



## 23 Summary

- 24 • Crop yields must increase to address food insecurity. Grain weight, determined by grain  
25 length and width, is an important yield component, but our understanding of the  
26 underlying genes and mechanisms is limited.
- 27 • We used genetic mapping and near isogenic lines (NILs) to identify, validate and fine  
28 map a major quantitative trait loci (QTL) on wheat chromosome 5A associated with grain  
29 weight. Detailed phenotypic characterisation of developing and mature grains from the  
30 NILs was performed.
- 31 • We identified a stable and robust QTL associated with a 6.9 % increase in grain weight.  
32 The positive interval leads to 4.0 % longer grains, with differences first visible twelve days  
33 post fertilization. This grain length effect was fine-mapped to a 4.3 cM interval. The locus  
34 also has a pleiotropic effect on grain width (1.5 %) during late grain development that  
35 determines the relative magnitude of the grain weight increase. Positive NILs have  
36 increased maternal pericarp cell length, an effect which is independent of absolute grain  
37 length.
- 38 • These results provide direct genetic evidence that pericarp cell length affects final grain  
39 size and weight in polyploid wheat. We propose that combining genes which control  
40 distinct biological mechanisms, such as cell expansion and proliferation, will enhance  
41 crop yields.

42

43 Key words: wheat, thousand grain weight, yield, grain size, grain length, cell size, pericarp, QTL

## 44 Introduction

45 By 2050, it is predicted that the human population will have exceeded nine billion people  
46 (United Nations, 2015). This is driving an increased demand for food production which is  
47 exacerbated by the use of crops for fuel and animal feed, and the pressures on agricultural  
48 systems resulting from climate change. With land for agricultural expansion being limited,  
49 increasing crop yields provides a sustainable route towards meeting this demand. However,  
50 rates of yield increase have slowed in recent years and are currently insufficient to achieve the

51 estimated doubling in crop production that will be required by 2050 (Tilman *et al.*, 2011; Ray *et*  
52 *al.*, 2013). Projections show that increasing productivity on existing farmlands would increase the  
53 available food supply and lower prices, significantly reducing the number of people at risk of  
54 hunger globally (Rosegrant *et al.*, 2013). With one in nine people currently living under food  
55 insecurity (FAO *et al.*, 2015), it is urgent that we identify ways to increase crop yields.

56

57 Final crop yield is a complex quantitative trait strongly influenced by interacting genetic and  
58 environmental factors. For cereal crops, seed/grain weight (measured as thousand grain weight  
59 (TGW)) is a major yield component and is more stably inherited than final yield itself (Kuchel *et*  
60 *al.*, 2007). Grain weight is largely defined by the size of individual grains and the morphometric  
61 components of grain area, length and width. A number of genes controlling these traits have  
62 been cloned from major grain weight quantitative trait loci (QTL) in rice (Fan *et al.*, 2006; Song *et*  
63 *al.*, 2007; Weng *et al.*, 2008; Wang *et al.*, 2012). For example, GW2, a RING-type E3 ubiquitin  
64 ligase acts as a negative regulator of cell division and was identified as the gene underlying a  
65 major QTL for rice grain width and weight (Song *et al.*, 2007). These studies, in addition to those  
66 in model species, have shown that seed size is controlled through diverse mechanisms and  
67 genetic pathways (reviewed in Xing & Zhang, 2010; Li & Li, 2015). In Arabidopsis, the  
68 *AINTEGUMENTA* (*ANT*) transcription factor increases seed size through increased cell  
69 proliferation (Mizukami & Fischer, 2000), whilst the *APETELA2* (*AP2*) transcription factor  
70 regulates seed size by limiting cell expansion (Ohto *et al.*, 2005). Other genes include those  
71 involved in phytohormone biosynthesis and signalling (Riefler *et al.*, 2006; Schruff *et al.*, 2006;  
72 Jiang *et al.*, 2013) and G-protein signalling pathways (Huang *et al.*, 2009). Interestingly, many of  
73 these genes have been shown to act maternally (reviewed in Li & Li, 2015) and it has been  
74 proposed that the seed coat/pericarp (a maternal tissue) sets an upper limit to the final size of  
75 the seed/grain (Adamski *et al.*, 2009; Hasan *et al.*, 2011; Xia *et al.*, 2013).

76 Despite these advances, our understanding of the control of grain size is more limited in  
77 important crop species such as wheat (*Triticum aestivum*). Wheat provides around 20% of the  
78 calories consumed by humans and more protein globally than all types of meat combined (FAO,

79 2017). Many QTL for grain weight and, more recently, individual grain size/shape components  
80 have been identified in wheat (Brescghello & Sorrells, 2007; Gegas *et al.*, 2010; Simmonds *et al.*,  
81 2014; Farre *et al.*, 2016; Kumar *et al.*, 2016). However, no mechanistic insight has been provided  
82 for these QTL, few have been validated (Simmonds *et al.*, 2014) and as yet, none have been  
83 cloned.

84 A major challenge to validate and define the mechanisms governing grain weight QTL in  
85 polyploid wheat has been that their effects are often subtle compared with QTL identified in  
86 diploid species such as rice (Uauy, 2017). One explanation is that wheat has a more limited  
87 capacity for increasing grain size than rice. An alternative, and more likely, scenario is that the  
88 effect of variation in an individual gene is masked by functional redundancy from  
89 homoeologous gene copies (Borrill *et al.*, 2015); bread wheat is a hexaploid species with three  
90 homoeologous genomes (A, B and D) that share between 96-98% sequence similarity across  
91 genes (Krasileva *et al.*, 2013). In addition, the size (17 Gb) and highly repetitive nature of the  
92 wheat genome has meant that, until recently, the genomic resources available in wheat have  
93 been limited. However, in the last few years there has been a radical change in the wheat  
94 genomics landscape with resources now including complete genome sequences and high  
95 quality gene models (IWGSC RefSeq v1.0; IWGSC, 2014; Clavijo *et al.*, 2017), transcriptomic  
96 databases (Pearce *et al.*, 2015b; Borrill *et al.*, 2016), high density single nucleotide polymorphism  
97 (SNP) arrays (Wang *et al.*, 2014; Winfield *et al.*, 2016) and exome-sequenced mutant populations  
98 (Krasileva *et al.*, 2017).

