference
206 in expression between the RAC875 and Kukri alleles for the gene(s) responsible for *qYDH.3BL*
207 effect. As the QTL was associated with early vigour, when no treatment had been applied, we
208 studied gene expression in seedlings grown in unstressed conditions. The expression profiles
209 of genes I, II, IV, V, VI, VIII and XII in RAC875 and the NILs carrying the RAC875 allele
210 were not significantly different from Kukri and the lines carrying the Kukri allele
211 (Supplementary Fig. 5a-g). Gene X (*TaSINA 3B*) was the only gene with a differential
212 expression pattern both between the parental lines and among the NILs (Fig. 5a). The gene was
213 consistently highly expressed and statistically different in the lines containing the Kukri-
214 Gladius allele compared to the lines containing the RAC875-Drysdale allele, in both shoot and
215 root tissues of seedlings, except for NIL2 in shoot tissue (Fig. 5a and 5e). By contrast, gene X
216 homeologs on chromosomes 3A and 3D had the same expression profile in the two parental
217 lines and the NILs independently of the allele that they contained and the tissue analyzed (Fig.
218 5b and 5d).
219 We also analyzed the expression profile of gene X in cDNA of RAC875 and Kukri grown in
220 well-watered conditions and cyclic drought[39]. Gene X was significantly over-expressed in
221 Kukri compared to RAC875 in both well-watered and drought treatments (Fig. 5c). Based on
222 homology with rice and *Brachypodium*, gene X is homologous to a *seven in absentia (SINA)*
223 gene encoding an E3 ubiquitin ligase protein, hereafter called *TaSINA*.

224 The translated Sanger sequencing of the *TaSINA* gene revealed two amino acid substitutions
225 between the RAC875 and Kukri protein sequences that were not predicted to affect the protein
226 function. We also found sequence polymorphisms in the promoter region with the predicted
227 *cis*-acting elements CANBNNAPA and HDZIP2ATATHB2 present in Kukri and absent in
228 RAC875 (Fig. 5g). In RAC875 and Drysdale promoter sequences, the insertion of a CCAC
229 domain 713 bp upstream of the start codon created a SBOXATRBCS (CACCTCCA) motif
230 and a -10PEHVPSBD (TATTCT) motif which were absent in the Kukri and Gladius promoter
231 sequences (Fig. 5g). The sequences of *TaSINA* homeologs on chromosomes 3A and 3D were
232 monomorphic between RAC875 and Kukri. The alignment of the TaSINA predicted protein
233 sequence and its homeologs revealed an amino acid change in the protein sequence of the 3A
234 copy (Fig. 5f). A SNP in the coding sequence of the 3A copy introduced the substitution of an
235 alanine to a valine at the 227 amino acid position that was predicted to be deleterious for the
236 protein function (Fig. 5e). This suggested that the 3A protein might be non-functional in both
237 RAC875 and Kukri, although we did find evidence of gene expression in the roots.
238 We studied the worldwide allelic distribution of *TaSINA* alleles using the two closest SNP
239 markers (ADW594 and ADW595). Alleles were homogeneously distributed among the lines,
240 with 53 % of the accessions carrying the same allele as RAC875 (Fig. 6). We looked at the
241 origins and years of release of the accessions in each allelic group to identify a potential pattern
242 of selection (Supplementary Table 1). The RAC875 allele was over-represented in accessions
243 originating from CIMMYT in Mexico and in Australia compared to the Kukri allele which was
244 more abundant in the germplasm originating from the Middle-East, South America and Asia
245 (Fig. 6). Both alleles were evenly represented in European and African wheats. The Kukri allele
246 was present in historical wheat varieties such as Du Toits, Federation, Ward's Prolific and
247 Hudson Early Purple Straw, released before 1900, and also in a large proportion of landraces
248 (Supplementary Table 1). The RAC875 allele appeared later in the panel's chronology, with

249 most of the lines released after 1950, and seemed to follow the migration of germplasm released
250 by CIMMYT during the green revolution of the 1960s.
251

252 Discussion

253 The RAC875 allele at *qYDH.3BL* increases early vigour, seminal root number, plant height,
254 spike length, above ground biomass, stem and spike biomass, spike number, number of
255 spikelets per spike and total grain number in deep soil. The association of *qYDH.3BL* with early
256 vigour suggests that the mechanisms underlying the QTL are effective at an early stage of plant
257 development, before abiotic stress is encountered. Early vigour is an important trait associated
258 with water uptake, preventing water loss from soil evapotranspiration to the profit of
259 transpiration[40]. We hypothesize that this increased early growth in plants carrying the RAC875
260 allele had no significant effect under optimal conditions, as observed in southern Australian
261 field trials in 2009[29], but was beneficial to the plant when temperatures increased.
262 As *qYDH.3BL* had been previously associated with differences in canopy temperature[11], a
263 surrogate for plant evaporative cooling, we used sap flow sensors to evaluate the transpiration
264 rates of whole plants[35]. We observed that sap flow responded to daily temperature variations,
265 but this response was abolished in lines carrying the RAC875 allele during heat stress late in
266 development. These results are consistent with the observations of Izanloo et al.[28] on the water-
267 management behaviour of RAC875 and Kukri parental lines under cyclic-drought. They
268 concluded that RAC875 reduced its water consumption at early stages of drought stress for
269 later consumption when the stress become more severe. Here, the RAC875 allele increased
270 plant biomass and water use at early, vegetative stages of development and reduced
271 transpiration in response to heat late in development without any negative impact on yield
272 components.

273 By fine mapping the QTL and by expression analysis of eight genes in the QTL interval, we
274 found that only the *TaSINA* gene showed consistent variation between lines carrying
275 contrasting alleles at the QTL. Beyond those identified in the reference assembly, no other gene
276 was present in the interval when we locally assembled RAC875 genomic sequence. Therefore,
277 *TaSINA* is a strong candidate for the QTL effect.
278 *SINA* genes encode for an E3 ubiquitin ligase protein that forms a complex with the E1
279 ubiquitin-activating enzyme and the E2 ubiquitin-conjugating enzyme which ubiquitinate
280 target proteins for degradation[41]. *SINA* genes were first reported in Drosophila associated with
281 the development of photoreceptor cell, R7, localized in the Drosophila eye[42]. Analyses of the
282 gene family in plants identified several copies of *SINA* in *Arabidopsis thaliana*[43], *Populus*
283 *trichocarpa*[44], *Oryza sativa*[43], *Zea mays*[43], *Physcomitrella patens*[43], *Medicago truncatula*[44] and
284 *Solanum lycopersicum*[45] with roles in plant development and abiotic stress tolerance[46,47]. No
285 *SINA* gene has previously been reported in bread wheat. In Arabidopsis, *SINAT5* ubiquitinates
286 a *NAC1* transcription factor to regulate the growth signalling hormone auxin[48]. Arabidopsis
287 plants overexpressing *SINAT5* had fewer lateral roots compared to the wild type, whereas
288 expression of a dominant-negative mutant (substitution Cys49-Ser) induced more lateral
289 roots[48]. The ectopic expression of this dominant negative form of *SINAT5* in *M. truncatula*
290 reduced root nodulation, but both root and shoot growth were more vigorous in young (*in vitro*)
291 plants after 20 days and in older plants, after eight weeks' growth[44]. We observed similar results
292 in our experiments when Kukri had fewer primary and seminal roots compared to RAC875
293 (Table 1): *TaSINA* was more strongly expressed in Kukri compared to RAC875 and early
294 vigour and biomass were increased in wheat plants when *TaSINA* was not expressed (Table 1,
295 Fig. 4). No change in *TaSINA* expression was observed for RAC875 and expression was
296 reduced in Kukri only after prolonged cyclic drought (25d) (Fig. 3b). This suggests that *TaSINA*

