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1 # Over-expression of the brassinosteroid gene *TaDWF4* increases wheat
2 productivity under low and sufficient nitrogen through enhanced carbon
3 assimilation

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11 **Abstract:**

12 There is a strong pressure to reduce nitrogen (N) fertiliser inputs while maintaining or increasing
13 current cereal crop yields. Brassinosteroids, (BR), are a group of phytohormones essential for
14 plant growth and development, that have been demonstrated to regulate several agronomic
15 traits. *DWF4* encodes a cytochrome P450 that catalyses a rate-limiting step in BR synthesis.
16 We show that overexpression of the dominant shoot expressed homoeologue *TaDWF4-B* in
17 wheat can increase plant productivity by up to 105% under a range of N levels on marginal
18 soils, resulting in increased N use efficiency (NUE). We show that a two to four-fold increase in
19 *TaDWF4* transcript levels enhances the responsiveness of genes regulated by N. The
20 productivity increases seen were primarily due to the maintenance of photosystem II operating
21 efficiency and carbon assimilation in plants when grown under limiting N conditions and not an
22 overall increase in photosynthesis capacity. The increased biomass production and yield per
23 plant in *TaDWF4* OE lines could be linked to modified carbon partitioning and changes in

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24 expression pattern of the growth regulator Target Of Rapamycin, offering a route towards

25 breeding for sustained yield and lower N inputs.

26

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27 **Introduction:**

28 Wheat (*Triticum aestivum* L.) is a major crop worldwide providing 23% of human dietary
29 protein[1]. In many parts of the world, on-farm wheat yields have plateaued since the mid-1990s,
30 although the yield capacity of cultivars has continued to increase[2]. Modern agricultural practices
31 include the development of high-yielding varieties of cereal grains, expansion of irrigation
32 infrastructure, modernization of management techniques, distribution of hybridized seeds, and
33 the addition of synthetic fertilisers and pesticides to increase and protect yields. However, this
34 approach has led to direct losses of reactive N to the aquatic environment which lower water
35 quality through eutrophication, coupled with the loss of N_2O and the high CO_2 emissions during
36 fertiliser production, so the environmental consequences are substantial[3–5]. As it has been
37 estimated that only 33% of N applied to a field is taken up by the crop, a multi-faceted approach
38 is crucial to reducing the necessary application of fertiliser as well as losses of the applied
39 fertiliser[6,7]. Improving NUE (defined as ratio of grain produced per unit of N supply) can be
40 achieved by improving yield under a constant N supply. Thus far, strategies to improve the yield
41 capacity of crop cultivars have focused on photosynthesis[8–10], with gains in biomass and yield
42 reaching 40%[11,12]. However, this is not sufficient and reducing our reliance on synthetic fertiliser
43 will also rely on identifying ways to reduce wheat N requirement, i.e. being able to produce
44 wheat with lower N input without a reduction in yield or grain quality[2]. While identifying genes
45 and pathways regulating specific aspects such as N uptake, assimilation or remobilization can
46 provide some advantages, approaches that target regulatory components of N metabolism are
47 more promising. In particular, understanding and identifying genes that can regulate N
48 responsiveness, i.e. the overall capacity of plants to induce morphological and physiological
49 changes according to the external availability of N, are eagerly sought[6,13].

50 Many domesticated crop species are polyploid and it is thought that the genome duplication
51 events and the resultant chromosome rearrangements have been a major driving force creating

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52 biological complexity, novelty and adaptation to environmental changes[14–16]. Wheat, an
53 allohexaploid with genome AABBDD, is the product of two ancient hybridization events: *Triticum*
54 *uratu* (A) and *Aegilops spp.* (B) hybridised 0.5 million years ago[17], to form the tetraploid *Triticum*
55 *turgidum*, which then underwent a second hybridisation ~10,000 years ago with *Aegilops*
56 *tauschii* (D) to form *Triticum aestivum*[18]. A more nuanced process of continuous diversification
57 and allopolyploid speciation to produce the network of polyploid *Triticum* wheats has also been
58 described[19] and with the advent of whole genome resequencing, the B-subgenome has been
59 shown to exhibit most variation with multiple alien introgressions and deletions[20]. The presence
60 of homeoalleles in hexaploid wheat increases both coding sequence variation *per se* and
61 regulatory variation with the new promoter and transcription factor combinations and functional
62 diversification of the duplicated genes[21,22]. This genome asymmetry and variation has
63 consequences for quality traits such as the control of wheat seed storage proteins, biotic and
64 abiotic traits, agronomic traits and response to pests and diseases[23–25].

65 Phytohormones are known to modify agronomically important traits, in particular
66 brassinosteroids (BR) [26,27]. BR have been shown to positively affect traits including tiller
67 number, leaf size and angle, photosynthesis and yield [28–31] plus other desirable traits, including
68 N use efficiency (NUE), disease resistance, and end use quality traits[31–34].

69 Manipulating levels of *DWF4/CYP90B*, the rate-limiting step in the BR synthesis pathway, has
70 been shown to increase yield, biomass production, tiller number and quality traits in diverse
71 plant species[30,31,34,35]. The mechanism of how altering BR levels impacts yield is still not
72 completely understood, but in many plant species an increased rate of carbon fixation has been
73 observed, suggesting that photosynthesis is key to the increased yields[30,34].

74 An explanation of how the BR pathway controls so many different agronomically important
75 phenotypes was shown through the direct interaction between BZR1 (Brassinazole Resistant 1),

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76 the transcription factor activating BR related genes, and the Target Of Rapamycin (TOR), the
77 growth regulator sensing the carbon (C) status of the plant[36,37]. Overexpression of TOR in
78 plants also mirrors many of the same phenotypes as modification of the BR pathway,
79 suggesting that TOR may be the mode of increased growth and yields shown by many BR over-
80 expressors. The BR and TOR pathways directly interact though the BR transcription factor
81 BZR1, and the TOR kinase. The TOR kinase can maintain the activation potential of BZR1 and
82 its role in growth promotion, by keeping the BZR1 protein stable[37]. Reducing *TOR* expression
83 by RNAi silencing led to a decreased ability of BR regulated genes to be upregulated, arrested
84 plant growth and, abolished the ability of increased BZR1 to promote growth when high levels of
85 sugar were present[38].

86 While many studies have shown that modification of the BR pathway can increase yields, plants
87 have typically been assessed under nutritionally replete conditions. In order to assess if the
88 same yield gains could be achieved in wheat grown on either marginal soils, or with lower
89 fertiliser inputs, we generated over-expression lines to test whether modification of DWF4 levels
90 could drive productivity gains and/or increase NUE.

91 **Results:**

92 ***The wheat DWF4 gene family has seven members***

93 To identify putative orthologs of DWF4/CYP90B in wheat, the amino acid sequence for the rice
94 DWF4/CYP90B (locus Os03g0227700) was used in a BLASTP search using wheat genomics
95 resources at Ensembl (http://plants.ensembl.org/Triticum_aestivum/Info/Index). Seven amino
96 acid sequences were identified with high homology to the rice DWF4 amino acid sequence (e
97 value < $1e^{-50}$, and > 70% homology), these were encoded by four sequences located on
98 chromosome 3 (one on chromosome 3A, one on chromosome 3B, two sequential copies on
99 chromosome 3D) and three sequences located on chromosome 4 (Suppl. Fig. 1a). The amino

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100 acid sequences were clustered by chromosome group (Suppl. Fig. 1a). Putative proteins
101 encoded by sequences on chromosome group 4 tended to be more similar to each other
102 (98.62% to 99.01% identity) compared to those on chromosome group 3 (87.97% to 95.77%
103 identity). Within chromosome group 3, TaDWF4-3D2 was the most distinct within the family
104 (Suppl. Table 1).

105 Comparison of the group 4 homoeologue putative protein sequences revealed that 4A
106 contains two large insertions when compared to the other homoeologues and OsDWF4 (Suppl.
107 Fig. 2). The first insertion is located between exons 1 and 2, and the second insertion is located
108 between exons 5 and 6, relative to the predicted CDS in the B and D homoeologues. The genes
109 on chromosome 4 showed the highest expression levels, in particular *TaDWF4-4B*, while low to
110 no expression could be seen for any of the genes on chromosome 3 in the wheat
111 expression.com databases[39] (Suppl. Fig. 1a). The expression patterns of the identified
112 homoeologues on chromosome 4 showed unbalanced expression with *TaDWF4-4B* dominating
113 expression in the shoot (70.9%), and *TaDWF4-4A* dominating expression in the spike (56.6%).
114 There was no dominant homeologue expressed in the roots as *TaDWF4-4A* and *TaDWF4-4B*
115 showed similar but higher expression in the roots than *TaDWF4-4D*.