99 In this study, we identified a stable and robust QTL for grain weight in hexaploid wheat, which is  
100 driven by an increase in grain length. The QTL affects cell expansion in the grain and acts to  
101 increase the length of cells in the pericarp (maternal seed coat). We genetically mapped the  
102 effect to an interval on chromosome 5A, and used the latest wheat genome sequences and gene  
103 models to define the genes within the physical space. This detailed characterisation of the QTL  
104 provides direct genetic evidence that pericarp cell expansion affects final grain size, offering new  
105 insights into the mechanisms controlling grain weight in polyploid wheat.

## 106 Methods

### 107 Plant material

108 A doubled haploid (DH) mapping population was developed from the cross between two UK  
109 hexaploid winter wheat cultivars, 'Charger' and 'Badger'. The population was created using the  
110 wheat x maize technique from F<sub>1</sub> plants (Laurie & Bennett, 1988) and comprised 129 individuals,  
111 92 of which were genotyped and used for evaluation. The 5A QTL was validated with the  
112 development of near isogenic lines (NILs). Two DH lines (CB53 and CB89) homozygous for the  
113 positive Badger loci across the complete linkage group were crossed to Charger and  
114 heterozygous F<sub>1</sub> plants were backcrossed to the Charger recurrent parent for four generations  
115 (BC<sub>4</sub>). Heterozygous plants were selected at each generation using markers *Xgwm293* and  
116 *Xgwm186*. After BC<sub>2</sub> and BC<sub>4</sub>, heterozygotes were self-pollinated and NILs homozygous for the  
117 alternative alleles across the interval were extracted (BC<sub>2</sub>F<sub>2</sub> and BC<sub>4</sub>F<sub>2</sub>). In total 10 BC<sub>2</sub> NILs were  
118 generated, six of which carried the *Xgwm293* to *Xgwm186* Badger positive interval. An additional  
119 twelve BC<sub>4</sub> NILs were generated from the two DH lines (six Badger and six Charger interval).  
120 Two representative BC<sub>4</sub> NILs with alternative haplotypes were genotyped with the 90K iSelect  
121 array (Wang *et al.*, 2014) to confirm the introgression and identify additional segregating  
122 genomic regions. Recombinant BC<sub>4</sub>F<sub>2</sub> plants between the flanking markers were also selected  
123 and self-pollinated for the development of homozygous BC<sub>4</sub>F<sub>3</sub> recombinant inbred lines (RILs).  
124 Screening 170 plants with flanking markers *Xgwm293* and *Xgwm186* yielded 60 recombinants  
125 within the interval, defining a genetic interval of 17.65 cM.

### 126 Genetic map construction and QTL analysis

127 The Charger x Badger genetic map was developed using simple sequence repeat (SSR) markers.  
128 From 650 SSR's tested, 239 from JIC/*psp* (Bryan *et al.*, 1997; Stephenson *et al.*, 1998), IPK  
129 Gatersleben/*gwm/gdm* (Roder *et al.*, 1998; Pestsova *et al.*, 2000), Wheat Microsatellite  
130 Consortium/*wmc* (<http://wheat.pw.usda.gov/ggpages/SSR/WMC/>), Beltsville Agricultural  
131 Research Station/*barc* (Song *et al.*, 2005) and INRA/*cfa/cfd* (Guyomarc'h *et al.*, 2002) collections  
132 were polymorphic between parental lines. Consensus maps (Somers *et al.*, 2004) were used to  
133 select 212 SSR markers which maximised genome coverage with an approximate marker density

134 of one SSR every 20 cM. In addition, nine sequence-tagged microsatellite profiling (STMP)  
135 markers (Hayden & Sharp, 2001) were incorporated into the map. To increase marker density, 75  
136 Kompetitive Allele Specific Primers (KASP) markers were utilised. Markers with assigned  
137 chromosome locations (Allen *et al.*, 2011) were targeted to fill gaps in the genetic map.

138 DNA extractions and genotyping procedures were performed as in Simmonds *et al.*, (2014).  
139 Likewise, map construction, QTL detection and multi-trait multi-environment (MTME) analysis  
140 was conducted as in Simmonds *et al.* (2014). Significant QTL effects were detected above a 2.5  
141 log-of-odds (LOD) threshold.

142 SSR and KASP markers used in the QTL analyses were positioned with respect to the newly  
143 released Chinese Spring sequence through a BLAST search of 100 to 300 bp encompassing each  
144 SNP against the IWGSC RefSeq v1.0 ([https://wheat-urgi.versailles.inra.fr/Seq-  
145 Repository/Assemblies](https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies)). In most cases, the order on the reference sequence agreed with the  
146 genetic order in the Charger x Badger population. For discrepancies, we used the genetic  
147 position to order markers. In cases of no hits to the RefSeq v1.0 assembly, we inferred a physical  
148 position based on the two closest markers and the relative distance of all three markers based  
149 on their centiMorgan positions. Similarly, physical positions of all iSelect SNPs were obtained  
150 using BLAST to align the surrounding sequence (201 bp) to the RefSeq v1.0 assembly. TGACv1  
151 gene models were positioned on RefSeqv1.0 with GMAP (Wu & Watanabe, 2005) using best hit  
152 position and 95% minimum similarity cut-off (David Swarbreck and Gemy Kaithakottil; Earlham  
153 Institute).

## 154 **Field evaluation and phenotyping**

155 The DH population was evaluated in the field in a randomised complete block design with three  
156 replications at five sites (Norwich and Sandringham, England; Balmonth, Scotland; Bohnshausen,  
157 Germany; and Froissy, France (Simmonds *et al.*, 2014)). The experiments were grown for three  
158 years (2001–2003) at Norwich and Sandringham, and two years (2002–2003) at the other three  
159 sites. The field trials were sown in large-scale yield plots (1.1 × 6 m) and treated with standard  
160 farm pesticide and fertiliser applications to reproduce commercial practise. All trials were sown

161 by grain number for comparable plant densities per plot ( $275 \text{ seeds} \cdot \text{m}^{-2}$ ). Plots were measured  
162 for final plot yield after adjustment for plot size, and TGW was calculated by counting and  
163 weighing 100 seeds from each plot.