297 expression is relatively insensitive to stress and that heat tolerance in wheat is more likely
298 conferred by increases in growth and water use when *TaSINA* expression is reduced.
299 The promoter sequence of RAC875 *TaSINA* contained a *cis*-acting element, SBOXATRCS
300 (CACCTCCA) also called S box, absent in the promoter of Kukri *TaSINA* (Fig. 5). This motif
301 is a putative ABSCISIC ACID INSENSITIVE-4 (ABI4) -binding site which negatively
302 regulates the *Conserved Modular Arrangement 5* genes, *CMA5,* in Arabidopsis in response to
303 sugar and ABA signals[49]. ABA is a phytohormone that plays a central role in different
304 physiological processes of plants, mediates gene expression in response to environmental
305 stimuli and regulates ABA-dependent responses in heat stress[50]. Sugar signalling also plays an
306 important role in mediating plant responses to environmental stimuli and mediates gene
307 expression associated to photosynthesis, carbon and nitrogen metabolism and secondary
308 metabolisms[51]. We hypothesize that the presence of the S box element and ABI4 binding site,
309 in the promoter sequence of *TaSINA,* contributes to the down-regulation of its expression in
310 RAC875 and might affect ABA and sugar signalling. In Kukri, the absence of the motif would
311 prevent the regulation of *TaSINA* and lead to gene expression and, consequently, the
312 degradation of its target proteins.
313 Following genotyping of 743 worldwide wheat accessions, we found that the RAC875 allele
314 was over-represented in the CIMMYT germplasm suggesting that the allele had been selected
315 through breeding for high yield under heat stress. The RAC875 allele was unexploited for
316 wheat breeding until the 1950s when it appears to have been recruited by the CIMMYT wheat
317 programme in Mexico and into Australia with the introduction of CIMMYT material. It is
318 surprising that RAC875 allele is not more prevalent in India and other Asian countries given
319 that CIMMYT germplasm, either as parents or directly as cultivars, has been estimated to
320 account for over 65% of production in India. This allele has not yet been exploited in other
321 regions where heat was not considered a threat until recently. With the increasing occurrence

322 of heat stress events, this allele could be beneficial in other regions with deep soil such as in
323 Europe and in the Punjab in India.
324

325 Materials and Methods

326 Plant materials

327 A set of 160 recombinant inbred lines (RILs) of the cross between RAC875 (RAC-
328 655//SR21/4*Lance/3/4*Bayonet) and Kukri (Madden/6*RAC-177//Grajo/76-ECN-44) were
329 used for the fine mapping of *qYDH.3BL*. A set of 70 RAC875 x Kukri RILs were selected for
330 their contrasting yield in the 2011[29] and 2012 field trials conducted in Mexico (Supplementary
331 Table 2) and based on their recombination point in the QTL interval.

332 Four near isogenic families derived from QTL-specific residual heterozygous lines from
333 RAC875 x Kukri RIL were developed to further study the mechanism underlying *qYDH.3BL*.
334 A second set of 44 RILs of the cross between Drysdale (Hartog*3/Quarrion) and Gladius
335 (RAC875/Krichauff//Excalibur/Kukri/3/RAC875/Krichauff/4/RAC875//Excalibur/Kukri)
336 (DG) was also studied. The lines were selected based on their recombination breakpoint in the
337 QTL interval and their extreme yield values during the 2009 and 2010 Mexican and New South
338 Wales field trials[52]. The four parental lines RAC875, Kukri, Drysdale and Gladius were
339 included in each trial.

340 Nulli-tetrasomic lines cv. Chinese Spring (LV-Szechuan) of the chromosome group 3 were
341 used to test the specificity of chromosome-specific primers. The method used to obtain nulli-
342 tetrasomic lines was previously described[53].

343 A diversity panel combining 743 landraces and modern varieties of hexaploid wheat was used
344 to identify the distribution of alleles at *qYDH.3BL*. 544 accessions were spring wheat types

345 originating from world-wide locations with more than one quarter from Australia. We also
346 used the INRA core collection which contains 372 worldwide accessions of winter wheat
347 mostly from Europe[54]. Pedigree, year of release and geographical origin of the lines were
348 retrieved from the wheat pedigree portal, www.wheatpedigree.net, and personal
349 communications (Peter Langridge, Margaret Pallotta).

350 **Genotyping and genetic map construction**

351 DNA was extracted as described previously[55]. Gene-based markers were designed using the
352 whole genome sequencing (WGS) data of the parental lines (RAC875, Kukri, Drysdale and
353 Gladius) generated by BioPlatforms Australia using Illumina HiSeq sequencing technology[33].
354 Sequence reads (100 bp) were aligned against the reference sequence IWGSC RefSeq v1.0 of
355 Chinese Spring (https://www.wheatgenome.org/) using DAWN. DAWN is a web interface that
356 integrates multiple datasets including IWGSC RefSeq v1.0, RNA sequencing data of five
357 tissues at three developmental stages[39] and WGS data of the parental lines. SNPs within
358 *qYDH.3BL* interval were identified with a minimum of five reads per line at a SNP position.
359 As regions with a high density of reads are more likely to be repetitive elements, SNPs with a
360 coverage higher than 50 reads were discarded.
361 Kompetitive Allele Specific PCR (KASP) markers were designed with the Kraken software
362 (LGC genomics, Middlesex, UK) on sequences of at least 100 bp spanning a SNP. Two allele-
363 specific forward primers were designed for each SNP in combination with a common reverse
364 primer. Each forward primer had a tail attached at the 5'end specific to a fluorophore. The
365 hybridization of one specific forward primer to the target sequence allowed the pairing of the
366 fluorophore present in the KASP mix to the primer tail releasing the quencher allowing
367 emission of fluorescence[56]. KASP assays were performed using the KASPline
368 (https://www.lgcgroup.com). KASP markers were prefixed AWG- and ADW- (Supplementary
369 Table 3).

370 The genotyping data of the KASP markers were added to RAC875 x Kukri and Drysdale x
371 Gladius RILs genetic maps previously generated by Bonneau et al.[29]. Genetic maps were
372 constructed using ASMap package[57] available in the software R. Genetic distance between
373 each marker was calculated using the Kosambi mapping function[58] which is based on a new
374 algorithm named MSTmap[59]. This algorithm can determine the markers order efficiently by
375 establishing a minimum spanning tree. Two markers, AWG594 and AWG595, were used for
376 studying the allelic distribution in the wheat diversity panel.

377 **Deep soil phenotyping platform**

378 Wheelie bins (100 x 57.5 x 51 cm) were filled with a mixed medium (1/3 coco peat, 1/3 sand
379 and 1/3 clay). Twenty-five plants were grown per bin, in a 5 rows x 5 columns grid with 10 cm
380 space between each plant. Trials were conducted at the Waite Campus, Urrbrae, Australia, in
381 a tunnel with a polyurethane cover. Meteorological data, air temperature and relative humidity
382 were recorded with a data logger (Kongin KG100, China). Gypsum blocks (MEA, Magill, SA,
383 Australia) were placed in the bins at two depths (10 and 40 cm) to record soil water potential
384 (Supplementary Fig. 3).