116

117 ***DWF4 gene duplications in wheat***

118 In diploid plant species where the DWF4 gene family has been characterized (including
119 Arabidopsis, rice, and maize (*Zea mays*), DWF4 is encoded by a single gene[28,40,41]. It is
120 interesting that in hexaploid wheat, cv. Chinese Spring, seven putative orthologs could be
121 identified, suggesting that there may have been one or possibly two duplication events: the first
122 duplication event leading to the presence of DWF4 genes on both chromosome 3 and

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123 chromosome 4 groups, and the second duplication event leading to two DWF4 genes on
124 chromosome 3D, as the three gene models are co-linear on chromosome 3D.

125 The *DWF4* inter-chromosomal duplication event in wheat, which led to the presence of the
126 DWF4 gene on both chromosome groups 3 and 4, cannot be found in crop or model species
127 such as Arabidopsis, rice, maize or brachypodium (*Brachypodium distachyon*) (Suppl. Figure
128 1b). In rice, the closest related proteins to OsDWF4 are other cytochrome P450s more
129 commonly known as CPD1 or CPD2 sharing 46.7 percent identity at the amino acid level
130 (Ensembl v 46). In Arabidopsis, the closest protein to AtDWF4 (AT3G50660) is also a
131 cytochrome P450, CYP72A1 sharing 36.1% identity at the amino acid level (Ensembl v 46). In
132 maize and brachypodium the closest related proteins are ZmCYP724A1 (Zm00001d003349)
133 with 50.5% identity and BdCYP724A1 (BRADI_5g12990v3) with 47.4% identity.

134 The inter-chromosomal duplication of the DWF4 genes can also be inferred for both *T.*
135 *urartu* and *Aegilops tauschii*, two of the three wheat progenitor species (progenitors of the A and
136 D genomes in *T. aestivum)*, as well as in the tetraploid *T. dicoccoides* (Suppl. Figure 1b). An
137 intra-chromosomal duplication in *Aegilops tauschii* on chromosome 3, supports the hypothesis
138 that these copies arose before the progenitors hybridized. Therefore, one gene duplication
139 event seems to have arisen before the progenitor species hybridized, and a second event after
140 the hybridization occurred to produce modern bread wheat.

141

142 ***Overexpression of TaDWF4-4B in wheat leads to the upregulation of BR responsive***
143 ***genes***

144 Given that *TaDWF4-4B* in Chinese spring showed the highest expression levels in the shoot
145 tissues identified in public RNAseq databases, we selected this homeologue for over-expression
146 in hexaploid wheat (Suppl. Figure 1). A total of forty independent T_0 plants were generated in

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147 the cv. Fielder, with *TaDWF4-4B* CDS expressed from the constitutive promoter, OsActin[42]. In
148 six out of nine T_0 plants with a single T-DNA insertion, *TaDWF4* transcript levels (including both
149 transgene and endogenous gene) were significantly increased from 1.6 to 4.9-fold compared to
150 wild type (Suppl. Figure 3a). In the four over-expressing (OE) lines showing the highest
151 *TaDWF4* transcript levels, we measured the transcript levels for EXORDIUM (EXO),
152 DWF3/CPD/CYP90A, BZR2 and BES1, as these genes either have been shown in Arabidopsis
153 to be upregulated by BR[43] or involved in the BR pathway[44–46]. *TaEXO* and *TaBES1* transcript
154 levels were significantly higher than WT in the four OE lines tested ($p < 0.05$) and followed the
155 same trend as those of *TaDWF4* (Suppl. Figure 3b). *TaBZR2* and *TaDWF3* did not show
156 significantly increased expression in OE wheat lines and this contrasts with previous reports in
157 other plant species of either *DWF4* overexpression or the application of exogenous BL [44,47].

158 ***Overexpression of TaDWF4-4B increases productivity under low to high N levels***

159 The four highest expressing transgenic lines (OE-1, OE-2, OE-3 and OE-4) and a
160 corresponding null segregant (WT) were grown in pots on a low fertility soil supplemented with
161 NH_4NO_3 to reach field-equivalent N levels of 70, 140 and 210 kg/ha N. A low fertility soil with
162 limited N was used as a substrate in order to demonstrate N deficiency symptoms. The lowest
163 level (70 kg/ha N) corresponds to a N deficiency and the highest level (210 kg/ha N) to the
164 agronomic level typical of current UK agronomic practice [48]. All four OE lines tested showed
165 significant increases in yield per plant when compared to the WT at all 3 N levels ($p = 0$; Figure
166 1a). There was also a large increase in above ground biomass ($p = 0$), ranging from a 45% to
167 101% increase in the OE lines compared to WT (Figure 1b). An overall increase in tiller number
168 ($p = 0$; Figure 1c), grain number per plant ($p = 0$; Figure 1e) and grain weight measured as
169 thousand grain weight (TGW) ($p = 0$; Figure 1f) were also observed. The OE lines also showed
170 a significant increase in harvest index (HI, defined as the proportion of biomass in the grain
171 divided by the total biomass) relative to WT ($p = 0$; Figure 1d). The significant change in yield

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172 per plant under all levels of N tested resulted in a significant increase ($p = 0$) in the NUE in OE
173 lines relative to WT (Suppl. Fig 5), as expected with yield increase. Full statistical comparisons
174 are included in Suppl Tables 2-7.

175 As anticipated, increasing N supply led to increased yield in both WT and OE lines. The
176 WT line showed a defined N dependent yield increase, with OE lines producing higher yield at
177 all N levels and generally mirroring the gains seen by WT. The converse effect was seen for
178 biomass where increased N levels decreased the differences seen in biomass; as N became
179 less limiting the differences in biomass decreased (Fig. 1b). Overexpression of *TaDWF4-B* in
180 cv. Fielder led to significantly increased growth compared to the WT even under low N
181 conditions, and also led to higher productivity per plant.

182 ***Nitrogen responsive genes in TaDWF4 OE lines show altered expression:***

183 At the whole plant level, OE lines appear to be highly responsive to low N levels, maintaining
184 yield increases under N-deficient to replete conditions. The expression of four genes known to
185 be differentially regulated under high or low N conditions in wheat were selected to test the N-
186 responsiveness at the transcript level of an OE line (OE-1) relative to WT[49,50]. The genes
187 selected encode the high and low affinity N uptake transporters (NRT2.1 and NRT1), a N
188 transporter involved in N translocation through the plant (NPF7.1), and glutamate
189 dehydrogenase (GHD2), an enzyme involved in N remobilization in response to limiting carbon
190 (C). All four genes tested show a clear increase in OE-1 and WT transcript levels under low N
191 compared to replete N conditions in both shoots and roots of 10 day-old seedlings grown in a
192 hydroponic system (Figure 2).

193 Transcript abundance in OE-1 shoot tissues under N limited conditions showed
194 significantly lower expression of *TaNRT1* ($p = 0$), *TaNPF7* ($p < 0.001$) and *TaGDH2* ($p < 0.001$),
195 but not *TaNRT2.1 ($p = 0.07$),* compared with WT. Under replete conditions, no significant

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196 differences were found between OE-1 and WT. A similar pattern was seen in root tissues under
197 N limited conditions with significantly lower expression of *TaNRT1* ($p = 0$), *TaNPF7* ($p < 0.001$),
198 *TaNRT2.1* ($p < 0.001$), and *TaGDH2* ($p = 0$), in the OE-1 line compared with WT. Under replete
199 conditions only *TaNRT1* and *TaNRT2.1* showed significantly lower expression in OE-1
200 compared with WT ($p < 0.01$, $p < 0.05$).

201 Given the putative role of DWF4 in N response we also measured the level of transcripts
202 for *TaDWF4* from chromosome 4 in the shoots and roots of WT Fielder plants grown under four
203 N levels (0, 1/3, 2/3 and full as a proxy for 0, 70, 140 and 210 kg/ha fertiliser application rate).
204 Transcript levels of *TaDWF4* were higher in the shoots relative to the roots of wheat plants
205 grown under the four N levels ($p < 0.05$, Suppl. Fig 6). Increased N concentrations led to higher
206 *TaDWF4* transcript levels in the shoots and lower transcript levels in the roots (Suppl. Fig 6, $p <$
207 0.05). The effect of N level on *TaDWF4* transcript levels was driven by the difference between
208 the lowest N levels. There were no significant differences in transcript levels observed amongst
209 the 1/3 N, 2/3 N and N full treatments. *TaDWF4* expression therefore appears to be responsive
210 to changes in N levels under very limiting N conditions promoting expression in the roots and
211 decreasing expression in the shoots in WT Fielder.

212 ***Increases in yield and biomass in TaDWF4 OE lines are independent of N uptake and***
213 ***translocation:***

214 To further understand how the OE lines were driving an increase in yields, the flux of N was
215 measured using ^{15}N tracer studies. Uptake and translocation of N was measured between the
216 four OE lines and the WT when grown under low N conditions for two weeks and supplied
217 with^{15}N to quantify short term uptake and translocation from the root to the shoot. No significant
218 differences were seen in the uptake or translocation of N between any of the lines tested (Figure
219 3a, b) (ANOVA, *n.s.*).