164 The NILs were evaluated at Norwich in 2012 and 2013 (10 BC<sub>2</sub> NILs), 2014 (12 BC<sub>4</sub> NILs) and  
165 2015 and 2016 (4 BC<sub>4</sub> NILs), while BC<sub>4</sub> RILs were analysed in 2014-2016. For both NIL and RIL  
166 experiments, a randomized complete block design was used with five replications. NILs were  
167 grown in large-scale yield plots (1.1 × 6 m), whereas RILs were grown in 1.1 × 1 m plots in 2014  
168 and large-scale yield plots in 2015 and 2016. Final grain yield (adjusted by plot size and  
169 moisture content) was determined for NILs across the five years. Developmental traits were also  
170 measured for NILs in 2012-2016, although not all traits were measured in each year (Supporting  
171 Information Table S1). For all NILs (2012-2016) and RILs (2014-2016), grain morphometric  
172 measurements (grain width, length, area) and TGW were recorded on the MARVIN grain  
173 analyser (GTA Sensorik GmbH, Germany) using approximately 400 grains obtained from the  
174 harvested grain samples. For all NILs (2012-2016), ten representative spikes per field plot were  
175 also measured for spike yield components (spikelet number, number of viable spikelets, spike  
176 length, grain number per spike, spike yield and seeds per spikelet), TGW and grain  
177 morphometric parameters. The data from the ten representative spikes was consistent with the  
178 whole plot values.

### 179 Grain developmental time courses

180 The BC<sub>4</sub> NILs grown in 2014-2016 were used for the grain developmental time courses. Two  
181 Charger (5A-) and two Badger (5A+) NILs were used, and the same NILs were used in all three  
182 years. We tagged 65 ears per NIL across each of four blocks in the field at full ear emergence  
183 (peduncle just visible) to ensure sampling at the same developmental stage. Ten spikes per NIL,  
184 per block, were sampled at each of five (2014) or six (2015-2016) time points. 2014 time points  
185 included 4, 8, 12, 18 and 27 days post anthesis (dpa). 2015 time points included anthesis (0 dpa),  
186 4, 7, 12, 19 and 26 dpa. 2016 time points included 0, 3, 8, 10, 15, 21 dpa. Ten grains were  
187 sampled from each spike from the outer florets (positions F1 and F2) of spikelets located in the  
188 middle of the spike. Grains were weighed to obtain fresh weight, assessed for morphometric

189 parameters (grain area, length and width) on the MARVIN grain analyser and then dried at 37 °C  
190 to constant weight (dry weight). For each block at each time point, a total of ~100 grains were  
191 sampled (10 spikes x 10 grains) per NIL. However, for the statistical analysis the average of each  
192 NIL within each block was used as the phenotypic value as the individual grains and spikes were  
193 considered as subsamples.

## 194 Cell size measurements

195 One representative 5A- and 5A+ BC<sub>4</sub> NIL was used for cell size measurements. We selected  
196 mature grains from three blocks of the 2015 harvest samples based on a variety of criteria. For  
197 each NIL, we selected nine grains of average grain length from the whole harvest sample from  
198 each block (groups 5A-/5A+ average). For the 5A- NIL, an additional nine grains were selected  
199 that had grain lengths equivalent to the average of the 5A+ NIL sample (5A- large). For the 5A+  
200 NIL an additional nine grains were selected that had grain lengths equivalent to the average of  
201 the 5A- NIL sample (5A+ small). We also selected grains of average length from three blocks of  
202 the 2016 harvest (nine grains were selected from each block per genotype). Grains were stuck  
203 crease-down on to 12.5mm diameter aluminium specimen stubs using 12 mm adhesive carbon  
204 tabs (both Agar Scientific), sputter coated with gold using an Agar high resolution sputter coater  
205 and imaged using a Zeiss Supra 55 SEM. The surface (pericarp) of each grain was imaged in the  
206 top and bottom half of the grain, with images taken in at least three positions in each half. All  
207 images were taken at a magnification of 500x. Cell length was measured using the Fiji  
208 distribution of ImageJ (Schindelin *et al.*, 2012) (Supporting Information Fig. S1). Cell number was  
209 estimated for each grain using average cell length/grain length. For the statistical analyses, we  
210 considered the average cell length of each individual grain as a subsample within the block.

## 211 Statistical analysis

212 DH lines homozygous across the genetic interval for the two major QTL, *Qtgw-cb.2B* (*Xgwm259-*  
213 *Xstm119tgag*) and *Qtgw-cb.5A* (*Xgwm443-XBS00000435*) were classified by genotype. Using this  
214 classification, general linear model analyses of variance (ANOVA) were performed for TGW  
215 incorporating environment and year as factors for each individual QTL, and for lines with both  
216 increasing alleles compared with those with neither. Pearson's correlation coefficient was



217 calculated to assess the correlation between yield and TGW. All analyses performed on DH lines  
218 was carried out using Minitab v17.3.1 (Minitab Inc.).

219 The NILs and RILs were evaluated using two-way ANOVAs, with the model including the  
220 interaction between environment and the 5A QTL. RIL groups were assigned as having a Charger  
221 or Badger like grain length phenotype using a post hoc Dunnett's test to compare with C- and  
222 B-control groups. Similarly, two-way ANOVAs, including genotype and block, were conducted  
223 for the developmental time courses and cell size measurements. Analyses were performed using  
224 GenStat 15<sup>th</sup> edition (VSN International) and R v3.2.5.

## 225 Results

### 226 A QTL on chromosome 5A is associated with increased grain weight

227 A genetic map was developed for the Charger x Badger DH population comprising of 296  
228 polymorphic molecular markers. Linkage analysis resulted in 32 linkage groups which were  
229 assigned to 21 chromosomes, covering a genetic distance of 1,296 cM. The only chromosome  
230 with no marker coverage was 6D.

231 QTL analysis identified two regions with consistent variation for TGW, chromosomes 2B (*Qtgw-*  
232 *cb.2B*) and 5A (*Qtgw-cb.5A*), based on the mean LOD score across environments (Fig. 1) and co-  
233 localization of significant QTL (Supporting Information Table S2). *Qtgw-cb.2B* was identified in 7  
234 of the 12 site/year environments, providing a mean of 11% of the explained variation when  
235 significantly expressed and a mean additive effect of 1.26 g/1000 grains, with Charger providing  
236 the increasing allele. The peak LOD for the QTL was located at markers *Xgwm148* and *Xgwm120*  
237 depending on the environment. *Qtgw-cb.5A* was also significant at 7 of the 12 environments and  
238 accounted for 15.5% of the phenotypic variation with a mean additive effect of 1.6 g/1000  
239 grains. The peak for *Qtgw-cb.5A* was defined by markers *Xgwm293* (20.6 cM) and *Xbarc180* (25.3  
240 cM; Fig. 2a), with Badger providing the increasing allele.