385 In 2014 and 2015, RILs and parental lines were grown in a resolved latinized incomplete block
386 using CycDesigN[60], plus one filler plant placed in the centre of the bin. The design took into
387 account the position of the lines according to a west/east axis and split the bins between an
388 inner and outer layers. Two trials were conducted in both 2014 and 2015. In 2014, RAC875 x
389 Kukri RILs were sown later than normal planting season (44 RILs sown on 11th of August and
390 20 RILs sown on 8th of September, instead of May) to phenotype plants under combined
391 drought and heat treatment. Plants were well-watered during the first month for both
392 treatments; watering was stopped at booting stage for stress treatments. In 2015, both trials

393 were conducted under dry and hot conditions: 30 RAC875 x Kukri RILs were sown on the 10th
394 of July, and 44 Drysdale x Gladius RILs were sown on the 18th of August.

395 In 2017, the NILs were grown in a single plant plot design with 15 replicates each for the
396 parental lines RAC875 and Kukri and 54 replicates of each pair of NIL. The design was
397 developed using the function "prDiGGer" in the DiGGer package in R for partially replicated
398 designs (Supplementary Fig. 6). Replicates were distributed within the bins so that the same
399 genotype was not present twice in the same row or column. In 2017, the plants were sown on
400 31st of July, watering was maintained until late booting in the beginning of October and then
401 stopped.

402 **Phenotypic evaluation in the deep soil-mimic system**

403 Plant developmental stages were scored using the Zadoks' scale around anthesis[61]. Early vigour
404 was scored using two methods. In 2014, during the first month after planting, photographs were
405 taken for each bin every week and early vigour were scored using a 1 (less vigorous) to 5 (more
406 vigorous) comparative scale. In 2015, early vigour was monitored by measuring the total leaf
407 area when a plant reached the four-leaves stage. The lengths and widths of each leaf on the
408 main tiller were measured to determine the individual leaf area. The total leaf area was
409 calculated as the sum of the leaf area (leaf width x leaf length x 0.8) of the second and third
410 leaves[62]. Chlorophyll content of the flag leaf was estimated with a SPAD-502L meter (Ozaka,
411 Japan) at three stages: booting, anthesis and grain filling. Three measurements per flag leaf at
412 each stage were taken to estimate the average value. Flag leaf area was measured from booting
413 to flowering time using the LI-3000C portable leaf area meter (LI-COR Inc., USA) in 2014. In
414 2015 flag leaf length and width were measured at flowering stage with a ruler. Stomatal density
415 was measured by taking a leaf imprint of the flag leaf adaxial face at anthesis using translucent
416 nail polish on a glass slide[63]. The slides were then analysed by microscopy using the Leica AS-

417 LMD Laser Microdissection Microscope (Wetzlar, Germany). Three pictures per sample were
418 taken and the guard cells number was counted for each.

419 Tiller number was recorded at four-leaf stage and before harvesting. Tiller abortion was
420 calculated at the end of the trial by subtracting the spike number from the tiller number. After
421 harvest, spikes were manually counted to evaluate the number of spikes per plant. Spikes and
422 stem biomass were weighed separately, and harvest index was determined by dividing grain
423 biomass by total above-ground biomass. Spike length was measured using a ruler before
424 counting the number of spikelets per spike for each spike. Seed number was measured using a
425 seed counter (Pfueffer GmBH, Germany) and then weighed to determine single grain weight.
426 In 2014, carbon and nitrogen isotopes discrimination of mature grains were measured on five
427 grains per plant. Grains were dried for one week at 60˚C and finely ground to 1 to 4 mg of
428 powder stored in tin capsules pressed (Sercon, gateway Crewe, UK). The samples were sent to
429 the University of California, Davis Stable Isotope Facility, University of California, for
430 analysis using continuous flow Isotope Ratio Mass Spectrometer (IRMS).

431 Root traits were also evaluated in the 2014 experiment in deep-soil bins by digging out the
432 roots at 10 cm depth after harvesting. The number of both seminal and nodal roots were
433 measured. Pictures of the root system were taken for each plant and analysed using the Image
434 J software[64]. The root growth angle of the angle formed by seminal roots and soil surface was
435 measured.

436 **Measurement of plant water use using sap flow sensors**

437 In 2017, SF-4/5 Micro Stem Sap Flow sensors (Edaphic Scientific, Port Macquarie, NSW,
438 Australia) were used in the wheelie bins on the NILs to measure ascending sap flow through
439 the main stem and calculate plant water use[35]. The measurements are non-destructive and
440 continuous. Each sensor contains a heater located between two temperature probes. Data were

441 automatically collected every 15 min after the initial warm up of the stem by the heater for five
442 min. The output voltage was proportional to the difference of temperatures between the two
443 probes. Sensors were installed on plants from mid-October when watering was stopped until
444 harvest and placed between the first and second node. Data were recorded from the beginning
445 of grain filling. Sensors were isolated with aluminium foil to avoid temperature fluctuations.
446 Sensors were calibrated by measuring stomatal conductance (g_s, $mmol.m^{-2}.s^{-1}$) of plants every
447 two hours from pre-dawn until after sunset with an AP4 leaf porometer (Delta-T devices,
448 Cambridge, UK). Collected raw sap flow data were normalized by setting a constant zero as
449 the average of readings for five hours during each preceding night and then averaged for every
450 hour to calculate the mean average of the sap flow data for each allele within a NIL pair. The
451 data were then plotted with the daily mean temperature. We also calculated the total
452 transpiration rate per day and compared it within each NIL pair between the lines carrying the
453 RAC875 allele and those with the Kukri allele.

454 **Early vigour imaging and water use analysis**

455 Uniformly sized seeds of the NIL3 and NIL4 allelic pairs (n=6) were sown in 150 mm pots
456 (one seed per pot) in cocopeat compost and grown in a climate-controlled glasshouse
457 containing an automated gravimetric watering and imaging platform previously described[65]
458 with day/night temperatures of 22/15°C. The plants were arranged in two lanes × 18 positions
459 using a randomised complete-block design. After 25 d, images of each plant from each NIL
460 and allelic pair were captured daily for the succeeding 25 d using the LemnaTec-Scanalyser
461 3D platform. Projected shoot area (PSA kpixel) was estimated as the sum of the areas from
462 three RGB camera views comprising two side views at 80° angles and one view from above.
463 Water use was computed using the smoothed values of watering amounts (mL) and water use
464 index for a given interval was the ratio in change between PSA and total water use in that
465 interval, with higher values corresponding to higher water use efficiency.

466 **QTL analysis**

467 The multi-environment QTL analysis of the RAC875 x Kukri RILs combined four field trials
468 conducted in Mexico, Ciudad de Obregon, in 2011[29] and 2012[52] and was performed as
469 described previously[29]. For the single-marker analysis, we generated the best predicted
470 unbiased estimates (BLUEs) for each trait using the model (1) and the asremlPlus package[66].
471 The normal distribution of the BLUEs was then evaluated using the Shapiro-Wilk test[67].
472 Single-marker analysis at each marker position was performed to test association with the
473 studied traits by a one-way ANOVA in R. For the trait which did not follow a normal
474 distribution, a Kruskal-Wallis test was used[68].