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220 To understand if the OE lines showed altered N content compared with WT over a
221 longer plant growth cycle, total N was measured in the grain and senesced flag leaf from plants
222 grown under three N levels to compare N taken up from the soil at the end of the life cycle. At
223 harvest, no significant differences in flag leaf N content could be measured in plants grown on
224 eq. 210 kg/ha N (Figure 3c), while the grain N content was significantly lower in the OE
225 compared to WT. The decrease in grain N content in OE lines compared to WT was also
226 measured when plants were grown under three different N levels (Figure 3d) (ANOVA $p < 0.05$).

227 ***TaDWF4-B overexpression maintains photosynthesis under N limitation:***

228 In rice, OE of *DWF4* led to an increase in leaf level photosynthetic C fixation[30]. Leaf level CO_2
229 assimilation per unit area was measured on the fourth expanded leaf in the OE-1 and WT lines
230 grown under both low (eq. 70 kg/ha) and high (eq. 210 kg/ha) N levels. No differences in CO_2
231 assimilation could be measured when OE-1 and WT plants were supplied with adequate N and
232 high light (Figure 4a). A significant increase in CO_2 assimilation over a range of CO_2
233 concentrations in the OE-1 line was observed when plants were grown under low N conditions
234 and high light (Figure 4b). The increase in CO_2 assimilation in OE-1 compared to WT under low
235 N was not driven by a significant difference in chlorophyll content, a major sink for N in a plant,
236 as both the OE-1 and WT line had similar levels of chlorophyll under both low and high N
237 (Figure 4c) ($p = 0.34$). Overall, this suggests that under low N conditions an increased
238 photosynthetic capacity may drive the yield and biomass gains measured in the OE lines.
239 However, this is not the case when plants are grown under N replete conditions.

240 To understand if the plants were able to maintain photosynthesis at a higher level when grown
241 under lower input conditions, plants were grown on the same low fertility soil as shown in Figure
242 1 supplemented with either 70 or 210 kg/ha equivalent N. Spot measurements of CO_2
243 assimilation were taken under growth chamber conditions, with light levels at 250 $\mu mol\ m^{-2}\ s^{-1}$

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244 and 400 ppm CO_2. Under these conditions a significant increase in CO_2 assimilation could be
245 measured in OE-1 compared to WT, under both N levels tested (Figure 5a) ($p < 0.05$).
246 Measurements of chlorophyll fluorescence were taken as proxy for the operating efficiency of
247 photosystem II ((Fm'-Fo') / Fm') on light adapted leaves from plants grown under low (70 kg/ha)
248 vs high (210 kg/ha) N conditions. OE-1 plants had a greater proportion of PSII centers available
249 to collect the saturating light pulse than that of WT plants under both low and high N conditions
250 (Figure 5b) ($p = 0$). While PSII efficiency declined in WT plants grown under low N conditions (p
251 <0.001), this was not the case for OE-1 plants.

252 To understand the effect of continued photosynthesis on C content, flag leaves of fully senesced
253 plants grown on 210 kg/ha equivalent N were measured. There was a significant increase (1 to
254 2%) in the flag leaf C content in OE-1 lines compared to WT ($p < 0.01$) (Suppl. Fig 7). Soluble
255 sugars were measured in fourteen-day old seedlings grown in hydroponic solution under low
256 (1/3, 70 kg/ha) or replete (Full, 210 kg/ha) conditions. Under replete N conditions, WT showed
257 significantly higher levels of both soluble sugars measured (D-fructose, D-glucose) ($p < 0.05$)
258 (Figure 6a-d), led mostly by large differences in the roots of both sugars measured. Under low
259 N conditions, there were similar levels of bother sugars per gram dry weight in the shoots of OE-
260 1 plants relative to WT plants. Although the distribution in the two tissues tested of the sugars is
261 different and significant differences were observed in both sugars in both tissues measured ($p <$
262 0.05).

263 Next, we tested whether the effect of *TaDWF4-B* overexpression on photosynthesis and soluble
264 sugar/C levels was mediated by TOR. Significantly higher transcript levels for *TaTOR* were
265 measured in the roots of OE-1 relative to WT, when plants were grown under both low and
266 replate N in hydroponics. *TaTOR* transcripts levels were higher in roots than shoots in OE-1 (p
267 < 0.05) and a significant difference in TOR expression was seen under both low and high N
268 growth conditions ($p < 0.05$) (Figure 6d, e). The effect of low N led to increased *TaTOR*

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269 expression in OE-1 shoots, however the converse was seen in WT shoots where *TaTOR*
270 transcripts levels were markedly reduced under low N. In roots OE-1 showed significantly higher
271 transcript levels than WT under both high and low N (Figure 6e).

272 ***Indirect selection has potentially increased BR activity in modern wheat varieties:***

273 To understand if the increased photosynthesis observed in the OE lines has already been
274 indirectly selected for in modern wheat cultivars, seven diverse spring wheat cultivars as well as
275 Fielder and OE-1 were tested for both their ability to maintain PSII operating efficiency under
276 limiting N levels, and to determine if this could be a factor in determining NUE differences. The
277 ability of the different varieties to maintain PSII electron transport could be seen in three
278 different varieties including the former elite UK spring wheat cultivar Paragon (Figure 7A)
279 suggesting that this trait may have been indirectly fixed via breeding. *TaDWF4* transcript levels
280 were lower in all cultivars tested compared to OE-1, and there was no significant difference
281 amongst cultivars (Figure 7b). The lack of selection for higher BR levels/DWF4 expression is
282 further supported as lines which maintained PSII reaction centers open had both high and low
283 NUE, where NUE is defined as yield on low N (eq. 70 kg/ha) divided by yield on high N (eq. 210
284 kg/ha) (Suppl. Fig 8).

285

286 **Discussion:**

287 In this study, we identified the functional orthologues of the rice DWF4 in wheat and generated
288 OE lines to understand whether modification of the BR pathway could be used to increase
289 wheat NUE and drive an increase in yield as seen in other crops with modified BR functions. We
290 identified seven putative orthologues in wheat, four on chromosome 3 plus three on
291 chromosome 4, and have shown that the putative orthologues on chromosome 3 had low level
292 to no expression, whereas the 4B homoeologue had the highest expression, based on data

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293 available from public wheat expression databases. Significant tissue type differences were
294 seen in the expression of the three homoeologues, suggesting that specific regulatory elements
295 may control BR levels in particular tissues to increase plant growth in wheat. The duplication of
296 putative orthologues on the two different chromosome groups also appears to have happened
297 before hexaploid bread wheat arose 5.5 Mya, as each of the progenitor species, durum wheat
298 and *T. dicoccoides* have the duplication[24]. Further support for the orthologues on chromosome
299 4 being similar to the previously characterized DWF4 is the high degree of synteny between
300 wheat chromosome group 4, and rice chromosome 3, on which OsDWF4 resides, extending
301 most of its length. In contrast, chromosome group 3 is the best conserved of all wheat
302 chromosome groups, along both short and long arms, compared with rice chromosome 1[51].

303 Based on the bioinformatic analysis the homoeologue on 4B was selected for creation of OE
304 lines in order to understand the role of increased BR expression on increasing yield and NUE.
305 Overexpression of *TaDWF4-B* resulted in dramatic increases in several agronomic measures
306 including yield and biomass across a range of N levels. Interestingly, total biomass levels of WT
307 plants started to approach those of OE lines as N levels increased, however this alone did not
308 lead to a decrease in the difference in yields suggesting that biomass alone is a proxy and not a
309 perfect measure of ultimate yield potential (Figure 1a, b). The OE lines showed significant
310 increases in yield at all tested N levels and seemed to respond as if N was not limiting growth
311 under low N. Similar results have been recently reported in maize overexpressing ZmDWF4
312 grown under field conditions although different N levels were not tested[52]. Uptake rates of N as
313 well as translocation rates did not differ between the OE or WT lines and similar levels of N
314 were found in most tissues measured outside of the grain, suggesting that the OE lines did not
315 demand an increased level of N to support the increased plant growth. The altered N sensing by
316 higher DWF4 expression was supported by the expression of N regulated genes which showed
317 a muted response to the removal of N in *TaDWF4-B* OE lines. This expression data suggests

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318 that BR overexpression can mitigate nutrient limitations and that other signals such as C or
319 soluble sugars are more important to drive yield gains while lowering the need for N input. This
320 difference in growth was not due to a difference in the photosynthetic capacity, which has been
321 previous suggested as the reason for increased yields[30]. Overall we observed assimilation rates
322 and levels of chlorophyll were similar at high N levels (Figure 4). The maintenance of C
323 assimilation under limited N conditions may contribute to the increased biomass and yield. The
324 increase in levels of soluble sugars observed in WT plants including glucose possibly suggests
325 an altered sensing of required nutrient signals to activate growth and development. This is
326 supported by the higher expression of *TaTOR* under both N levels tested suggesting that
327 *TaDWF4* OE lines maintain growth, where WT would slow down. Further work is required to
328 understand the exact regulation of soluble sugars and other nutrient signals which mediates the
329 increase in growth in DWF4 overexpressing lines. Based on the data generated here it is
330 difficult to determine whether the increase in BR levels allowed growth to continue due to
331 altered levels of soluble sugars, or whether the lack of BR pathway inhibition allowed the
332 photosynthetic pathways to produce additional sugars and fuel further growth. Data collected
333 here suggests that higher BR levels in wheat can redirect normal feedback inhibition of N
334 limitation supporting continued plant growth. It would be interesting to determine if similar effects
335 are observed with other common nutrient limitations such as phosphate or potassium.