241 Analysis of DH lines homozygous across the wider QTL regions for both *Qtgw-cb.2B* (*Xgwm259-*  
242 *Xstm119tgag*) and *Qtgw-cb.5A* (*Xgwm443-XBS00000435*) demonstrated that the increasing  
243 alleles of each individual QTL provided a significant 4.1% and 5.5% increase in TGW ( $P < 0.001$ ),

244 respectively. DH lines containing both QTL (n=9) produced a 10% increase ( $P < 0.001$ ) over lines  
245 with neither (n=10), suggesting *Qtgw-cb.2B* and *Qtgw-cb.5A* are additive when combined.

246 There was a significant correlation ( $P < 0.001$ ) between grain yield and TGW across all datasets,  
247 however significant QTL were only co-located for both traits in France 2003 (2B) and England-  
248 Norwich 2002/Scotland 2002(5A) (Supporting Information Table S3). This suggests that although  
249 TGW was an important component regulating yield in this DH population, it was also influenced  
250 by other yield components. As *Qtgw-cb.5A* had a larger mean additive effect and accounted for  
251 more of the phenotypic variation than *Qtgw-cb.2B*, we selected *Qtgw-cb.5A* for further analyses.

### 252 Multi-Trait Multi-Environment (MTME) analysis defines *Xgwm293* as the peak 253 marker of *Qtgw-cb.5A*

254 MTME analysis was conducted on chromosome 5A for both TGW and grain yield. For TGW,  
255 markers above the significance threshold ( $LOD > 2.5$ ) ranged from *Xgwm293* (20.6 cM) to  
256 *XBS00015653* (33.7 cM) with the peak being at *Xgwm293* (Fig. 2b,c). At least one of the markers  
257 within the identified region was significant at each of the twelve environments, with Badger  
258 always providing the beneficial alleles. For yield, MTME analysis identified a significant QTL in  
259 the *Qtgw-cb.5A* region, with the peak marker (*Xgwm293*) being the same as for TGW. Significant  
260 increases in the additive effect of Badger were observed in seven environments (Supporting  
261 Information Fig. S2), contrasting to only two in the previous single-environment analysis. It is  
262 worth noting that in two environments (England-Sandringham 2001 and 2003), the alternative  
263 parent Charger had a borderline significant effect on yield in the MTME analysis. Taken together,  
264 these results suggest that the Badger *Qtgw-cb.5A* interval is associated with a consistent effect  
265 on TGW across environments which often, but not always, translates into a yield benefit.

### 266 Near isogenic lines (NILs) differing for *Qtgw-cb.5A* show a 6.9% difference in TGW

267 To independently validate and further investigate the effect of *Qtgw-cb.5A* (referred to hereafter  
268 as 5A QTL) on TGW, BC<sub>2</sub> and BC<sub>4</sub> NILs differing for the QTL region were developed using  
269 markers *Xgwm293* and *Xgwm186* and Charger as the recurrent parent. Pairs of BC<sub>4</sub> NILs carrying  
270 the Charger (5A-) or Badger (5A+) segment were genotyped using the iSelect 90K SNP array  
271 (Wang et al, 2014) and found to be 97.2% similar, only showing variation in 221 markers across

272 the 5A QTL, compared with 7,973 SNPs between the parents (Fig. 1, inner tracks). These NILs  
273 therefore provide a valuable resource for specifically studying the effects of the 5A QTL in more  
274 depth.

275 Across five years of replicated field trials 5A+ NILs showed an average increase in TGW of 6.92%  
276 ( $P < 0.001$ ) ranging from 4.00 to 9.28% (Table 1), and significant in all years. The difference in  
277 TGW was associated with a yield increase of 1.28% in 5A+ NILs across all years, although this  
278 effect was not significant ( $P = 0.093$ ). The effect varied across years with a significant yield  
279 increase of 2.17% ( $P = 0.046$ ) in 2014 and non-significant effects of between 0.02 to 1.72% in the  
280 other four years. The positive effect of the QTL on yield was similarly subtle in the DH population  
281 as described previously.

282 We measured the NILs for a series of spike yield component traits to determine possible  
283 pleiotropic effects associated with the 5A+ TGW effect. Within most years, there was no  
284 significant effect of the 5A+ allele on spike yield components such as spikelet number, seeds per  
285 spikelet or grain number per spike (Supporting Information Table S4). However, when all years  
286 were analysed together, we observed a significant reduction in grain number (-3.55%,  $P = 0.04$ )  
287 and seeds per spikelet (-3.37%,  $P = 0.015$ ) associated with the 5A+ interval. This statistical  
288 significance was driven by a particularly strong negative effect in 2016 as grain number and  
289 seeds per spikelet were non-significant in the preceding four seasons (2012-15). Overall,  
290 however, the 5A+ interval is associated with a consistent small decrease in these spike yield  
291 components.

292 Taking into account the 6.92% effect of the 5A+ QTL on TGW and the tendency for decreases in  
293 some spike yield components, the overall spike yield increased by 2.33% ( $P = 0.032$ ) across the  
294 five years. However, similar to grain number and seeds per spikelet, the statistical significance is  
295 driven by a single year (2014) despite overall positive effects in another three years (2012, 2013,  
296 and 2015). We also measured tiller numbers and found a significant reduction of 4 tillers per m  
297 in the 5A + NILs across two years ( $P = 0.008$ ) (Supporting Information Table S1). No effect was  
298 seen for spikelet number and additional phenology traits (Supporting Information Table S4).  
299 Taken together, these results suggest that the 5A+ interval has a consistent positive effect on

300 TGW and that the effects on yield are modulated by a series of smaller compensating negative  
301 effects on yield components such as grain number, seeds per spike and tiller number.