475 (1) Var[Y] = (((Rep/Bin)*(Zone/Side))/Position)

476 **Anchoring of *qYDH.3BL* onto the wheat reference sequence**

477 Flanking markers at the QTL were used to find sequence similarity with the reference sequence
478 RefSeq v1.0 of Chinese Spring by BLASTN (e-value -10; # hit return =100). The 1.5 Mbp
479 sequence delimited by AWG43_1 and AWG38 was extracted using Fetch-seq, an in-house
480 sequence retriever that allows the extraction of sub-sequences from the IWGSC RefSeq v1.0
481 using coordinates. Gene annotations were retrieved using the coordinated interval in the
482 browser available on the URGI website (https://wheat-urgi.versailles.inra.fr/). Genomic
483 sequences of the annotated genes were used to find similarities with *Brachypodium distachyon*
484 v3.1 and the *Oryza sativa* v7 reference genomes to identify putative gene function by BLASTN
485 (default settings) in phytozome (https://phytozome.jgi.doe.gov/).

486 **Targeted assembly of the region in RAC875**

487 Initial, stringent alignments of WGS reads of RAC875[33] indicated that approximately 150 kbp
488 region in Chinese Spring RefSeq 1.0 (from adw477 to adw594) may either be absent or highly
489 divergent in RAC875. Further, more relaxed alignments of RAC875 paired-end (PE) and mate-
490 pair (MP) data to RefSeq v1.0 failed to produce any evidence for the region being deleted.

491 Reads and their mates which remained unaligned and those which aligned within the region of
492 interests were k-merized, k=64 using KMC2[69]. We then assembled k-mers occurring three or
493 more times in the input data into unitigs i.e. contigs unambiguously supported by k-mers, using
494 yakat kextend[70]. Unitigs were scaffolded with the complete MP and PE datasets using sspace[71].
495 The resulting scaffolds were aligned to RefSeq v1.0 using BLASTn to exclude those derived
496 from other regions of the genome and to identify a subset likely to be derived from the region
497 of interest. The total length of 80 scaffolds putatively identified as coming from the region of
498 interest adds up to ~200 Kbp and the longest scaffold covers 36 Kbp. Scaffolds with a
499 minimum length of 10 Kbp were then annotated using TriAnnot[72].

500 **Gene sequencing and promoter analysis**

501 *TaSINA* chromosome specific primers were designed with Primer 3 available in Geneious
502 (Biomatters, Auckland, New Zealand) (Supplementary Table 3). Nulli-tetrasomic lines[53]
503 specific to the group 3 chromosome were used to test the specificity of the primers through
504 amplification of the amplicon fragment. PCR samples were Sanger sequenced at the Australian
505 Genome Research Facility Ltd (http://www.agrf.org.au/). Oligo sequences were conserved in
506 RAC875, Kukri and Chinese Spring. Promoter sequences of the RAC875 and Kukri *TaSINA*
507 were annotated using the Plant Cis-acting Regulatory DNA Elements (PLACE) database which
508 includes sequence motifs reported as cis-acting regulatory DNA elements[73]. Protein domains,
509 residues and motifs of *TaSINA* were identified using Prosite[74] and Pfam
510 (http://beta.supfam.org/).

511 **Gene expression analysis**

512 The wheat-drought experiment cDNA series of RAC875 and Kukri was previously described[39].
513 Additionally, we used two-week-old seedlings of the NIL pairs for gene expression analysis.
514 Seedlings were grown in an air-conditioned glasshouse in pot trays filled with a potting mix
515 (coco-peat). Total RNA for both root and shoot tissues was extracted using the Spectrum™

516 Plant Total RNA Kit (Sigma-Alridch, Carlsbad, California, USA) using the manufacturer's
517 recommended protocol. cDNAs were synthetised using the SuperScript IV First-Strand
518 Synthesis System (Invitrogen, Sigma-Alridch, Carlsbad, California, USA) as per the
519 manufacturer's instructions and used for qPCR. qPCR primers targeting 70-200 bp amplicon
520 sequences were designed to target the chromosome specific gene copy (Supplementary Table
521 3). Specificity was tested with nulli-tetrasomic lines. qPCR assays were performed using the
522 Kapa SYBR Fast Universal 2X qPCR Master Mix (Geneworks, Thebarton, South Australia,
523 Australia) on the QuantStudio 6 Flex (Applied Biosystems, Foster City, CA, USA) following
524 these steps: 95 ºC for 3 min; followed by 40 cycles at 95 ºC for 20 sec, 63 ºC for 20 sec, 72 ºC
525 for 15 sec. The initial run was then followed by a melting curve at 95 ºC for 15 sec, 60 ºC for
526 1 min increasing by 0.05 ºC per cycle up to 95 ºC. Three technical replicates per sample and
527 per gene and four reference genes including *TaActin*, *TaCyclophilin*, *TaGAPDH*
528 (glyceraldehyde-3-phosphate dehydrogenase) and elongation factor (*TaEFα*), were included in
529 the analysis for normalization of gene expression.

530

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Author contributions

PaT and PK performed the genotyping, QPCR and plant phenotyping experiments, developed the NIL and PaT analysed data. JB developed molecular markers, ran the trial on RIL in Ciudad de Obregon and generated the RIL fine genetic map. CB developed the experimental design of the deep soil-mimic and imaging experiments and wrote the R scripts to analyse the data. RS did the local assembly of RAC875 genomic sequence. UB supervised the DAWN analysis and the annotation of the genomic sequences performed by PaT. PT analysed the sap flow data. PaT, JB, PL, PT, and DF designed the experiments, interpreted the results and co-wrote the paper.

Competing interests

The authors declare no competing interests.

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738

739 # Figure Legends

740 **Fig. 1. Genomic structure of *qYDH.3BL* in wheat. a,** QTL analysis of the Drysdale x Gladius
741 recombinant inbred lines (RILs) in 5 field trials in Australia in 2009 and 2010. **b**, QTL analysis
742 of RAC875 x Kukri (RILs) in 4 field trials conducted in 2011 and 2012 in Ciudad de Obregon,
743 Mexico, delineating the QTL peak between the markers AWG43_1 and AWG38. **c,** Physical
744 interval of *qYDH.3BL* in RAC875 defined an interval of ~ 1 Mbp. HC: high confidence gene
745 models, LC: low confidence gene models. The orange, hatched rectangle highlights the interval
746 where RAC875 local assembly has been done. **d,** Genotypes of RAC875 x Kukri RILs with
747 residual heterozygosity at *qYDH.3BL* used to generate near-isogenic lines (NIL).