336 Finally it has been suggested that breeding for increased BR levels could improve overall yields
337 and improve the tolerance of crop species to various abiotic and biotic stress[28,30,35,41].
338 Observations here also suggest that wheat breeders are not selecting for increased DWF4
339 expression as DWF4 expression was not a significantly higher in the more modern spring wheat
340 varieties tested (Figure 7). There were significant differences in DWF4 expression between the
341 varieties tested as well as in response to N levels suggesting that variation may exist allowing
342 for selection for higher expression in wheat shoots.

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343 Overall, we have shown that OE of *TaDWF4* in wheat leads to plants which grow as if they need
344 less N, increasing overall NUE. The plants do not sense N as a limitation to growth and keep
345 growing under lower N levels. Based on these results we propose that a doubling of *TaDWF4*
346 expression could allow farmers to reduce application of N fertilizer by up to 70 kg/ha whilst still
347 producing the same amount of yield per plant as currently grown at standard 210 kg/ha N input
348 level. When this 70 kg/ha saving is multiplied by the estimated land in which wheat is grown in
349 the UK (1.69 million ha in 2019) there is the potential to reduce CO_2 released into the
350 atmosphere from fertilizer production by over 100,000 t [5,53,54]. This is equivalent to the energy
351 needed to power and heat more than 15,000 UK homes for one year [55]. Here we show that it is
352 possible to increase levels of DWF4 in wheat through genetic modification but that there is also
353 potential to exploit native variation via traditional breeding. This could potentially have a large
354 impact on wheat crop management, reducing fertiliser demands whilst maintaining levels of
355 productivity.

356

357 **Materials and Methods:**

358 **Gene identification:** The *OsDWF4* coding sequence from NCBI (XP_015633105.1) was used
359 as a query for BLAST searches of the wheat genome (IWGSC 2014) and expressed sequence
360 tag (EST) databases in GenBank (http://www.ncbi.nlm.nih.gov/), Ensembl
361 (http://plants.ensembl.org/index.html) and Komugi (https://shigen.nig.ac.jp/wheat/komugi/).
362 Gene prediction was carried out using FGENESH (www.softberry.com). Protein domain
363 prediction was found using InterProScan (https://www.ebi.ac.uk/interpro/). Identification of
364 DWF4 like genes was based on sequences in Ensembl (version 42) using BLASTP and using
365 an e value cut off of $< 1e^{-50}$.

366 **Plasmid construction for genetic modification:**

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367 *TaDWF4-B* was synthesized from the public sequence available for the wheat cultivar Chinese
368 Spring with attL1 and attL2 sites for direct recombination into a binary gateway vector. All
369 primers used in this study are listed in Supplemental Table 1. *TaDWF4-B* was then recombined
370 into the binary vector pSc4ActR1R2 using a Gateway LR Clonase II Kit (Thermofisher) to create
371 pMM90. *TaDWF4-B* was expressed *in planta* from the rice *Actin* promoter and transcripts
372 terminated by the *Agrobacterium tumefaciens* nopaline synthase terminator (tNOS)[42].

373 pMM90 was verified by restriction digest and sequencing before being electro-transformed into
374 *A. tumefaciens* strain EHA105 Plasmids were re-isolated from Agrobacterium cultures and
375 verified by restriction digest prior to use in wheat transformation experiments[56].

376 **Plant materials**

377 Wheat cv. Fielder (USA) was used for genetic transformation experiments. The spring wheat
378 varieties Baj-1 (India), Cadenza (UK), CDC Landmark (Canada), CDC Stanley (Canada),
379 Lancer (Australia), Paragon (UK) and Weebill (Mexico) were obtained from the Germplasm
380 Resource Unit, UK (www.seedstor.ac.uk/search-browseaccessions.php?idCollection=35), and
381 used in photosynthetic measurements.

382 **Plant growth conditions:**

383 Wheat cv. Fielder plants were grown in controlled environment chambers (Conviron) at 20 $^{\circ}$C
384 day/15 $^{\circ}$C night with a 16 hr day photoperiod (approximately 400 µE m^{-2} s^{-1}) for harvest of
385 immature embryos for the transformation experiments.

386 Transgenic lines and corresponding null segregants were grown on TS5 low fertility soil to
387 control total nitrogen with a starting nitrogen level of 0.1 mg/l (Bourne Amenity, Kent, UK).
388 Ammonium nitrate was then added to reach a final concentration in the pots which contained
389 roughly 750 g of dry soil in each pot. Equivalent to field fertiliser application of 70, 140 or 210

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390 kg/ha N which equates to 23.3, 46.6 or 70 mg/pot. Each pot also received 4.2 mg Ca, 2.7 mg K,
391 0.62 mg Mg, 0.04 mg P, 0.56 mg S, 0.008 mg B, 0.13 mg Fe, 0.015 mg Mn, 0.0012 mg Cu,
392 0.0024 Mo, 0.0045 Zn, 0.00 mg Na, and 0.63 mg Cl per pot. Plants were grown in a climate
393 controlled glasshouse with supplemental light for a 16 hr day and 20°C/15°C day night
394 temperatures.

395 For transcript abundance measurements and sugar content, wheat seedlings were grown for
396 ten days in 2.2 L pots containing Magnavaca solution containing 3 µM KH_2PO_4, 3.52 mM
397 $Ca(NO_3)^2$, 0.58 mM, KCl, 0.58 mM K_2SO_4, 0.56 mM KNO_3, 0.86 mM $Mg(NO_3)_2$ 0.13 mM H_3BO_3,
398 5 µM $MnCl_2$, 0.4 µM Na_2MoO_4, 10 µM $ZnSO_4$, 0.3 µM $CuSO_4$, $Fe(NO_3)_3$ and 2 mM MES (pH 5.5)
399 and supplemented with either 0, 0.4 mM, 0.8 mM or 1.3 mM NH_4NO_3. Plants were harvested on
400 the tenth day separating tissue in to roots and shoots for analysis.

401 Plants were grown on Magnavaca solution containing either 0 or 1.3 mM NH_4NO_3 for ten days in
402 controlled environment chambers (Conviron) at 20 $^{\circ}$C day/15 $^{\circ}$C night with a 16 hr day
403 photoperiod (approximately 400 µE m^{-2} s^{-1}).

404 **Wheat transformation:**

405 Immature seeds were collected 14-20 days post-anthesis (dpa) and immature wheat embryos
406 were isolated and co-cultivated with *Agrobacterium tumefaciens* for 2 days in the dark[57].
407 Subsequent removal of the embryonic axis and tissue culture was performed as previously
408 described[58]. Individual plantlets were hardened off following transfer to Jiffy-7 pellets (LBS
409 Horticulture), potted up into 9 cm plant pots containing M2 compost plus 5g/l slow release
410 fertiliser (Osmocote Exact 15:9:9) and grown to maturity and seed harvest in controlled
411 environment chambers, as above.

412 **DNA analysis of transformed wheat plants:**

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413 Plantlets that regenerated under G418 selection in tissue culture, were tested for the presence
414 of the *nptII* gene using QPCR. The *nptII* copy number was assayed relative to a single copy
415 wheat gene amplicon, GaMyb, normalized to a known single copy wheat line[59]. Primers and
416 Taqman probes were used at a concentration of 10 μM in a 10 μl multiplex reaction using
417 ABsolute Blue qPCR ROX mix (Thermofisher) with the standard run conditions for the ABI 7900
418 HT. The relative quantification, $\Delta\Delta^{CT}$, values were calculated to determine *nptII* copy number in
419 the T_0 and subsequent generations[60]. Homozygous and null transgenic lines were identified on
420 the basis of *nptII* copy number and segregation analysis. WT Fielder plants were null
421 segregates.