### 302 The TGW increase in 5A+ NILs is primarily due to increased grain length

303 TGW is determined by individual components including physical parameters such as grain  
304 length and width. To understand the relative contribution of these components to the increase  
305 in TGW, NILs were assessed for these grain morphometric parameters (length, width and area)  
306 using a 2D imaging system (Table 1). 5A+ NILs had significantly increased grain length ( $P <$   
307  $0.001$ ), width ( $P < 0.001$ ) and area ( $P < 0.001$ ) compared to 5A- NILs across all years with the  
308 exception of width in 2016. On average, the 5A+ QTL increased grain length by 4.04% ( $P <$   
309  $0.001$ ), ranging from 3.58 to 4.72% ( $P < 0.001$  in all years). The effect on width was smaller,  
310 averaging 1.45% ( $P < 0.001$ ; range 0.27 to 2.35%) and significant in four out of five years (Table  
311 1). The effects on length and width combined to increase grain area by an average of 5.41% ( $P <$   
312  $0.001$ ), significant in all five years. These results were based on combine harvested grain samples  
313 and were also confirmed in ten representative single ear samples taken before harvest. TGW of  
314 the ten spikes correlated strongly with the whole plot samples ( $r = 0.84$ ,  $P < 0.001$ ) and showed a  
315 similar difference between NILs (6.00%,  $P < 0.001$ ; Supporting Information Table S4). Across  
316 datasets, the effect of the 5A+ QTL on grain length was more than twice the size of the effect on  
317 grain width. This fact, together with the more consistent effect on grain length across years  
318 (Coefficient of variation length = 10.6%; width = 55.3%; TGW = 27.8%) suggests that the  
319 increase in grain length is the main factor driving the increase in grain area and TGW.

320 We compared the distribution of grain length and width using data from individual seeds to  
321 determine whether the QTL affects all grains uniformly. Violin plots for length showed variation  
322 in distribution shape among years (Fig. 3). However, within years the 5A- and 5A+ grain length  
323 distributions were very similar in shape, suggesting that the QTL affects all grains uniformly and  
324 in a stable manner across the ear and within spikelets. In all years, the 5A+ grain length  
325 distributions were shifted higher than the 5A- NILs with an increase in longer grains and fewer  
326 shorter grains, in addition to the higher average grain length (Fig. 3). Grain width distributions  
327 were also very similar in shape within years, but had a less pronounced shift between NILs

328 (Supporting Information Fig. S3) consistent with the overall smaller effect of the 5A QTL on grain  
329 width.

### 330 The 5A QTL region acts during grain development to increase grain length

331 To determine when differences in grain morphometric parameters between NILs are first  
332 established, we conducted grain development time courses of two 5A- and two 5A+ BC<sub>4</sub> NILs.  
333 Grains were sampled in 2014, 2015 and 2016 from field plots at anthesis and at five further time  
334 points across grain development until the difference in grain size had been fully established.  
335 Data from 2015 is shown in Fig. 4 as a representative year (samples taken at anthesis (0 dpa), 4,  
336 7, 12, 19 and 26 dpa). The first significant difference in grain length was observed at 12 dpa with  
337 5A+ NILs having 1.5% longer grains than 5A- NILs ( $p = 0.034$ ). This effect increased to 4.4 % at  
338 19 dpa ( $P < 0.001$ ) and was maintained at 26 dpa (4.5 % increase,  $P < 0.001$ ; Fig. 4a). No  
339 significant effects on grain width were observed until 26 dpa when 5A+ NILs increased grain  
340 width by 1.7 % ( $P = 0.015$ ; Fig. 4b). Significant differences in grain area were detected at 19 dpa  
341 (5.7 % increase;  $P < 0.001$ ; data not shown) and this difference was maintained at the final time  
342 point 26 dpa (6.1 %,  $P < 0.001$ ). By the final time point 5A+ NILs also had significantly heavier  
343 grains (3.7%,  $P = 0.01$ ; Fig. 4c). These effects were all consistent with the grain size and weight  
344 differences observed in mature grains in 2015 (Table 1) and were also observed in 2014 and  
345 2016 (Supporting Information Figs. S4,S5). The fact that the effects on width, area and weight  
346 are all after the first significant difference on grain length in all three years further supports grain  
347 length as the main factor driving the increase in grain weight.

### 348 5A+ NILs have increased pericarp cell length independent of absolute grain 349 length

350 We used scanning electron microscopy (SEM) to image pericarp cells and determine cell size of  
351 BC<sub>4</sub> 5A- and 5A+ grains. Mature grains from the 2015 field experiment were selected from a 5A-  
352 and 5A+ NIL pair based on their grain length and using a variety of criteria to allow for distinct  
353 comparisons (Fig. 5). First, we compared grains of average length from the 5A- and 5A+ NIL  
354 distributions (Fig. 5a). We found that average 5A+ grains had an 8.33 % significant increase in  
355 mean cell length ( $P = 0.049$ ) compared to average 5A- grains and that this was reflected in a  
356 shift in the whole distribution of 5A+ cell lengths (Fig. 5a). Next, we compared cell lengths in

357 grains of the same size from 5A- and 5A+ NILs. We selected relatively long grains from the 5A-  
358 NIL distribution (Fig. 5b; orange) that had the same grain length as the average 5A+ grains. This  
359 comparison showed that 5A+ grains still had longer cells (9.53%,  $P = 0.015$ ) regardless of the  
360 fact that the grain length of the two groups were the same (6.8 mm; Fig. 5b). We also made the  
361 opposite comparison by selecting relatively short grains from the 5A+ NIL distribution (Fig. 5c;  
362 green) and comparing them with average 5A- grains. Similar to before, the 5A+ grains had  
363 longer cells (8.61%), although this effect was borderline non-significant ( $P = 0.053$ ; Fig. 5c).  
364 Finally, a comparison of long 5A- grains and short 5A+ grains again showed that cells were  
365 longer in 5A+ grains (9.81%,  $P = 0.011$ ), even though the 5A+ grains used in this comparison  
366 were 7.65% shorter than the 5A- grains. Within genotype comparisons of cell length between  
367 grains of different lengths showed no significant differences in mean cell length (Supporting  
368 Information Fig. S6). The results were confirmed in 2016 where average 5A+ grains had a 24.6 %  
369 significant increase in mean cell length compared to average 5A- grains ( $P < 0.001$ ; Supporting  
370 Information Fig. S7). These results indicate that the 5A+ region from Badger increases the length  
371 of pericarp cells independent of absolute grain length. Using grain length and mean cell length  
372 to calculate cell number, we determined that the average length grains of both 5A- and 5A+  
373 had the same number of cells in 2015. However, in 2016, 5A- NILs had significantly more cells  
374 than 5A+ NILs (Fig. S8).