748

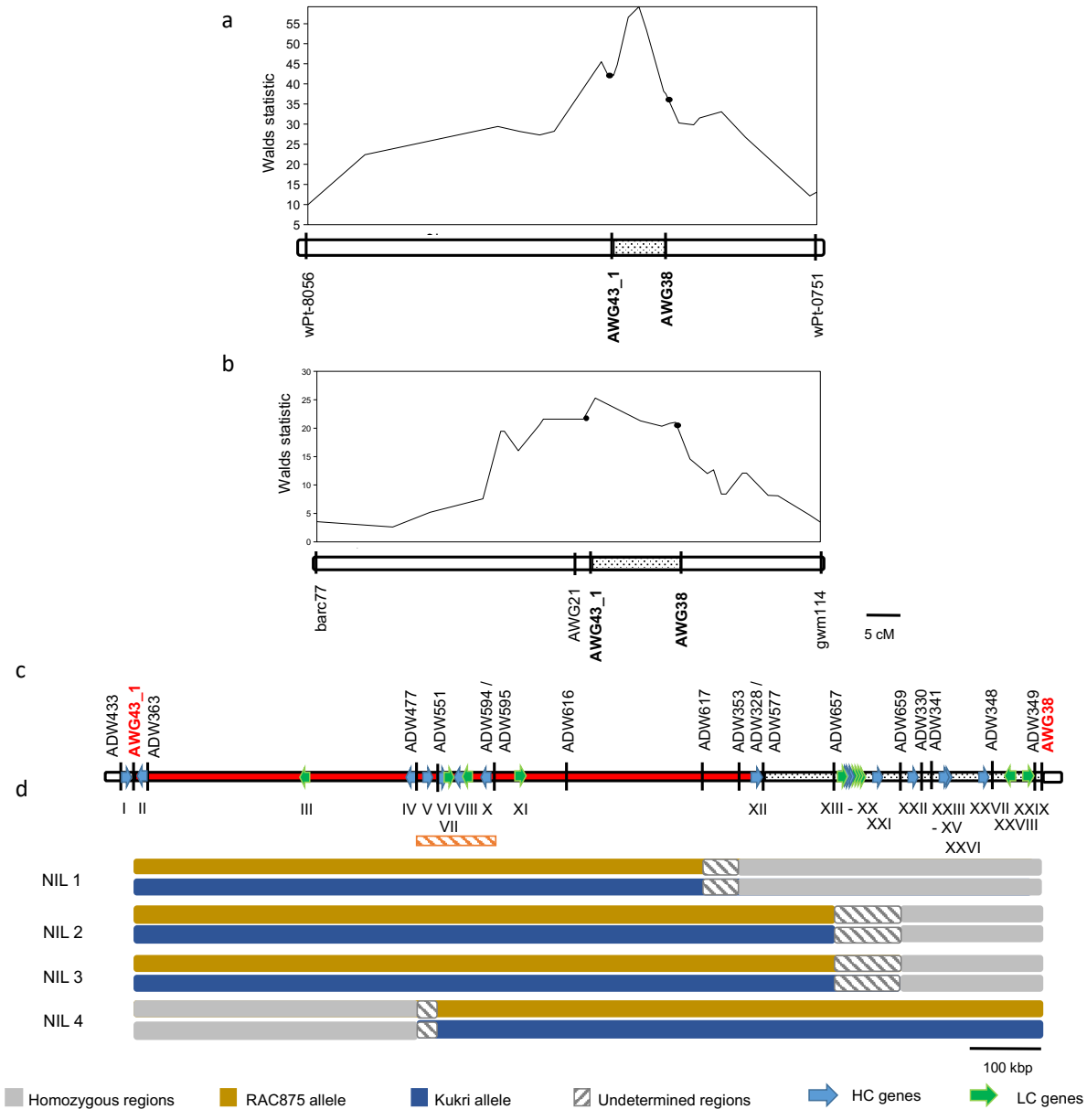
749 **Fig. 2. Single marker analysis in 30 RAC875 x Kukri RILs and 44 Drysdale x Gladius**
750 **RILs segregating for *qYDH.3BL* and grown in dry and hot conditions in the deep-soil**
751 **platform (2015). a,** Positive effects were associated with the RAC875 allele while negative
752 effects were associated with the Kukri allele. **b,** Positive effects were associated with the
753 Drysdale allele while negative effects were associated with the Gladius allele. * : p value <
754 0.05; ** : p value < 0.01

755

756 **Fig. 3. Regression of normalized sap flow of NIL3 and NIL4 families against**
757 **temperature. a**, temperate days. **b**, cool days. **c**, hot days. AA = RAC875 allele (gold), BB =

758 Kukri allele (blue). Differences between AA and BB alleles were non-significant on
759 temperate and cool days. *** : p<0.001 between AA and BB alleles on hot days.
760
761 **Fig. 4. Early vigour and water use of NIL grown in unstressed conditions. a,** estimated
762 shoot area (kpixel) and **b,** absolute growth rate (kpixel/ day) of young NIL3 (solid line) and
763 NIL4 (hatched line) family plants. AA = RAC875 allele (gold), BB = Kukri allele (blue). **c,**
764 water use (mL/ day) and **d**, water use index (shoot area kpixel/ mL).
765
766 **Fig. 5. Expression analysis and gene structure of *TaSINA* and its homeologs.**
767 **a**, **e** Expression analysis of *TaSINA* 3B in seedling shoot and root tissues of RAC875, Kukri
768 and NILs (**a**) and in Gladius and Drysdale (**e**). **c**, Expression of *TaSINA* 3B in RAC875 and
769 Kukri under well-watered and cyclic drought conditions described in [39]. Expression analysis of
770 *TaSINA* 3A (**b**) and *TaSINA* 3D (**d**). "A' corresponds to the RAC875 allele and the "B"
771 corresponds to the Kukri allele. *: p value < 0.05; ** : p value < 0.01; *** : p value < 0.001. **f**,
772 Exon-intron structure of *TaSINA* 3A and 3D. **g**, Exon-intron structure and promoter annotation
773 of *TaSINA* 3B. Yellow boxes indicate the position of cis-acting elements present in the
774 promoter region; green triangle, SNP position inducing the alanine to valine amino acid
775 change; orange boxes represent the exons and the grey boxes the UTRs.
776
777 **Fig. 6. Allelic distribution at *TaSINA* in a diversity panel of 743 *T. aestivum* accessions**
778 **genotyped with KASP markers ADW594 and ADW595.**
779