422 **Transcript level analysis:**

423 Total RNA was isolated from both roots and shoots for each nitrogen treatment using an
424 RNeasy Kit (Qiagen). Following treatment with DNaseI (Thermofisher), cDNA synthesis was
425 conducted on 500 ng of total RNA using Omniscript RT Kit (Qiagen). The cDNA was diluted 1:2
426 with water and 0.5 μL was used as template in each RT-PCR reaction. Transcripts levels were
427 quantified using SYBR Green JumpStartTaq ReadyMix (SIGMA) with the standard run
428 conditions for the ABI 7900 HT. Three technical replicates were performed on each of the three
429 biological replicates. Two reference genes *TaUbiquitin* and *TaEF1α* were used for the
430 normalization using the $\Delta\Delta^{CT}$. The sequence of primers used in QPCR assays are shown in
431 Table S1.

432 **Whole plant measurements:**

433 Total shoot dry weight, seed weight (yield per plant), seed number, seed size, and tiller number
434 were measured at maturity and following two weeks of drying at 35 °C. Biological replicates
435 each contained 15 plants per line and were grown until seed maturation and grown in two

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436 separate experiments. NUE was calculated as yield per plant divided by the amount of
437 ammonium nitrate added.

438 **C and N content determination, N uptake:**

439 Wheat tissues (leaf or grains) were dried at 75 °C for 48 h before grinding. Four grains were
440 placed in 2 mL microfuge tubes with 2 x 5 mm diameter stainless steel beads and shaken in a
441 genogrinder until a fine powder was obtained. Dried and ground samples were carefully
442 weighed (0.5 mg) into tin capsules, sealed and loaded into the auto-sampler. Samples were
443 analyzed for percentage carbon, percentage nitrogen, $^{12}C/^{13}C$ ($\delta^{13}C$) and $^{14}N/^{15}N$ ($\delta^{15}N$) using a
444 Costech Elemental Analyzer attached to a Thermo DELTA V mass spectrometer in continuous
445 flow mode.

446 To measure N uptake, roots from 2 week old seedlings were exposed to $^{15}NH_4{}^{15}NO_3$ for 5 min,
447 then washed in 0.1 mM $CaSO_4$ for 1 min, harvested, and dried at 70 °C for 48 h. N content and
448 isotopic levels were analyzed as described above. The excess ^{15}N was calculated based on
449 measurements of $\delta^{15}N$ and tissue N%. First the absolute isotope ratio (R) was calculated for
450 labelled samples and controls, using $R_{standard}$ (the absolute value of the natural abundance of ^{15}N
451 in atmospheric $N_{2;}$ 1).

452 (1) $R_{sample\ or\ control} = [(\delta^{15}N/1000)+1] \times R_{standard}$

453 Then, molar factional abundance (F) and mass-based factional abundance (MF) were
454 calculated (2,3,4)

455 (2) $F= R_{sample\ or\ control}/(R_{sample\ or\ control}+1)$

456 (3) $MF= (F \times 15) \times /[(F \times 15)+ ((1-F)\times 14)]$

457 (4) $\Delta MF = MF_{sample} - MF_{control}$

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458 The excess ^{15}N in mg in a total tissue was calculated as in (5)

459 (5) Excess ^{15}N (g)= ΔMF x Tissue dw (g) x Tissue N%/100

460 **Chlorophyll measurements**

461 Leaf chlorophyll content was determined using the method developed by Hiscox et al.[61].
462 Chlorophyll was extracted from 100mg of fresh leaf tissue from six independent plants in 10mL
463 DMSO, mixed for 30 mins and then placed at 4 °C overnight. Extracts were diluted 1:2 with
464 DMSO before absorbance measurements at 645 and 663 nm (spectrophotometer Jenway
465 model 7315, Staffordshire, UK).

466 **Leaf gas exchange**

467 Leaf level photosynthesis and stomatal conductance were measured on the youngest fully
468 expanded leaf from the main tiller of at least six wheat plants at the tillering stage (GS23) grown
469 in a controlled environment chamber with 16hr day and 20 °C/15 °C day night temperatures.
470 Photosynthesis and stomatal conductance were measured using a portable infrared gas
471 analyzer (Licor 6400XP, Licor Environmental). Gas exchange was measured at a PPFD of 1500
472 μmol m^{-2} s^{-1} (using the LI-COR 6400 LED light source), block temperature was maintained at 22
473 °C, while humidity was maintained close to 60%. Each leaf was clamped in the Licor chamber,
474 and let to acclimate until CO_2 concentrations in the chamber reached a steady state for 5 min.
475 Gas exchange measurements were recorded under a range of CO_2 concentrations (400, 300,
476 200, 100, 400, 600, 800, 1000, 1200, 400 ppm).

477 **Chlorophyll fluorescence measurements and A/C_i curve analysis:**

478 An LI-6800 portable photosynthesis infrared gas analyzer system (LI-COR) equipped with a
479 multiphase flash fluorimeter was used to assess physiological differences for photosynthetic
480 parameters between transgenic and WT wheat plants. Measurements were taken on the fourth

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481 leaf of plants grown on TS5 low fertility soil to control total nitrogen with a starting nitrogen level
482 of 0.1 mg/l (Bourne Amenity, Kent, UK). Ammonium nitrate was then added to reach a final
483 concentration in the pots equivalent to field fertiliser application of 70 or 210 kg/ha N. Plants
484 were grown in a climate-controlled chamber with supplemented light (250 $\mu mol.m^{-2}.s^{-1}$) for a 16
485 hr day and 20 $^{\circ}C$/15 $^{\circ}C$ day night temperatures. For chlorophyll fluorescence measurements
486 (Fm'-Fo') / Fm') leaves from at least six plants were pulsed four times to acclimatize tissues and
487 a steady state reading taken. For measurements of the A/C_i curve was measured on six plants
488 for each treatment. Ca reference values were 400, 400, 300, 200, 100, 50, 25, 400, 400, 400,
489 600, 800, 1,000, 1,200, and 400 $\mu L\ L^{-1}$, with a saturating rectangular pulse of 12,000 $\mu mol\ m^{-2}$
490 s^{-1} at each reference point. All leaves were also normalized for the amount of area of the
491 measuring disk. Measurements were carried out on consecutive days between 1 and 8 h
492 postdawn, measuring three plants total selected at random from each treatment per day.

493 **Sugar measurements**:

494 Freeze-dried samples were ground to a fine powder with a pestle and mortar. A 50 mg sub
495 sample of leaf tissue and 25 mg of root tissue were extracted in 1 and 2 ml of ultra-pure water,
496 (Elgastat UHQPS), respectively. The extracts were mixed for 2 minutes on a miximatic
497 (Jencons) and incubated for 60 minutes in a water bath at 60 °C and mixed again. The solutions
498 were centrifuged (Sigma 4-16 KS) at 4500 g for 20 minutes. Five hundred microlitres of the
499 supernatant was pipetted into a Thomson 0.45 µm PTFE filter vial (Thames Restek).

500 Five microliters of supernatant were injected into a Waters Alliance 2695 HPLC. Sugars were
501 separated on an Ultra amino 100 Á 5 µm 250 x 4.6 mm column (Thames Restek, UK) and
502 detected with a Waters 2414 refractive index detector. The mobile phase was [80:20]
503 [acetonitrile: water] with a flow rate of 1 ml min^{-1}, the column was heated to 35 °C. Standards of
504 known amounts of the sugars were injected into the HPLC and Empower™ 3 software was used

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505 to produce linear calibration curves in the range of 0.625-10 µg for fructose, glucose, all curves
506 had r^2 greater than 0.998. These calibration curves were used to determine the concentration of
507 the sugars found in the samples. Sugar standards were analar grade (Sigma Aldrich UK),
508 solvents were HPLC grade (Fisher scientific).

509 **Statistical analyses:** Normal distribution of the data and equality of variance were verified
510 using Shapiro and Levene tests (Lawstat package[62]), respectively. Analysis of variance
511 (ANOVAs) or Wilcox Tests were run using the aov and TukeyHSD functions or wilcox.test
512 function in the R environment with the null hypothesis of no difference between lines[63]. Tukey's
513 post hoc test was added to identify each significant interaction between the lines tested. Data
514 was plotted using R ggplot2[64].

515 **Phylogenic trees:** Trees were made using MEGA X[65]. Amino acid sequences were obtained
516 from Ensembl. Amino acid sequences were aligned using the MUSCLE algorithm as part of
517 MEGA X and the tree was constructed using the maximum likelihood method, Jones-Taylor-
518 Thornton (JTT) model, with 500 bootstrap replications.

519

520 **Reporting summary.** Further information on research design is available in the Nature
521 Research Reporting Summary linked to this article.

522

523 **Data availability.** Data and seed are available upon request from the corresponding author.
524 Seed materials will be transferred under MTA.

525

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531

532 **Author contributions.** MC and SB generated the transgenic wheat plants. MM performed the
533 molecular and biochemical analysis, plus growth and gas-exchange experiments. SMS
534 performed ^{15}N uptake experiments. MM and SMS performed data analysis on their
535 contributions. MM designed the experiments and wrote the manuscript with input and
536 contributions from SMS, HG, ARB and EJW. All authors read and approved the final
537 manuscript.