### 375 The grain length QTL maps to a 75 Mb / 4.3 cM genetic interval

376 We used a set of 60 homozygous RILs to map the grain length phenotype to a narrower genetic  
377 interval within the 5A QTL region (17.65 cM, 367 Mbp). KASP markers were developed for 25  
378 additional SNPs between the two original QTL flanking markers (*Xgwm293* and *Xgwm186*; Fig.  
379 6a) based on data from the iSelect genotyping of BC<sub>4</sub> NILs and 820K Axiom Array genotyping of  
380 Charger and Badger (Winfield *et al.*, 2016). Based on the genotype of these 25 markers, 49 of the  
381 RILs were assigned to eleven distinct recombination groups represented as graphical genotypes  
382 in Fig. 6a. Control RILs were selected based on having either the Charger (5A-) or Badger (5A+)  
383 genotypes across the interval (C-control and B-control, respectively).

384 RILs were phenotyped for grain length in three field seasons and we found significant  
385 differences between RIL groups ( $P < 0.001$ ). The overall average grain length of the B-control  
386 group was 4.06 % higher than the C-control group ( $P < 0.001$ ; Fig. 6b), consistent with the  
387 differences in grain length observed between the NILs (Table 1). Each RIL group was classified  
388 based on Dunnett's tests to both control groups: for example, a RIL group was classified as  
389 Charger-like only if it was both significantly different to the B-control *and* non-significantly  
390 different to the C-control. Using this classification, we assigned unambiguously the eleven RIL  
391 groups to a parental type and genetically mapped the grain length phenotype between markers  
392 *XBS00182017* and *XBA00228977* (Fig. 6). This represents a genetic distance of 4.32 cM  
393 corresponding to a physical interval of 74.6 Mb in the Chinese Spring RefSeq v1.0 sequence.  
394 This 74.6 Mb interval contains 811 TGACv1 gene models (Clavijo *et al.*, 2017) based on *in silico*  
395 mapping to the Chinese Spring reference (Supporting Information File S1). We analysed the  
396 expression profile of these genes on the wheat expVIP expression platform (Borrill *et al.*, 2016)  
397 and found that 439 of these genes are expressed ( $> 2$  transcripts per million (tpm)) in at least  
398 one grain RNA-seq sample ( $n=147$ ). The developmental time courses suggest that the 5A QTL  
399 acts at around 12 dpa and we found 405 of these transcripts expressed in grain samples taken at  
400 around this time (4-15 dpa,  $n = 59$ ), with 298 genes expressed in the pericarp tissue (Pearce *et*  
401 *al.*, 2015a).

## 402 Discussion

403 In this study we identified a stable and robust QTL associated with a 6.9 % increase in grain  
404 weight. This increase is driven by longer grains associated with increased pericarp cell length. In  
405 wheat and barley pericarp cell division decreases shortly after fertilization (2 to 6 days; Drea *et*  
406 *al.*, 2005; Radchuk *et al.*, 2011) and cell expansion plays the predominant role in increasing  
407 pericarp size during grain development. Our results are consistent with a role of the 5A gene on  
408 pericarp cell expansion given that significant differences in grain size are only observed twelve  
409 days after fertilization, once cell expansion has begun. However, we cannot discard an  
410 overlapping late effect on cell division given the conflicting results in final pericarp cell number  
411 between years.

412 Overall, our results suggest that the gene underlying this locus regulates, either directly or  
413 indirectly, cell expansion in the pericarp (seed coat), a mechanism that is known to be a key  
414 determinant of grain/seed size in several species. Some genes, such as expansins and XTH  
415 (xyloglucan endotransglucosylase/hydrolases), affect cell expansion directly by physically  
416 modifying or “loosening” the cell wall (reviewed in Cosgrove, 2005), and the expression of these  
417 enzymes has been associated with pericarp cell expansion in wheat and barley (Lizana *et al.*,  
418 2010; Radchuk *et al.*, 2011; Munoz & Calderini, 2015). Other genes regulate pericarp/seed coat  
419 cell size indirectly, for example through the regulation of sugar metabolism and subsequent  
420 accumulation in the vacuole (Ohto *et al.*, 2005) and endoreduplication (Chevalier *et al.*, 2014).  
421 Our results provide direct genetic evidence that pericarp cell expansion affects final grain size  
422 and weight in polyploid wheat.

423 The maternal control of grain/seed size has been well documented in rice and Arabidopsis (Li &  
424 Li, 2015), as well as in wheat through physiological and genetic studies (Hasan *et al.*, 2011;  
425 Simmonds *et al.*, 2016). This can affect cell proliferation and/or cell expansion of maternal  
426 tissues, such as the wheat pericarp, both pre- and post-fertilisation (Garcia *et al.*, 2005; Adamski  
427 *et al.*, 2009; Ma *et al.*, 2016). For example, *GW2* in rice and its orthologue in Arabidopsis (*DA2*)  
428 affect grain/seed size through suppression of cell proliferation (Song *et al.*, 2007; Xia *et al.*,  
429 2013). Similarly in wheat, a knock-out mutant of the *GW2* orthologue has larger carpels than  
430 wild-type plants suggesting that the gene acts on maternal tissue pre-fertilisation (Simmonds *et*  
431 *al.*, 2016). The effect of the wheat *GW2* gene on cell size and number has not been determined  
432 however.

433 The direct assignment of the 5A effect to the maternal parent will require additional studies,  
434 including analysis of F<sub>1</sub> hybrids from reciprocal crosses. These studies are not routinely  
435 performed in wheat given that the phenotypic variation between individual F<sub>1</sub> grains often  
436 surpasses the relatively subtle phenotypic effects of most grain size QTL (usually less than 5% in  
437 wheat). The identification of a robust effect on pericarp cell length in this study, which is  
438 independent of the individual grain size, opens up a new approach to explore these parent-of-  
439 origin effects in polyploid wheat.