780 Fig. 1



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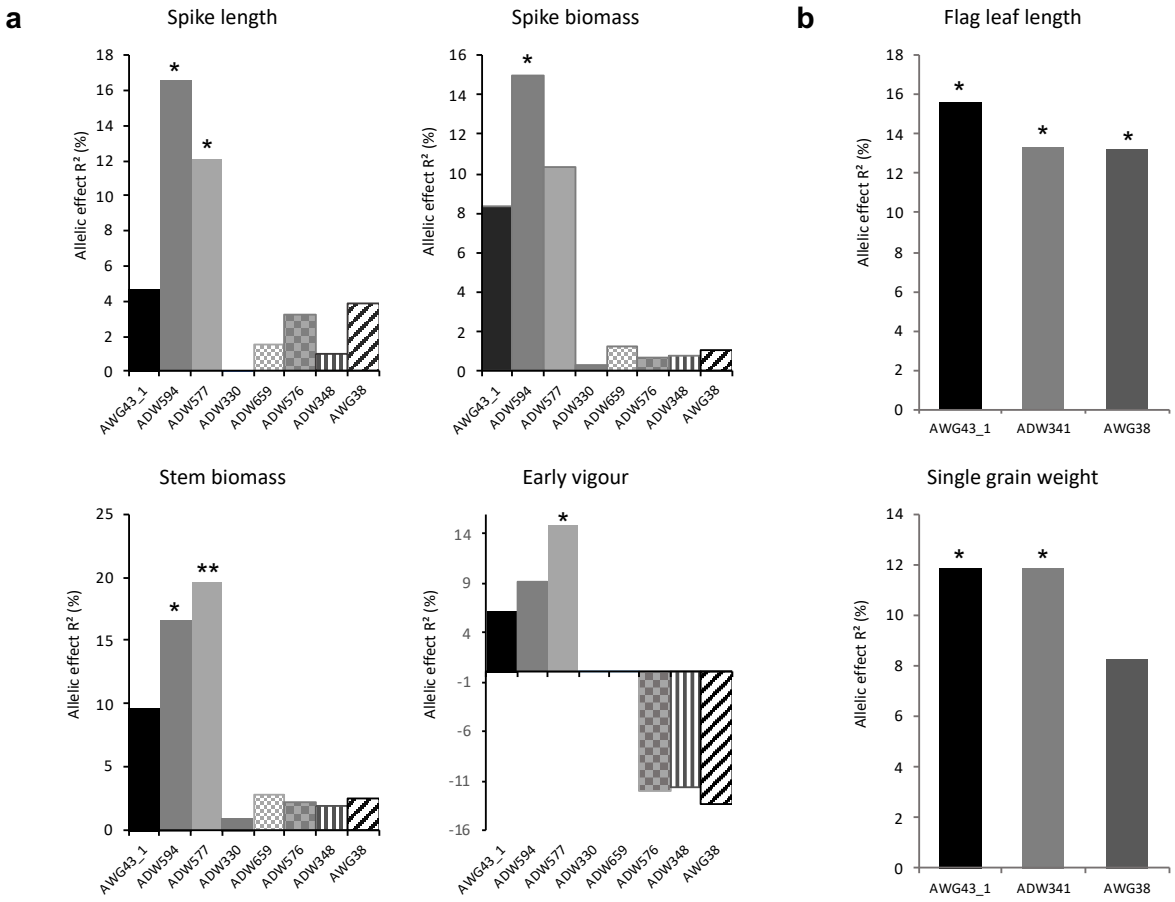
782

783 Fig. 2



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785

786

Fig. 3

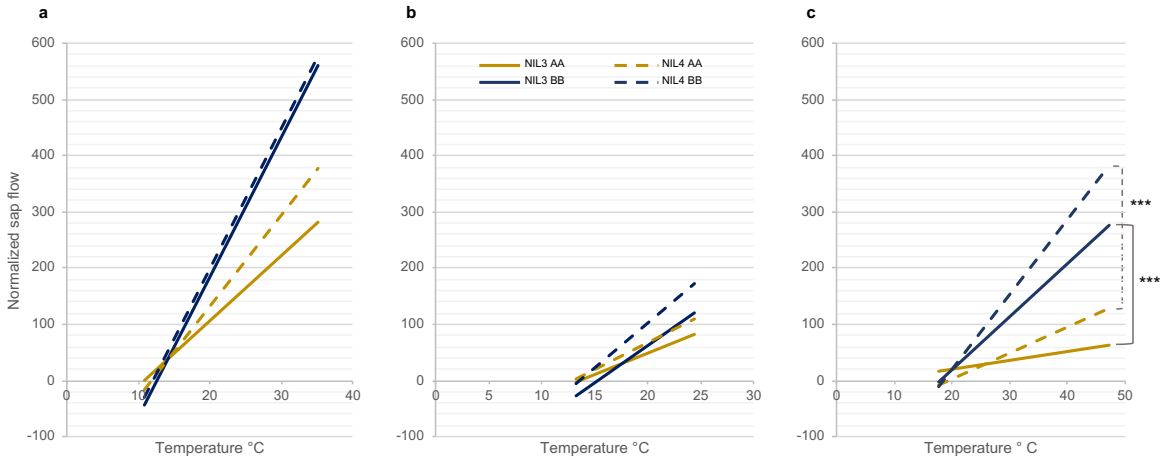

a
b
c
Normalized sap flow
Temperature °C
Temperature °C
Temperature ° C
NIL3 AA
NIL3 BB
NIL4 AA
NIL4 BB
***
***

Fig. 4

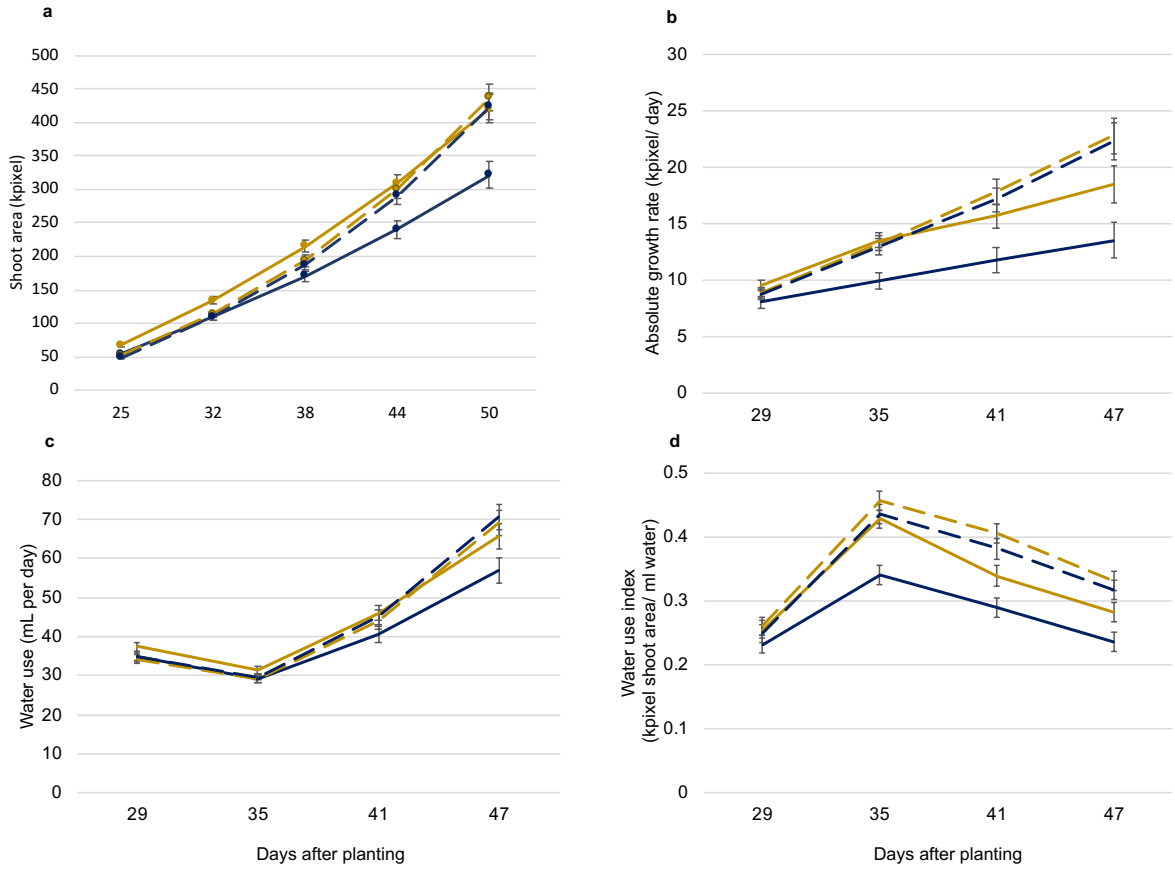

a
Shoot area (kpixel)
500
450
400
350
300
250
200
150
100
50
0
25
32
38
44
50
b
Absolute growth rate (kpixel/ day)
30
25
20
15
10
5
0
29
35
41
47
c
Water use (mL per day)
80
70
60
50
40
30
20
10
0
29
35
41
47
Days after planting
d
Water use index
(kpixel shoot area/ ml water)
0.5
0.4
0.3
0.2
0.1
0
29
35
41
47
Days after planting