538

539 **Competing interests.** The authors declare no competing interests.

540

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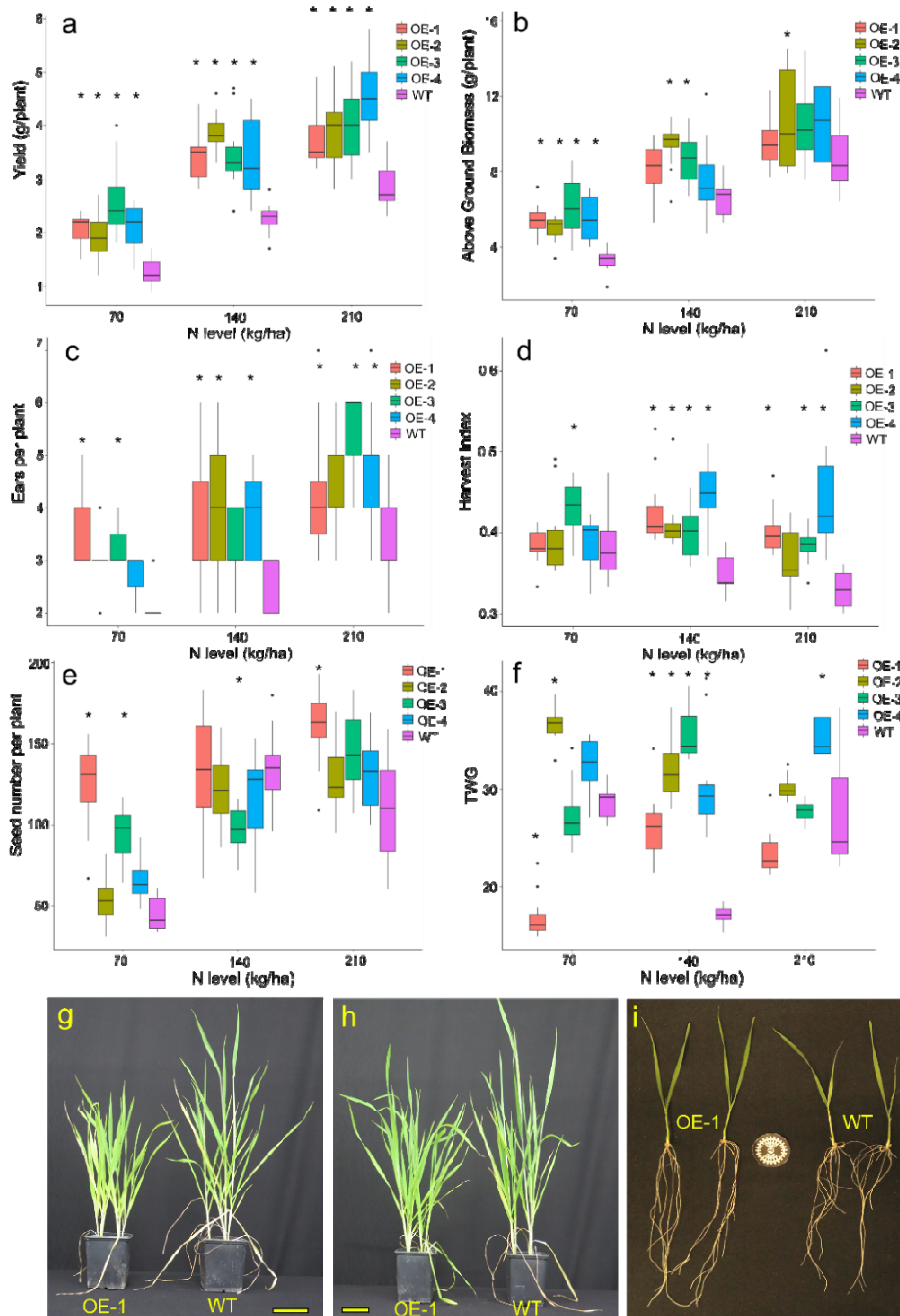

696

697 Figure 1: TaDWF4-B overexpressing lines show higher yield, above ground biomass, seed
698 number per plant and TGW and Harvest Index when grown under three N levels (eq. 70, 140,
699 210, kg/ha). Data are shown as mean values (central line), lower and upper quartiles (box),
700 minimum and maximum values (whiskers) and outliers as individual points. Fifteen plants were
701 grown per replicate for each genotype by N level combination. The overall plant growth
702 experiment was replicated twice; data from one replicate is shown. The statistical analysis was
703 performed with ANOVA and post hoc Tukey test, asterisk denotes p val <0.05. a) yield (g) per

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704 plant; b) above ground biomass (g) per plant; c) ears per plant; d) Harvest Index; e) seed
705 number per plant; f) Thousand Grain Weight per plant (TGW); g) OE-1 (left) and WT (right)
706 plants grown on 70 kg/ha equivalent; h) OE-1 (left) and WT (right) plants grown on 210 kg/ha
707 equivalent i); 10 day old plants from OE-1 (left) and WT (right) grown in hydroponic solution
708 under at 70 kg/ha N. Yellow bars in g and h = 10 cm, size standard in i = 40 mm diameter. Full
709 statistical comparisons are included in Suppl. Tables S2-7.

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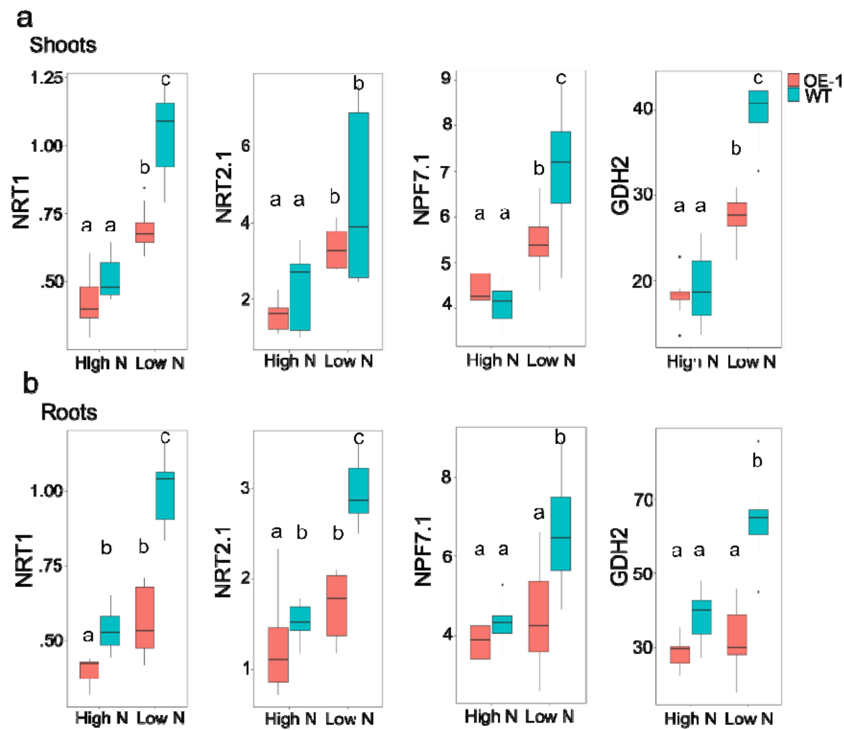

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714 Figure 2: Transcript abundance for N regulated genes in shoots and roots of ten day old OE-1
715 and WT seedlings under N limiting and N replete conditions. Transcript levels for NRT1,
716 NRT2.1, NPF7.1, GDH2 genes in wheat are shown relative to the expression of *TaUbi* under
717 low (LN) and high nitrogen (HN) in hydroponic solution in a) shoot; and b) root tissues. Data are
718 shown as mean values (central line), lower and upper quartiles (box), minimum and maximum
719 values (whiskers) and outliers as individual points. The statistical analysis was performed with
720 ANOVA and post hoc Tukey test, letters correspond to significant differences between transcript
721 levels of either line under either treatment ($p < 0.05$).