440 It has been proposed, in multiple species, that the size of the pericarp/seed coat determines  
441 final grain size by restricting endosperm growth (Calderini *et al.*, 1999; Adamski *et al.*, 2009;  
442 Hasan *et al.*, 2011). This is analogous to the way in which grain size in rice is limited by the size  
443 of the spikelet hull (Song *et al.*, 2005). Both the length (Lizana *et al.*, 2010; Hasan *et al.*, 2011) and  
444 the width (Gegas *et al.*, 2010; Simmonds *et al.*, 2016) of the pericarp have been proposed as key  
445 determinants of final grain weight in wheat. Our results provide genetic evidence for the  
446 importance of the maternal pericarp tissue and show that length is the underlying component  
447 for the 5A locus. Across three years, the difference in grain length between NILs was the first  
448 grain size component difference to be established. Only after this, did we observe any  
449 differences in grain width, weight or grain filling rate. These differences in grain length were  
450 extremely consistent across years (despite average TGW values ranging from 39.8 to 50.3 g)  
451 compared to the more variable differences in grain width and weight. Based on these results we  
452 hypothesise that the 5A locus increases grain weight by a primary effect on grain length, which  
453 confers the potential for further enhancements by pleiotropic effects on grain width. The grain  
454 length effect is genetically controlled and stable across environments, whereas the pleiotropic  
455 effect on grain width occurs later in grain development and is more environmentally dependent  
456 and variable. The final magnitude of the 5A grain weight increase (ranging from 4.0 to 9.3 %) is  
457 thus determined by the extent to which the late stage pleiotropic effect on grain width is  
458 manifested and the potential exploited. This could explain why the grain width increase was  
459 significantly correlated with the increase in TGW ( $r = 0.98$ ,  $p = 0.004$ ) whilst grain length was not  
460 ( $r = 0.71$ ,  $p = 0.18$ ).

461 By dissecting TGW to a more stable yield component (grain length) we were able to classify RILs  
462 in a qualitative/binary manner (i.e. "short" or "long" grains) which enabled the fine mapping of  
463 the 5A locus to a genetic distance of 4.3 cM. We identified roughly 400 genes in this interval  
464 that are expressed in the grain, several of which have annotations associated with genes  
465 implicated in the control of grain/seed size. Although it is premature to speculate on potential  
466 candidate genes, identification of the causal polymorphism will provide functional insight into  
467 the specific mechanism by which pericarp cell size and grain weight are controlled in polyploid  
468 wheat.

469 The consistent effect of the 5A locus on grain length and weight did not always translate into  
470 increased yield. In the original DH analysis, the 5A TGW effect co-located with final yield in  
471 seven of the twelve environments. This overall positive trend was also reflected in the NILs,  
472 although yield increases were only significant in 2014. We concluded that the effects on yield  
473 are modulated by a series of smaller negative effects on other yield components which could be  
474 due to additional genes within the broader 5A region. Alternatively, it could be that the full  
475 potential of the grain length effect will be realised only under certain environments or in  
476 combination with other genes.

477 By understanding the biological mechanism by which the 5A locus achieves increased grain size,  
478 hypotheses can be generated to combine genes in an informed and targeted way. For example,  
479 we are combining the 5A grain length/pericarp cell expansion effect with the *TaGW2* mutants  
480 which affect grain width (presumably through pericarp cell proliferation) to determine if they act  
481 in an additive or synergistic manner. Identifying the 5A gene will also allow the function of the  
482 homoeologous copies on chromosomes 5B and 5D to be determined. This is important since  
483 the effects of grain weight QTL in polyploid wheat are often very subtle compared to those in  
484 diploid species (Borrill *et al.*, 2015; Uauy, 2017). Modulating the function of all three homoeologs  
485 simultaneously holds the potential to expand the range of phenotypic variation and achieve  
486 effects comparable to those in diploids e.g. *NAM-B1* (Uauy *et al.*, 2006; Avni *et al.*, 2014; Liang *et al.*,  
487 2014). Ultimately, identifying the genes and alleles that control specific yield components  
488 and understanding how they interact amongst them and with the environment will allow  
489 breeders to manipulate and fine-tune wheat yield in novel ways.

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## 498 Author contributions

499 JB conducted the developmental time courses, fine-mapping, analysed the data and wrote the  
500 manuscript. JSi developed the germplasm used in this study, performed phenotypic assessments  
501 and QTL analyses, analysed the data and wrote the manuscript. FM conducted cell size  
502 measurements. MLW led the mapping of the Charger x Badger DH population. JSn coordinated  
503 and conceived the DH population field trials. CU conceived the experiments, analysed the data  
504 and wrote the manuscript. All authors read and approved the final manuscript.

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658 **Table**

659 **Table 1: Mean Thousand Grain Weight (TGW), yield and grain morphometric parameters of 5A NILs**

Year	Genotype	TGW (g)	Yield (kg/plot)	Grain area (mm <sup>2</sup> )	Grain length (mm)	Grain width (mm)
2012	5A-	38.027	4.408	18.755	6.625	3.475
	5A+	41.554	4.437	19.930	6.900	3.557
		9.28% <sup>***</sup>	0.66% <sup>NS</sup>	6.26% <sup>***</sup>	4.15% <sup>***</sup>	2.35% <sup>**</sup>
2013	5A-	40.772	6.157	19.969	6.705	3.674
	5A+	43.544	6.159	20.979	6.963	3.727
		6.80% <sup>***</sup>	0.02% <sup>NS</sup>	5.06% <sup>***</sup>	3.86% <sup>***</sup>	1.44% <sup>***</sup>
2014	5A-	47.368	6.495	21.493	6.798	3.930
	5A+	50.729	6.636	22.579	7.063	3.979
		7.09% <sup>***</sup>	2.17% <sup>*</sup>	5.05% <sup>***</sup>	3.90% <sup>***</sup>	1.25% <sup>**</sup>
2015	5A-	42.734	7.582	18.044	6.426	3.479
	5A+	46.201	7.712	19.293	6.730	3.554
		8.11% <sup>***</sup>	1.72% <sup>NS</sup>	6.93% <sup>***</sup>	4.72% <sup>***</sup>	2.16% <sup>***</sup>
2016	5A-	49.292	5.974	19.829	6.580	3.735
	5A+	51.266	6.064	20.610	6.816	3.745
		4.00% <sup>*</sup>	1.50% <sup>NS</sup>	3.94% <sup>**</sup>	3.58% <sup>***</sup>	0.27% <sup>NS</sup>
Overall	5A-	43.639	6.123	19.618	6.627	3.659
	5A+	46.659	6.201	20.678	6.894	3.712
		6.92% <sup>***</sup>	1.28% <sup>NS</sup>	5.41% <sup>***</sup>	4.04% <sup>***</sup>	1.45% <sup>***1</sup>

660

<sup>1</sup> %s indicate amount gained in 5A+ NILs compared with 5A- NILs. Superscripts indicate significance determined by ANOVA for either each year, or across all years (final row). ie. NS = Non-significant, \* = P < 0.05, \*\* = P < 0.01, \*\*\* = P < 0.001. 2012-13 = BC<sub>2</sub>-NILs, 2014-16 = BC<sub>4</sub>-NILs.