Fig. 5

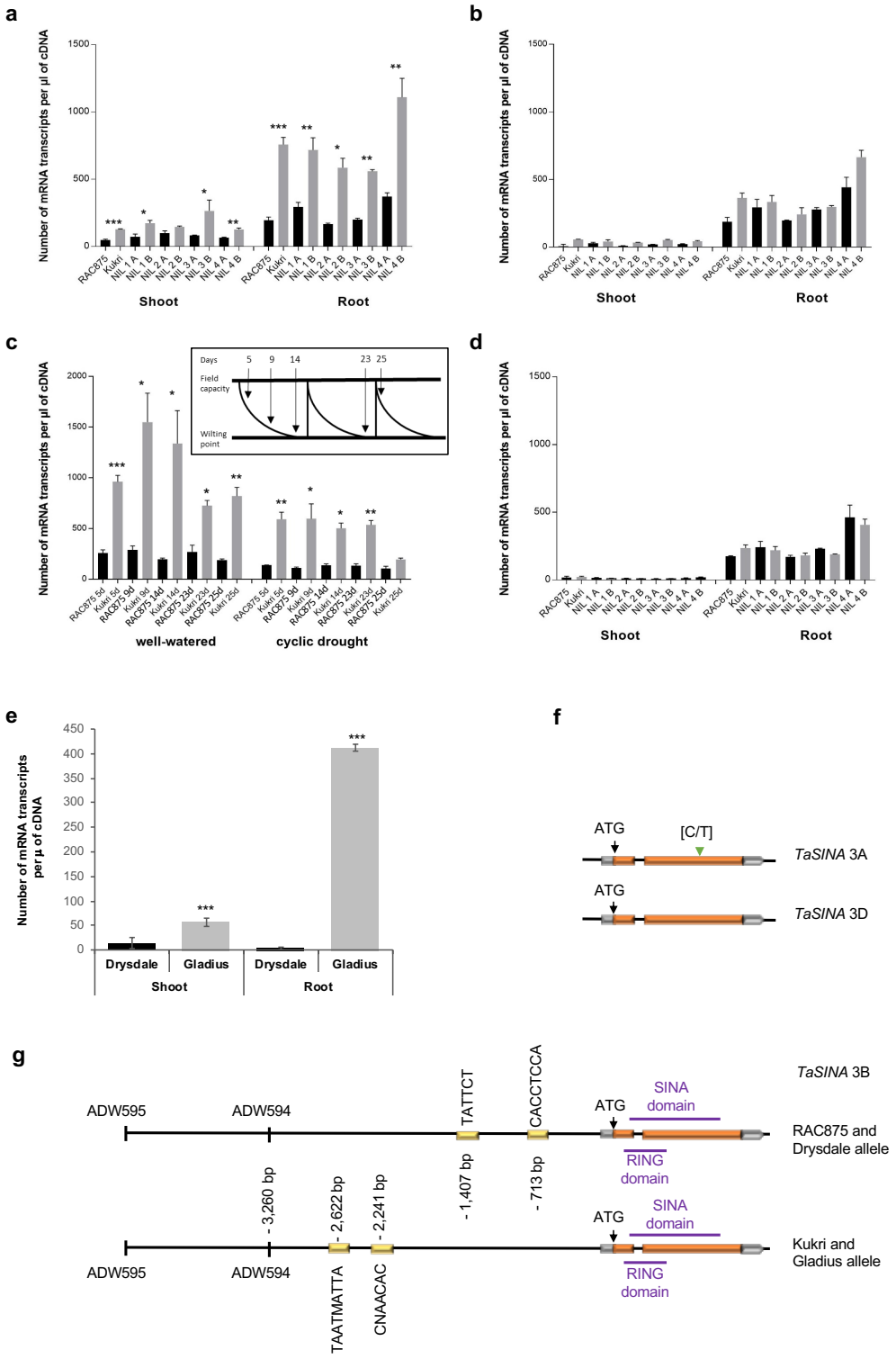
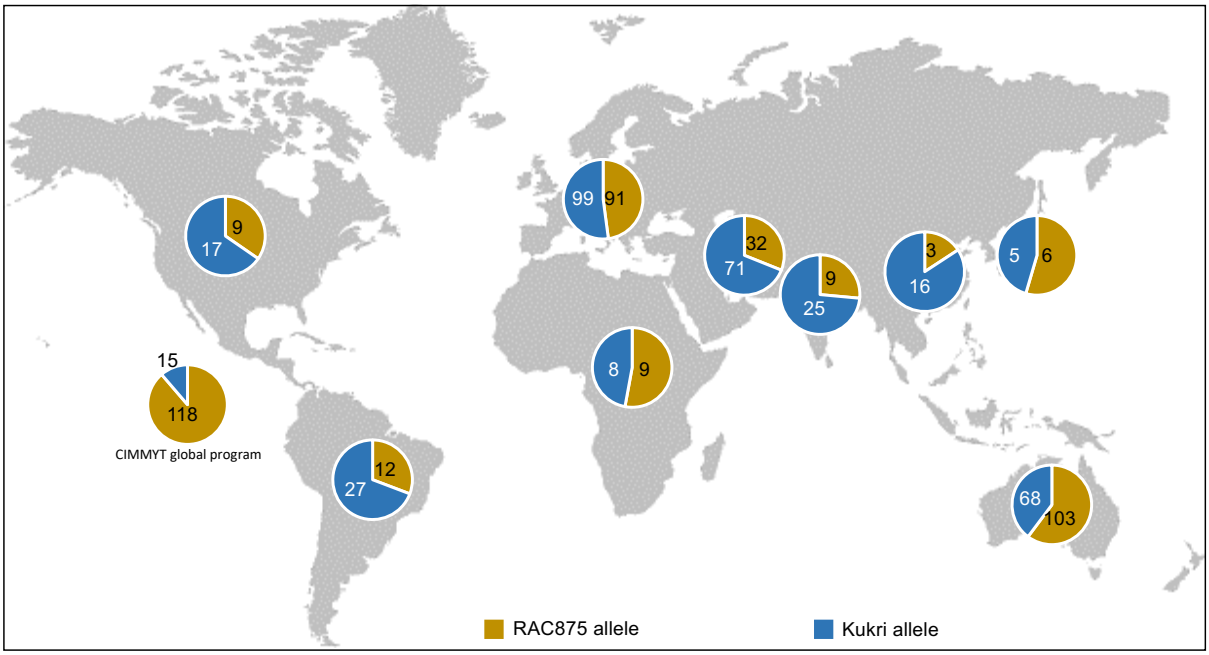

a
Number of mRNA transcripts per µl of cDNA
Shoot
Root
b
Number of mRNA transcripts per µl of cDNA
Shoot
Root
c
Number of mRNA transcripts per µl of cDNA
Days
Field capacity
Wilting point
well-watered
cyclic drought
d
Number of mRNA transcripts per µl of cDNA
Shoot
Root
e
Number of mRNA transcripts per µ of cDNA
Drysdale
Gladius
Shoot
Root
f
ATG
[C/T]
TaSINA 3A
ATG
TaSINA 3D
g
TaSINA 3B
ADW595
ADW594
TATTCT
CACCTCCA
ATG
SINA domain
RAC875 and Drysdale allele
RING domain
- 3,260 bp
- 2,622 bp
- 2,241 bp
- 1,407 bp
- 713 bp
ATG
SINA domain
Kukri and Gladius allele
RING domain
ADW595
ADW594
TAATMATTA
CNAACAC