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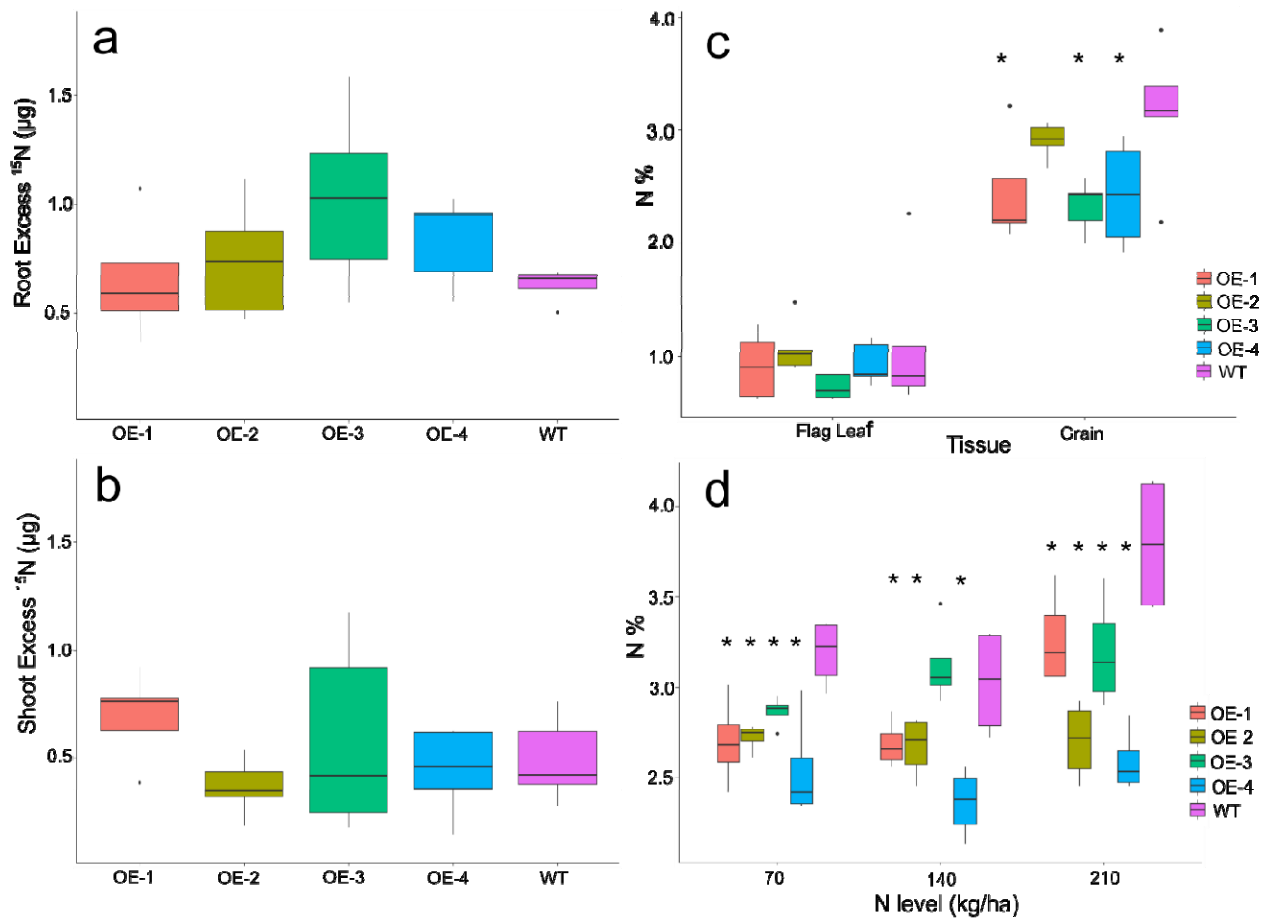

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726 Figure 3: Nitrogen flux and concentrations of TaDWF4-B overexpression plants. a) Uptake of
727 ^{15}N in roots of overexpression lines relative to WT roots; b) Translocation of ^{15}N from the soil to
728 the shoot in five minutes of uptake in OE lines relative to WT; c) Percentage N of flag leaves
729 and grains grown on 210 kg/ha at harvest; d) N content of grain grown on three N levels. Data
730 are shown as mean values (central line), lower and upper quartiles (box), minimum and
731 maximum values (whiskers) and outliers as individual points. The statistical analysis was
732 performed with ANOVA and post hoc Tukey test. Asterisks indicate a significant difference (p
733 <0.05) between WT and an OE line at the same N level.

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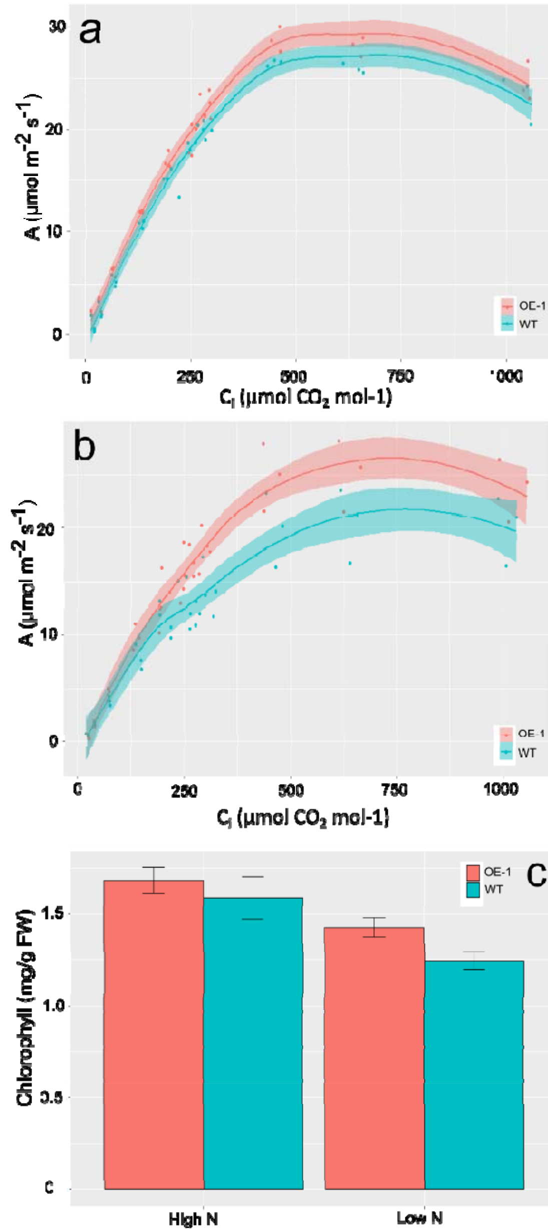

735

736 Figure 4: Photosynthetic C assimilation in the leaves of DWF4-B over-expression lines and WT
737 under high (210 kg/ha) and low (70 kg/ha) N levels grown in growth chamber with a light
738 intensity of 1250 µmol m^{-2} s^{-1} and CO_2 level of 400 ppm: a) A/Ci curve of OE-1 and WT plants
739 grown on under high N conditions (equivalent N 210 kg/ha) or b) under low N conditions
740 (equivalent N 70 kg/ha). c) Chlorophyll content in the leaves of plants used for A/Ci
741 measurements. In panels a and b the shading represents the 95% confidence interval. In panel
742 c the bars represent the mean and ± SD. from six plants measured on the fourth fully expanded
743 leaf.

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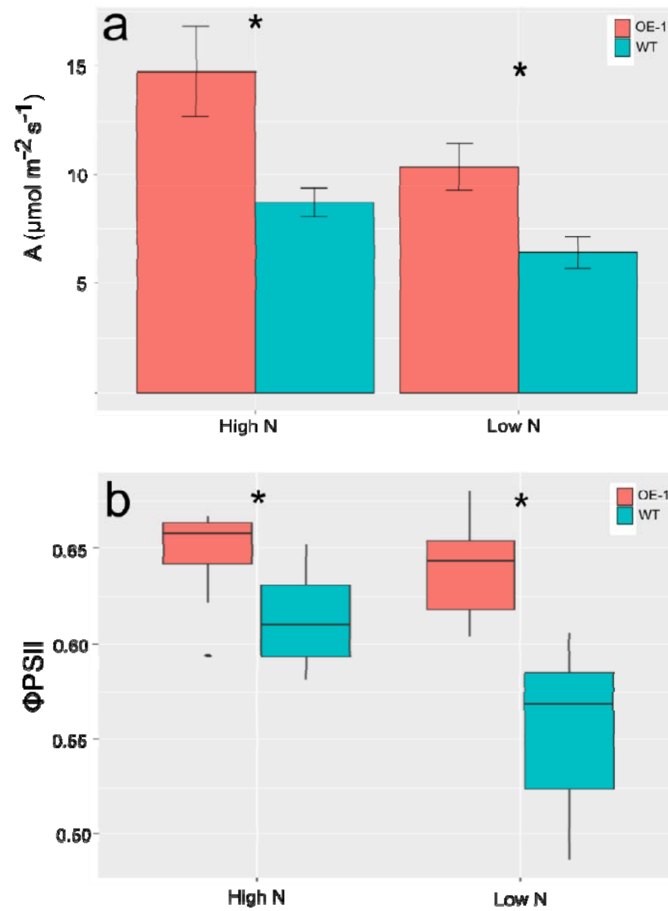

746

747 Figure 5: Photosynthetic performance of OE-1 or WT plants under low light conditions. a) Spot
748 measurements of C assimilation in OE-1 or WT under grown in growth chamber conditions
749 which include a light intensity of 250 μmol m^{-2} s^{-1} and CO2 level of 400 ppm. b) Operational
750 PSII efficiency (Fv'/Fm') in light adapted plants grown on two different N levels. Plants were
751 grown on either (Low) 70 or (High) 210 kg/ha N equivalent and the fourth leaf was measured for
752 PSII activity. The statistical analysis was performed with ANOVA and post hoc Tukey test.
753 Data shown represent the mean ± SD from six plants measured on the fourth leaf of when fully
754 expanded leaf. Asterisk in panels a and b indicates a significant difference ($p < 0.05$) between
755 WT and OE-1 at the same N level.