## 661 Figure legends

### 662 **Figure 1: QTL analysis and NIL development**

663 Circos diagram showing the whole genome quantitative trait loci (QTL) scan and single  
664 nucleotide polymorphism (SNP) variation. Outer track is the mean log-of-odds (LOD) score for  
665 thousand grain weight (TGW) across all environments measured. The red line shows a LOD  
666 threshold of 2.5. Wheat chromosome groups are represented in different colours beneath the  
667 QTL scans. The most significant and stable QTL identified was on chromosome 5A (boxed  
668 segment). Inner tracks correspond to heatmaps representing the number of iSelect SNPs in 30  
669 Mb windows showing variation between Charger and Badger, parents of the doubled haploid  
670 population (outer); or a representative pair of 5A-/5A+ NILs (innermost). Physical positions of all  
671 markers (including those used in the QTL scan and iSelect markers) were determined using the  
672 IWGSC RefSeq v1.0 sequence.

673

### 674 **Figure 2: Chromosome 5A genetic/physical map and TGW MTME analysis**

675 (a) Genetic and physical map of chromosome 5A. The left-hand side represents the genetic map,  
676 comprised of two linkage groups with calculated distances between markers in cM (Linkage  
677 group 1: 0 -74.4 cM, Linkage group 2: 0 - 30.2 cM). The right-hand side represents the physical  
678 map according to the Chinese Spring IWGSC RefSeq v1.0 sequence. Markers highlighted in  
679 orange indicate those used for NIL development. (b) Multi-trait multi-environment (MTME) QTL  
680 analysis of the 5A QTL for thousand grain weight (TGW) across Linkage group 1. The red line  
681 indicates a log-of-odds (LOD) threshold of 2.5. (c) Markers with significant additive effects are  
682 shown for each environment for those markers above the LOD threshold in (b). The intensity of  
683 the colour (yellow to brown) indicates the level of the significance as indicated by the legend. E-  
684 N = England-Norwich, E-S = England, Sandringham, F = France, G = Germany, Sc = Scotland.

685

### 686 **Figure 3: Distribution of grain length of NILs from whole plot samples**

687 Violin plots showing the distribution of individual seed measurements of grain length across the  
688 five field experiments of BC<sub>2</sub> (2012-2013) and BC<sub>4</sub> (2014-2016) near isogenic lines (NILs). Purple  
689 = 5A+ NILs, grey plots = 5A- NILs. All within year comparisons between NILs were significant (P  
690 < 0.001).

691

#### 692 **Figure 4: Grain development time course of 5A- and 5A+ NILs**

693 Grain length (a), grain width (b) and grain dry weight (c) of 5A- (grey, dashed line) and 5A+  
694 (purple, solid line) BC<sub>4</sub> near isogenic lines (NILs) during grain development with samples taken at  
695 anthesis (0 days post anthesis (dpa)), 4, 7, 12, 19 and 26 dpa in 2015 field trials. \* = P < 0.05, \*\* =  
696 P < 0.01, \*\*\* = P < 0.001. Error bars show standard error of the mean.

697

#### 698 **Figure 5: Comparisons of cell length between 5A- and 5A+ NILs**

699 Density plots of cell length measurements from 27 grains per genotype group; dashed line  
700 represents the mean. "Grain length" insets show the average grain length of each group of  
701 grains used for measurements. The increase in cell length of 5A+ near isogenic lines (NILs)  
702 relative to cell length of 5A- grains is shown as a percentage along with the P values calculated  
703 using ANOVA to compare means of the two groups displayed. (a) Grains of average length from  
704 5A- and 5A+ NILs, (b) average 5A+ grains and equivalent 5A- grains, (c) average 5A- grains and  
705 equivalent 5A+ grains, (d) long 5A- grains (length equivalent to average 5A+ grains) and short  
706 5A+ grains (grain length equivalent to average 5A- grains).

707

#### 708 **Figure 6: Grain length maps to a 4.3 cM interval on chromosome 5A**

709 (a) Graphical genotypes of recombinant inbred lines (RIL) groups with the number of lines in  
710 each group shown in parentheses. RILs were grouped based on their genotypes defined by  
711 having either the Charger-like (grey) or Badger-like (purple) allele at each marker shown across  
712 the interval. Markers highlighted in orange indicate markers used for NIL development. (b)  
713 ANOVA adjusted mean grain length of RIL groups across all experiments. Bars are coloured

714 based on a Charger- or Badger-like phenotype, determined by Dunnett's test. Purple = Badger –  
715 like, grey = Charger – like. Error bars represent standard error of the mean.

## 716 Supporting information

717 **Table S1: Developmental traits of 5A NILs**

718 **Figure S1: Example of SEM image for cell size measurements**

719 **Table S2: Significant QTL identified for TGW in the Charger x Badger doubled haploid population**

720 **Table S3: Significant QTL identified for yield in the Charger x Badger doubled haploid population**

721 **Figure S2: Yield MTME graph**

722 **Table S4: Spike yield components 10 representative single ear samples of 5A NILs**

723 **Figure S3: Distribution of grain width of NILs from whole plot samples**

724 **Figure S4: Grain development time course of 5A NILs (2014 field trials)**

725 **Figure S5: Grain development time course of 5A NILs (2016 field trials)**

726 **Figure S6: Comparisons of cell length within genotypes between different sized groups of grains**

727 **Figure S7: Comparisons of cell length between 5A+ and 5A- NILs in 2016**

728 **Figure S8: Comparisons of cell number in 2015 and 2016**

729 **File S1: TGACv1 genes in the fine mapped interval and associated expression data**

Number of SNPs