Fig. 6


9
17
99
91
32
71
9
25
3
16
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6
15
118
CIMMYT global program
8
9
12
27
68
103
RAC875 allele
Kukri allele

801 # Tables

802 **Table 1|** Effects of the RAC875 allele at *qYDH.3BL* in 20 RAC875 x Kukri RILs and four
803 lines (RAC875, Kukri, Drysdale and Gladius) sown in September 2014 in the deep soil-mimic
804 platform (n = 3).

Phenotypic traits	Allelic effect* (%)	*p* value
Early vigour per plant	19.3	0.03
Plant height	20.8	0.03
Spike number per plant	20.8	0.03
Grain number	21.3	0.02
Spike biomass	21.3	0.02
Relative chlorophyll content in flag leaf	21.6	0.02
Spikelet per spike	25.1	0.01
Seminal roots	27.2	0.01
Stem biomass	27.6	0.01
Spike length	29.0	0.01

805 *allelic effect calculated as the % of variance explained by the RAC875 compared to the Kukri allele.

806 **Table 2| Genes annotated in the Chinese Spring reference sequence v1.0 690 Kbp interval of *qYDH.3BL*.** The putative function of each gene

807 was retrieved by homology with rice and brachypodium. HC = high confidence gene; LC = low confidence gene

	Gene ID	Position (bp)	Putative function based on rice orthologues	E value	Putative function based on brachypodium orthologues	E value	Expression data*
I	TraesCS3B01G570900HC	802845950 - 802848503	LOC_Os10g33910.1_ Mitochondrial import inner membrane translocase subunit Tim16	4.1 e-42	Bradi2g61480_mitochondrial import inner membrane translocase subunit TIM16	1.7 e-55	expressed
II	TraesCS3B01G571000HC	802848874 - 802852363	LOC_OS02g17280_Gamma-secretase subunit APH-1B	4.3 e-131	Bradi3g10110_endopeptidase activity	8.5 e-135	expressed
III	TraesCS3B01G851200LC	803363524 - 803364407	-	-	-	-	-
IV	TraesCS3B01G572500HC	803511461 - 803513157	LOC_Os11g14410_ Polygalacturonase	8.5 e-142	Bradi2g57427_ Polygalacturonase / Pectinase	8.2 e-87	-
V	TraesCS3B01G572600HC	803523701 - 803525213	LOC_Os01g72720_ expressed protein	8.9 e-11	Bradi2g61275_PF03478 Protein of unknown function	4.8 e-56	expressed
VI	TraesCS3B01G572700HC	803540158 - 803540583	-	-	Bradi1g36100_Glycosyl hydrolase, subfamily GH 28	1.6 e-22	-
VII	TraesCS3B01G851300LC	803580461 - 803581032	LOC_Os01g72620_ expressed protein	1.6 e-22	-	-	-
VIII	TraesCS3B01G572800HC	803583095 - 803583731	LOC_Os01g59540_GRF zinc finger family protein	1.1 e-4	Bradi5g21617	1.5 e-24	-
IX	TraesCS3B01G851400LC	803586310 - 803587263	LOC_Os07g42000_expressed protein	1.8 e-14	-	-	-
X	TraesCS3B01G572900HC	803628595 - 803630088	LOC_Os01g03170_seven in absentia protein family protein	3.3 e-34	Bradi1g27970_ubiquitin-protein ligase activity	6 e-57	expressed
XI	TraesCS3B01G851500LC	803715648 - 803716915	-	-	-	-	-
XII	TraesCS3B01G573000HC	804008796 - 804009926	LOC_Os01g03170_seven in absentia protein family protein	2.7 e-31	Bradi1g27970_ubiquitin-protein ligase activity	1.7 e-48	-

808 *Expression data extracted from www.wheat-expression.com and in-house database

809 **Table 3| Analysis of variance of the NIL carrying the RAC875 or Kukri allele at *qYDH.3B* and grown in wheelie bins under drought and**

810 **heat stress.** The values represent the mean ± standard deviation.

811

Traits	Early vigour (cm^2)	Spike biomass (g)	Stem biomass (g)	Grain weight per plant (g)	Grain number	Number of spikelets per spike	Single grain weight (mg)	Average spike length	Spike number	Tiller number	Plant height (cm)
NIL2-AA	25.52 ± 3.59	1.95 ± 0.74	2.74 ± 0.72	1.08 ± 0.39	49.63 ± 16.30	18.04 ± 0.48	19.93 ± 3.81	9.23 ± 0.30	1.78 ± 0.83	3.67 ± 0.87	52.22 ± 3.85
NIL2-BB	23.75 ± 4.05	1.21 ± 0.34	1.81 ± 0.43	0.64 ± 0.20	37.75 ± 7.70	17.48 ± 0.47	16.65 ± 3.2	8.87 ± 0.51	1.38 ± 0.52	3 ± 0.53	48.39 ± 4.91
df	16	16	16	16	15	16	16	16	16	16	16
F-test	ns	*	**	*	ns	*	ns	ns	ns	ns	ns
NIL3-AA	19.40 ± 2.81	1.77 ± 0.46	3.01 ± 0.78	1.17 ± 0.31	40.63 ± 10.2	16.25 ± 0.94	29.17 ± 5.23	8.89 ± 2.98	1.58 ± 0.51	3.62 ± 0.51	54.68 ± 5.11
HIF3-BB	17.66 ± 2.63	1.46 ± 0.97	2.58 ± 1.26	0.83 ± 0.60	31.08 ± 17.6	16.07 ± 1.17	22.5 ± 5.65	8.64 ± 2.94	1.64 ± 0.84	3.5 ± 1.02	50.9 ± 6.01
df	26	25	25	24	23	25	24	25	25	26	25
F-test	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns
NIL4-AA	24.81 ± 2.71	2.38 ± 0.91	3.05 ± 0.9	1.29 ± 0.55	67.36 ± 13.74	16.3 ± 0.39	17.10 ± 2.80	8.03 ± 0.41	2.75 ± 0.62	3.58 ± 0.79	54.05 ± 4.56
NIL4-BB	22.49 ± 2.76	1.69 ± 0.54	2.26 ± 0.55	0.96 ± 0.32	51.45 ± 14.44	15.74 ± 0.81	18.56 ± 2.92	7.88 ± 0.44	1.91 ± 0.7	3.27 ± 0.79	51.73 ± 5.62
df	22	22	22	21	21	22	22	21	22	22	22
F-test	ns	*	*	ns	*	*	ns	ns	**	ns	ns

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