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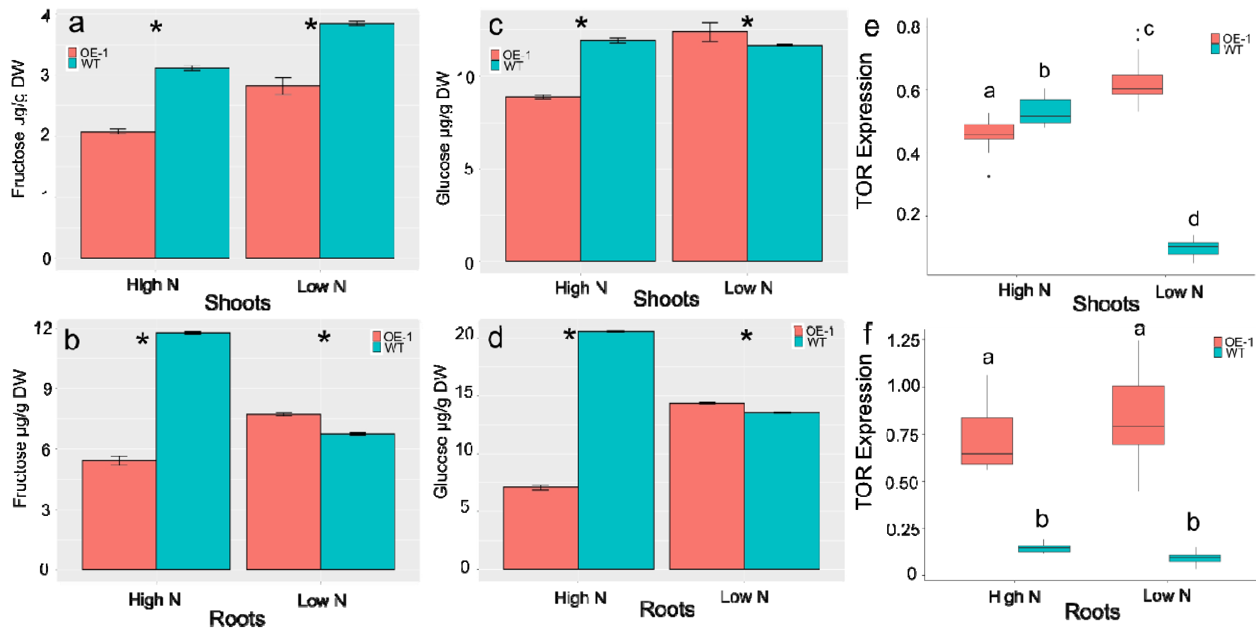

Figure 6: Root and shoot glucose and fructose content in OE-1 and WT wheat plants grown under high N or low N conditions in hydroponic solution, leads to increased transcript levels of *TaTOR*. Soluble sugars (fructose (a,b), glucose (c,d)) were extracted from wheat leaves of 14-day old plants grown High N (Full N) or Low N (0 N). The transcript abundance of *TaTOR* was measured in the shoots (e) and roots (f) in both OE-1 and WT 14-day old wheat plants grown under High N or Low N, expression shown is relative to *TaUbi*. n = 3 plants for each treatment and line tested. The statistical analysis in all panels was performed with ANOVA and post hoc Tukey test. Data in panels a, b and c represent the mean and ± SD Asterisk indicates a significant difference ($p < 0.05$) between WT and OE-1 at the same N level. Data in panels d and e are shown as mean values (central line), lower and upper quartiles (box), minimum and maximum values (whiskers) and outliers as individual points. Letters in panels d and e indicate significant differences ($p < 0.05$) amongst both the line and the treatment.

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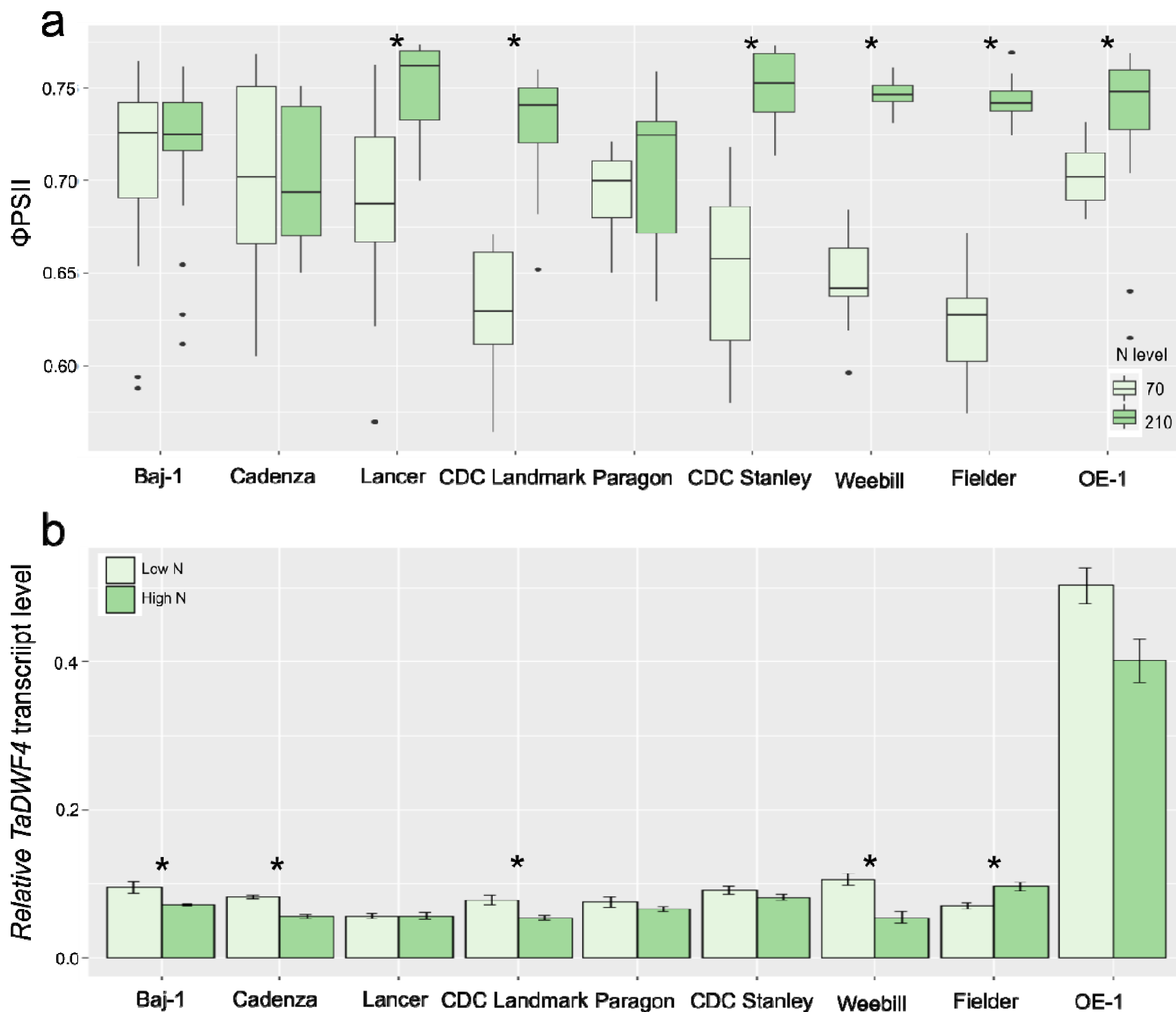

774

775 Figure 7: Evidence for indirect selection for BR indirectly through breeding: a) Operational PSII
776 efficiency in light adapted plants grown on two different N levels. Plants were grown on either 70
777 or 210 kg/ha N equivalent and the fourth leaf was measured for PSII activity. b) Expression of
778 *TaDWF4* in the shoots of 10-day old wheat plants grown under low N (LN=0 N) or high N
779 (HN=Full N), expression shown is relative to *TaUbi* in each cultivar. Data in panel a are shown
780 as the mean values (central line), lower and upper quartiles (box), minimum and maximum
781 values (whiskers) and outliers as individual points. Data in panel b are shown as the mean and
782 ± SD. n = 3 plants for each treatment and line tested. Statistical analysis was performed with
783 ANOVA and post hoc Tukey test, asterisk indicates a significant difference between low and
784 high N levels ($p < 0.05$).